Comparative study of lactulose production through electro-activation technology versus a chemical isomerization process using lactose, whey and whey permeate as feedstocks and valorization of the electro-activated materials to produce valuable metabolites using a kefir culture and Kluyveromyces marxianus

Thèse

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RÉSUMÉ

Le lactosérum et le perméat de lactosérum (WP) sont les principaux sous-produits du processus de fabrication du fromage et de la caséine. Ils sont considérés comme des polluants environnementaux en raison de leur charge organique élevée caractérisée par une haute demande biologique et chimique en oxygène. Ils créent un problème majeur d'élimination pour l'industrie laitière en raison des grands volumes de leur production annuelle. Par conséquent, il y a une demande constante de développer une approche durable pour leur utilisation afin d'éviter la pollution de l'environnement. Dans ce contexte, cette étude visait à comparer la technologie d'électro-activation (EA) à un processus d'isomérisation chimique, à alcalinité équivalente de la solution, pour produire du lactulose, qui est un prébiotique reconnu et éprouvé, en utilisant du lactose pur, du lactosérum et du perméat de lactosérum, comme matières premières sources de lactose, et de valoriser les produits électro-activés en produisant des métabolites à haute valeur ajoutée en utilisant une culture de kéfir et une culture pure de *Kluyveromyces marxianus* comme approche intégrée pour la valorisation complète de ces résidus de l'industrie laitière. La technologie d'électro-activation a été appliquée pour isomériser le lactose en lactulose dans un réacteur d'électro-activation modulé par des membranes échangeuses d'anions et de cations. L'électro-isomérisation du lactose en lactulose a été réalisée en utilisant des solutions de lactose (5, 10, 15 et 20 % p/v), de lactosérum (7, 14 et 21 % p/v) et de perméat de lactosérum (6, 12 et 18 % p/v) sous des intensités de courant électrique de 300, 600 et 900 mA pendant 60 min avec un intervalle d'échantillonnage de 5 min. L'isomérisation chimique conventionnelle a été réalisée à une alcalinité de la solution équivalente au KOH correspondant à celle mesurée dans les substrats électro-activés (lactose, lactosérum et perméat de lactosérum) à chaque intervalle de 5 min en utilisant de la poudre de KOH comme catalyseur à température ambiante (22 ± 2 °C). Les résultats obtenus ont montré que la production de lactulose en utilisant l'approche par électro-activation dépendait de l'intensité du courant électrique, de la concentration de la solution soumise à l'électro-activation et du temps de réaction. Les rendements les plus élevés de lactulose sont de 38 % en utilisant une solution de lactose de 10 % électro-activé pendant 40 min sous 900 mA, de 32 % en utilisant une solution de 7 % de lactosérum électro-activé sous 900 mA pendant 60 min et de 37 % en utilisant une solution de 6 % de perméat de lactosérum électro-activé sous 900 mA pendant 50 min. Parallèlement, les résultats ont montré qu'avec

une approche chimique conventionnelle avec du KOH comme catalyseur, les rendements de lactulose étaient de ~27 % en utilisant une solution de 10 % de lactose pendant 60 min et de 25,47 % en utilisant une solution de 6 % de perméat de lactosérum pendant 50 min. Cependant, aucune formation de lactulose n'a été observée en utilisant du lactosérum dans le procédé chimique conventionnel à une alcalinité équivalente de la solution traitée par électro-activation. Les résultats de cette étude ont révélé que la technologie d'électro-activation est plus efficace pour la production du lactulose à partir du lactose pur, du lactosérum et du perméat de lactosérum par rapport au processus d'isomérisation chimique conventionnelle. Par la suite, la faisabilité d'utiliser les substrats à base de lactose électro-activé, du lactosérum électro-activé et du perméat de lactosérum électro-activé comme sources de carbone pour produire de la biomasse riche en protéines et métabolites à valeur commerciale élevée comme des acides organiques (lactique, acétique, citrique et propionique) et des biomolécules aux propriétés aromatiques et gustatives a été étudiée en utilisant une culture microbienne mixte provenant de grains de kéfir comme ferment et une culture pure de *Kluyveromyces marxianus*. La fermentation a été réalisée pendant 96 h à 30 °C en utilisant les substrats électro-activés et non électro-activés du lactose, du lactosérum et du perméat de lactosérum. Les résultats obtenus ont montré que les substrats électro-activés ont permis d'atteindre une croissance de la biomasse la plus élevée en un temps de fermentation réduit comparativement aux substrats non électro-activés en utilisant la culture de kéfir comme agent de fermentation. La croissance cellulaire la plus élevée (6,04 g/L) a été obtenue dans le lactosérum électro-activé après 72 h, qui était 1,7 fois supérieure à ce qui était obtenu dans le milieu clostridien renforcé (RCM). De plus, le lactosérum électro-activé a permis de produire un maximum de 8,46, 3,97, 0,60 et 1,02 g/L d'acide lactique, acétique, citrique et propionique, respectivement. De plus, le lactosérum électro-activé a permis la production de kéfiran la plus élevée de 2,99 g/L, suivi par le lactosérum (2,67 g/L), le perméat de lactosérum électro-activé (2,31 g/L), le perméat de lactosérum (1,88 g/L), le milieu RCM (1,42 g /L), le lactose électro-activé (1,37 g/L) et le lactose (0,91 g/L). Les résultats ont également démontré que divers composés aromatiques volatils étaient produits au cours de la fermentation du lactosérum électro-activé, ce qui peut améliorer les caractéristiques organoleptiques et la qualité sensorielle des produits fermentés. Également, *K. marxianus* a également montré une production satisfaisante de la biomasse dans tous les substrats utilisés et que le lactosérum électro-activé a permis

d’atteindre une biomasse maximale (4,23 g/L) après 96 h de fermentation, suivie du milieu standard YM (4,85 g/L). La biomasse produite avait une teneur élevée en protéines et en lipides (24,43-57,83 et 15,44-25,64 %, respectivement) dépendamment des substrats utilisés et des conditions de fermentation. Plusieurs acides organiques majeurs comme les acides lactique, acétique, citrique et propionique ont été produits pendant la fermentation sur tous les milieux, avec des différences significatives entre les substrats électro-activés et non électro-activés. De plus, *K. marxianus* a produit divers composés aromatiques volatils aux propriétés organoleptiques appréciées. Le milieu de culture YM a entraîné la plus faible production d'éthanol (8,42 g/L à 48 h) tandis que la plus forte production d'éthanol a été produite dans le lactosérum non électro-activé (28,13 g/L à 48 h), suivi du lactose (27,85 g/ L à 48 h), du lactose électro-activé (26,77 g/L à 36 h), du perméat de lactosérum (25,99 à 72 h), du perméat de lactosérum électro-activé (24,66 g/L à 36 h) et du lactosérum électro-activé (22,06 g/L à 48 h). De plus, un maximum de 393,85 à 988,22 mg/L de 2-phényléthanol a été atteint, selon les substrats utilisés. Par conséquent, les résultats de ce projet suggèrent que la technologie d’électro-activation peut être une approche durable émergente permettant d’atteindre le double objectif de production de lactulose, un prébiotique reconnu et éprouvé, et de valorisation intégrale du lactosérum et de ses dérivés en utilisant des bioprocédés à base de culture de kéfir et de *K. marxianus* pour produire des métabolites à valeur commerciale élevée pour différentes applications; y compris pour l'industrie de l'alimentation humaine et animale. Ainsi, les connaissances obtenues dans ce projet pourront servir à améliorer la valorisation du lactosérum.

Mots-clés: Électro-activation; isomérisation chimique; lactosérum; perméat de lactosérum; substrats à base de lactosérum électro-activé; bioprocédés; kéfir; *Kluyveromyces marxianus*.

ABSTRACT

Whey and whey permeate (WP) are the main agro-industrial by-products from cheese or casein production process that are regarded as environmental pollutants because of their high organic load (high biochemical and chemical oxygen demand) and are creating a major disposal problem for the dairy industry. Consequently, there is a serious demand of developing a sustainable approach for their utilization to evade environmental pollution. In this context, the study was intended to compare the electro-activation (EA) technology with a chemical isomerization process at equivalent solution alkalinity to produce a prebiotic lactulose using lactose, whey, and WP as feedstocks and to valorize the electro-activated materials into valuable metabolites using a whole Kefir culture and a pure culture of *Kluyveromyces marxianus* as an integrated approach for complete valorization of these waste products. The EA technique was applied to isomerize lactose into lactulose in an EA reactor modulated by anion and cation exchange membranes. Electro-isomerization of lactose into lactulose was performed by using lactose (5, 10, 15, and 20%, w/v), whey (7, 14, and 21%, w/v), and WP (6, 12, and 18%, w/v) solutions under current intensities of 300, 600, and 900 mA during 60 min with a sampling interval of 5 min. The conventional chemical isomerization was carried out at the KOH-equivalent solution alkalinity corresponding to that measured in the electro-activated lactose (EA-lactose), electro-activated whey (EA-whey), electro-activated whey permeate (EA-WP) solutions at each 5 min interval using KOH powder as a catalyst at ambient temperature (22 ± 2 ℃). The results showed that the production of lactulose using the EA approach was current intensity-, solution concentration-, and reaction time-dependent. The highest lactulose yields of 38 (at 40 min for a 900 mA and 10% lactose solution), 32 (at 60 min for a 900 mA and 7% whey solution), and 36.98% (at 50 min for a 900 mA and 6% WP solution) were achieved for lactose, whey, and WP, respectively. Whereas the maximum lactulose yields of ~27 (at 60 min for 10% lactose solution) and 25.47% (at 50 min for 6% WP solution) were obtained for lactose and WP, respectively. However, no lactulose was produced for whey using the chemical process at the equivalent solution alkalinity as in the EA technique. The outcomes of this study revealed that the EA technology is a more efficient technique for the enhanced production of lactulose from lactose, whey, and WP compared to the convention chemical isomerization process. Thereafter, the feasibility of using electro-activated whey-based substrates including EA-

lactose, EA-whey, EA-WP as carbon sources to produce protein enriched biomass and valuable metabolites including organic acids (i.e., lactic, acetic, citric, and propionic acids) and biomolecules with aroma and flavor properties was studied using a mixed microbiota originated from whole kefir grains as a starter culture and a pure culture of *Kluyveromyces marxianus* ATCC 64884. Fermentation was performed for 96 h at 30 °C using both electro-activated (EA) and non-electroactivated (non-EA) substances of lactose, whey, and WP. The results showed that the EA-substrates achieved a higher biomass growth in a reduced fermentation time than their non-EA mediums using the kefir culture. The highest cell growth (6.04 g/L) was obtained for EA-whey after 72 h which was even 1.7-fold higher than a standard nutrition broth, the reinforced clostridial medium (RCM). Furthermore, EA-whey produced a maximum of 8.46, 3.97, 0.60, and 1.02 g/L of lactic, acetic, citric, and propionic acid, respectively. Moreover, EA-whey achieved the highest kefiran production of 2.99 g/L, followed by the whey (2.67 g/L), EA-WP (2.31 g/L), WP (1.88 g/L), RCM broth (1.42 g/L), EA-lactose (1.37 g/L), and lactose (0.91 g/L). The results also demonstrated that various aromatic volatile compounds were produced during the fermentation of EA-whey, which may increase the organoleptic characteristic/sensory quality of the fermented products. Nevertheless, *K. marxianus* also demonstrated a satisfactory biomass growth in all substrates used and EA-whey achieved a maximum biomass (4.23 g/L) at 96 h of fermentation followed by YM broth (4.85 g/L). The produced biomass had high protein and lipid content (24.43-57.83, and 15.44-25.64%) depending on the used substrates and fermentation conditions. Several major organic acids including lactic, acetic, citric, propionic acids were produced during the fermentation on all media, with significant differences between electro-activated and non-electro-activated substrates. Furthermore, *K. marxianus* produced various volatile aroma compounds with valued organoleptic properties. The YM-broth resulted in the lowest ethanol production (8.42 g/L at 48 h) while the highest ethanol was produced in the non-electro-activated whey (28.13 g/L at 48 h), followed by lactose (27.85 g/L at 48 h), EA-lactose (26.77 g/L at 36 h), WP (25.99 at 72 h), EA-WP (24.66 g/L at 36 h), EA-Whey (22.06 g/L at 48 h). Moreover, a maximum of 393.85 to 988.22 mg/L of 2-phenylethanol was achieved, depending on the substrates used. Therefore, the results of this work suggest that the EA technology can be an emergent sustainable technology for achieving dual objectives of prebiotic lactulose production and concurrent valorization of whey and its derivatives in

Kefir culture and *K. marxianus* driven bioprocesses to produce valuable metabolites for different applications including in food and feed industry. Thus, this knowledge is not only helpful to reduce the production cost of dairy industries, but also provide an eco-friendly alternative for the disposal of whey/WP as a part of integrated approach for complete valorization.

Keywords: Electro-activation; chemical isomerization; whey; whey permeate; electro-activated whey-based substrates; bioprocess and biproducts; Kefir microbiota; *Kluyveromyces marxianus*.

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Liste des abréviations, sigles, acronymes

AAB: Acetic acid bacteria

AAS: Atomic absorption spectrometry

AEM: Anionic exchange membrane

ANOVA: Analysis of the Variance

ATCC : American Type Culture Collection

BOD: Biological oxygen demand

CEM: Cationic exchange membrane

COD: Chemical oxygen demand

DC: Direct current

DI: De-ionized

EA: Electro-activation

EDTA: ethylenediaminetetraacetic acid

EA-WP: Electro-activated whey permeate

EA-Whey or EAW: Electro-activated whey

EPS: Exopolymeric substances (Extracellular polymeric substances)

FAMES: Fatty acid methyl esters

FDA: Food and Drug Administration

Fr: Froude number

GC-FID: Gas chromatography-flame ionization detector

GC-MS: Gas chromatography mass spectroscopy

GOS: Galactooligo saccharide

GRAS: Generally Regarded As Safe

HPLC: High Performance Liquid Chromatography

LAB: Lactic acid bacteria

LA: Lobry de Bruyn-Alberda van Ekenstein

MRPs: Maillard reaction products

OD: Optical density

ORP: Oxidation-reduction potential

PE: Phenylethanol

QPS: Qualified Presumption of Safely

RCM : Reinforced clostridial medium

SCP: Single cell protein

SCO: Single cell oil

UV: Ultraviolet

V/V: Volume/Volume

WHO: World Health Organization

W/V: Weight/Volume

WP: Whey permeate

YM: Yeast and Mold

Dédicaces

This thesis is profoundly dedicated…

To my loving father for earning an honest living for us and for supporting and encouraging me to believe in myself, and giving me the strength to move forward;

To my sweet mother for being a strong and gentle heart who taught me to trust ALLAH, believe in hard work, and that so much could be done with little;

To my brothers who have always given me the energy to continue;

To my sister, brother-in-law, and my beloved nephews and niece who have never stopped loving me;

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Avant-propos

This thesis is submitted to the Faculty of Graduate and Postdoctoral Studies of Université Laval to meet the requirements for obtaining the Doctor of Philosophy (Ph. D) degree in the Soil and Environment at the Faculty of Agricultural and Food Sciences. This doctoral thesis is composed of seven chapters and the results are presented in the form of scientific articles published or submitted in international peer-reviewed journals. Indeed, the research activities were carried out by the candidate with close collaboration and supervision from the research director and co-director.

The first chapter represents the literature review, which aims to provide the essential elements for a good understanding of the problematic of this doctoral thesis. This chapter published in 'International Dairy Journal' and 'International Journal of Food Microbiology' journals entitled "Production of prebiotic lactulose through isomerization of lactose as a part of integrated approach through whey and whey permeate complete valorization: A review" by Ahasanul Karim and Mohammed Aider and "*Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology" by Ahasanul Karim, Natela Gerliani and Mohammed Aider, respectively.

The second chapter presents the problematic and research gap, the hypothesis, and the objectives of the research. Most of the experimental work and the results obtained have been published or submitted for publication in appropriate scientific journals.

The third chapter presents the article entitled "Sustainable electroisomerization of lactose into lactulose and comparison with the chemical isomerization at equivalent solution alkalinity" published in 'ACS omega' by Ahasanul Karim and Mohammed Aider.

The fourth chapter presents another article entitled "Sustainable valorization of whey by electroactivation technology for in situ isomerization of lactose into lactulose: comparison between electroactivation and chemical processes at equivalent solution alkalinity" published in 'ACS omega' by Ahasanul Karim and Mohammed Aider.

The fifth chapter presents the third experimental article entitled "Contribution to the process development for lactulose production through complete valorization of whey permeate by using electro-activation technology versus a chemical isomerization process" published in 'ACS omega' by Ahasanul Karim and Mohammed Aider.

The sixth chapter presents the article entitled “Comprehensive utilization of electro-activated whey-based media in the cell growth, metabolites production and aroma compounds synthesis using a starter culture originated from kefir grain” submitted to ‘International Dairy Journal’ by Ahasanul Karim and Mohammed Aider and currently under review process (INDA-D-21-00474).

The seventh chapter presents the article entitled “Bioconversion of electro-activated lactose, whey and whey permeate to produce single cell protein, ethanol, aroma volatiles, organic acids and fat by *Kluyveromyces marxianus*” by Ahasanul Karim and Mohammed Aider is currently in submission process.

The last part constitutes a general conclusion including a summary of the main results obtained in the context of this research work and several perspectives, followed by the list of references and appendix. Ahasanul Karim is the first author of all the articles presented here above and was responsible for the planning, experimental design and interpretation of experimental work, analysis of results and writing of articles under the direct supervision of Professor Mohammed Aïder who was responsible for the scientific supervision of the project, experimental design, correction, revision and submission of manuscripts.

Introduction

The modern world is confronting with several pressing issues including wastewater generation, energy crisis, and global warming, etc. However, the environmental pollution through wastewater generation and depletion of energy are the most important issues for human society that cannot be avoided due to the urbanization, economic development, increasing population growth, and food production practices (Nayak et al., 2016, Chowdhary et al., 2018). Particularly, rapid industrialization combined with the population growth has led to huge biomass production. Recently, a report of World Bank has demonstrated that more than two billion tons of biomass residues including agricultural residues, food processing waste, etc. are produced every year globally, and this amount is expected to reach to 3.4 billion by 2050, representing a 70% increase (Sekoai et al., 2021). Valorization of biomass, e.g., food by-products into sustainable energy development/edible materials is one of the main interesting strategies of the 21st century and is being accelerated all over the world to combat the critical issues like environmental pollution, energy and food crisis, and depletion of fossil fuels (Arancon et al., 2013). In this context, the utilization of biomass residues/wastewater as suitable feedstocks (due to their high accessibility, reduced operational costs, and rich nutritional composition) to produce alternative value-added resources could be considered as an environmentally sustainable and economically viable approach (Abdel-Shafy and Mansour, 2018).

Whey and whey permeate (WP) are the main by-products in the cheese or casein production process. Whey is the liquid residue remaining after milk has been curdled and strained and WP is a co-product which is persisted after recovering whey proteins from liquid whey by ultrafiltration-diafiltration. The global production of whey is estimated to 190×10^6 ton/year, and Canada produces 4% of the total whey (Kaur et al., 2020, Ramos et al., 2021). In 2019 for example, Canada reported as one of the ten major cheese manufacturer countries, recording 5.2×10^5 tons of cheese (Valdez Castillo et al., 2021). Typically, a volume of ≈9 kg of whey is generated for the production of 1 kg of cheese (Park and Haenlein, 2013). Thus, it can be estimated that the production of whey was approximately 4.7×10^6 tons in Canada in 2019 (Valdez Castillo et al., 2021). According to the Quebec dairy council Inc., Canada produces about 372,000 tonnes of cheese annually, including 200,000 tonnes in Quebec, resulting in the production of 2,100,000 tonnes of whey in Canada (1,200,000 tonnes in

Quebec, which is more than 50%). The major constituents of whey/WP is lactose (and its derivatives), minerals, and other small molecules such as peptides and free amino acids (Yadav et al., 2015c). Discarding raw cheese whey or/and WP creates huge disposal problems because they have high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) with a value of 60,000-80,000 and 30,000-50,000 mg/L O_2, respectively, due to their high lactose concentration (Djouab and Aïder, 2019b). Dumping of whey on the arable land creates serious pollution concerns to the surrounding environment by modifying the physicochemical characteristics of soil and resulted in reduced crop yield. Furthermore, land spreading of whey may lead to the construction of different compounds or salt complexes in the soil, which can damage the soil features and influence the growth of plants (Ryan and Walsh, 2016). In addition, whey can reduce the redox potential of soil, winch results in the solubilisation of Mn and Fe present in the soil and thus, it may potentially contaminating ground water supplies (Ryan and Walsh, 2016). Nevertheless, discharging of whey into water bodies reduces dissolved oxygen, hinders biodegradability, and causes a huge risk to aquatic life, environment and human health (Ghaly et al., 2007b). Therefore, this remains a huge challenge for the dairy industries and researchers to develop economical and sustainable approaches of complete valorization of these industrial co-products, and that is why industries are multiplying different processes to produce various products with good commercial value utilizing these low cost co-products (Yadav et al., 2015c).

Whey/WP could be valorised into value-added ingredients since its high lactose content can be utilized as a cheap and readily available source in order to synthesis of non-absorbable lactose derivatives such as lactosucrose, lactitol, and lactobionic acids, etc. (Paseephol et al., 2008b). Seemingly, the industries are adopting various approaches to make the process economically viable, however, the commercial value of these valuable products remained low due to a complex and highly expensive process. Nowadays, the demand of lactulose production is increasing tremendously because of its bifidogenic (prebiotic) functionality with many applications in food, nutraceuticals, and pharmaceutical industries (Kareb and Aïder, 2018, Djouab and Aïder, 2019a). Currently, the commercial lactulose is produced through the chemical synthesis from lactose by following an isomerization reaction in an alkaline medium according to the Lobry de Bruyn-Alberda van Ekenstein (LA) transformation (Aider and de Halleux, 2007, Kareb and Aïder, 2018). This process is

generally characterized by huge challenge for low yield of lactulose, and subsequent by-products formation such as epilactose, galactose, glucose, isosaccharinic acid due to the high level of lactose degradation. However, presence of any side products are undesirable, especially for food, pharmaceutical, and medical applications (Kareb and Aïder, 2018). Recently, an emerging technology called electro-activation (EA) has been introduced and attracted particular attention for the isomerization of lactose into lactulose without adding any alkalinizing chemicals (Aider and Gimenez-Vidal, 2012, Kareb and Aïder, 2018). The mechanism of the EA process is based on the self-generating acid and alkaline conditions following electrolysis of water molecules at the solution/anode and solution/cathode interface, respectively (Aissa and Aïder, 2013a). Although a higher yield of lactulose was reported by the EA technology in some recent studies compared to the other methods (Djouab and Aïder, 2019b, a), this is imperative to perform a comparative study of different methods for a better comprehension of selecting an efficient method.

Furthermore, whey/WP can be valorized into value added products by utilizing these low-cost by-products as carbon and nitrogen sources for microbial assimilation (Yadav et al., 2015c). The microbial conversion of whey/WP to valuable fermented metabolites such as organic acids, enzymes, volatile flavour substances, and single-cell proteins could be a sustainable approach for whey management and valorization (Kaur et al., 2020), which can be a simple approach of treatment for increasing the net value of food industry co-products (Gardner, 1989, Siso, 1996). However, raw whey/WP is a tricky-type of waste by-product to handle, which can be quickly acidified because of its high organic content with inadequate bicarbonate alkalinity (Seo et al., 2014a). Several recent studies (Kareb et al., 2017c, Kareb and Aïder, 2018) demonstrated that the electroactivation of whey/WP could convert lactose into lactulose and other simple sugars such as galactose, glucose, and fructose at a desired alkaline condition, which in turn may promote microbial growth by ease assimilation of readily available monosaccharides and prebiotic lactulose (Kareb et al., 2017c). Therefore, it is postulated that the challenges of raw whey utilization could be defeated by using electro-activated whey and/or WP (EA-whey/WP) to produce value-added metabolites via fermentation. In the fermentation process, different microorganisms including pure cultures and mixed culture consortia were used to produce various fermented products using whey-based substrates (Yadav et al., 2015a, Carota et al., 2017a, Kareb et al., 2018a). The mixed

cultures are regarded robust inocula because they are able to utilize wide range of substrates, more tolerant to the environmental fluctuations, easy to obtain in nature, applicable in large scale, and less susceptible to contamination (Karim et al., 2018a, Islam et al., 2020). In contrast, a pure culture may react much differently in cultivation media than when it is combined with other species. Thus, it is also vital to understand how an individual microbe may contribute to produce a specific metabolite in the fermentation medium.

The objective of this thesis was to develop a process for producing prebiotic lactulose through isomerization of lactose *in situ* of whey/WP using the EA technology as a part of integrated approach for complete valorization of whey/WP without any fractionation and/or upstream and/or downstream treatment to combat its environmental negative impact. The end-product, lactulose is a high value-added prebiotic with antioxidant properties that can be used as an additive in industries food and nutraceuticals. To do this, the effect of different physicochemical parameters such as pH, alkalinity, temperature, ion migration, and oxidation-reduction potential on the conversion of lactose into lactulose was correlated with the lactulose formation rate to understand the involved process mechanism of action. Specifically, this study was intended to compare the efficiency of lactose isomerization into lactulose between the EA process and the chemical method based on the equivalent solution alkalinity using lactose, whey, and WP as substrates for the isomerization process. Nevertheless, this work was envisioned to explore the feasibility and viability of a novel fermentation process by utilizing several electro-activated whey-based substrates including EA-lactose, EA-whey, and EA-WP as the potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids, exopolysaccharides, and volatile compounds using a mixed culture originated from kefir grain as a starter culture and a pure nonconventional dairy yeast, *Kluyveromyces marxianus*.

Chapitre 1: Literature review

1.1 Production of prebiotic lactulose through isomerization of lactose as a part of integrated approach for whey and whey permeate complete valorization: A review

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RESUMÉ

Le lactosérum, un sous-produit de fabrication de fromages et de caséine, est généralement considéré comme un polluant environnemental en raison de sa charge organique élevée, résultant principalement des nutriments résiduels du lait. La production de lactosérum augmente progressivement en raison de la forte demande de produits dérivés du lait. Cela crée un énorme problème d'élimination pour les industries laitières et exige des solutions respectueuses de l'environnement. Dans ce contexte, plusieurs efforts ont été déployés pour transformer le lactosérum en produits à valeur ajoutée; notamment la poudre de lactosérum, les protéines de lactosérum, la poudre de perméat de lactosérum, le bioéthanol, les biopolymères, les bioprotéines, l'électricité, les enzymes, le biohydrogène, les acides organiques et les prébiotiques. Plus précisément, la conversion du lactosérum en produit contenant de la lactulose, un disaccharide prébiotique, pourrait être avantageuse compte tenu de sa teneur élevée en lactose. La demande mondiale de production de lactulose est d'un grand intérêt de nos jours en raison de son effet bifidogène avec diverses applications dans l'industrie pharmaceutique, nutraceutique et alimentaire. Traditionnellement, le lactosérum ou le perméat de lactosérum est transformé en lactulose par une réaction d'isomérisation en suivant une méthode enzymatique ou chimique. Récemment, l'électro-isomérisation du lactose par la technologie d'électro-activation (EA) est apparue comme une approche potentiellement économique et respectueuse de l'environnement. Dans le but de mieux comprendre la synthèse du lactulose grâce à la valorisation durable du lactosérum, cette revue fait la synthèse des techniques d'isomérisation couramment utilisées ainsi que leurs limites d'utilisation. Tout d'abord, une étude descriptive et complète est présentée pour expliquer les caractéristiques du lactosérum et son potentiel pour la production de différents bioproduits dérivés. Par la suite, l'efficacité de la technologie d'électro-activation en solution, comme approche émergente par comparaison aux techniques conventionnelles, pour produire du lactulose grâce à l'utilisation efficace du lactosérum est discutée. Ainsi, cette revue propose de fournir une compréhension plus large pour utiliser la technologie d'électro-activation comme approche efficace pour la production de lactulose grâce à la valorisation intégrale du lactosérum.

Mots-clés: Valorisation du lactosérum; Production de lactulose; Isomérisation chimique; Méthode enzymatique; Électro-activation.

ABSTRACT

Cheese whey, a by-product of casein or cheese manufacturing process, is usually considered as an environmental pollutant due to its elevated organic load, mostly results from the residual nutrients of milk. Whey production is gradually increasing because of the high demand of milk-derived products. It creates a huge disposal problem for dairy industries and demands eco-friendly solutions. In this context, several efforts have been carried out to transform whey into added value products including whey powder, whey proteins, whey permeate, bioethanol, biopolymers, bioproteins, electricity, enzymes, biohydrogen, lactic acids, and prebiotics. Specifically, the conversion of whey into prebiotic lactulose could be advantageous considering its high contents of lactose. The demand of lactulose production is of great interest nowadays due to its bifidogenic functionality with various applications in the pharmaceutical, nutraceuticals, and food industries. Traditionally, whey or whey permeate is transformed into lactulose through an isomerization reaction by following either an enzymatic or a chemical method. Recently, electro-isomerization of lactose by electro-activation (EA) technology has been emerged as an economic and environmentally friendly approach. With an intention to gain deeper insights into lactulose synthesis through sustainable valorization of whey, this review complies commonly practiced isomerization techniques along with their limitations. First, a descriptive and comprehensive survey is presented to explain the characteristics of whey and its potential for producing different bioproducts. Thereafter, the efficacy of a potential nonconventional technology, i.e., EA over traditional techniques to produce lactulose through the effective utilization of whey and their process mechanisms are reviewed. Thus, this review argues to deliver a broader understanding to utilize an effective technique for lactulose production through valorization of whey.

Keywords: Whey valorization; Lactulose production; Chemical isomerization; Enzymatic method; Electro-activation.

1.1.1 Introduction

Food processing industries, such as the dairy and cheese manufacturing plants, produce a huge volume of liquid waste including cheese whey. The whey is a liquid stream that is generated as a byproduct during the coagulation of milk casein or cheese making processes. Production of cheese whey is gradually rising due to the increasing demand of milk and milk derived products, showing around 2% growth rate per annum. Notably, the production of only one kilogram of cheese creates about nine kilograms of whey (Djouab and Aïder, 2019a, Schmidt et al., 2020). Global production of cheese whey was estimated at about 180-190 × 10^6 tons per year, and 50% of which is simply discarded as dairy effluent (Mollea et al., 2013, Yadav et al., 2015c). The production of this huge volume of whey postures a serious problem in the environment because of its high organic load including a high chemical (~60,000-80,000 mg/L) and biochemical (~30,000-50,000 mg/L) oxygen demand (Djouab and Aïder, 2019b). Therefore, the development of sustainable and eco-friendly ways is certainly required to mange this large volume of whey for environmental and economical resilience.

Various technical attempts have been made to transform whey into added valu products for minimizing the disposal problem. These technological advancements have accelerated the utilization of whey and about 50% of total production is being processed into different vale-added products including whey protein, whey powder, whey permeate (WP), bioprotein, biopolymers, bioethanol, hydrogen, methane, electricity, and probiotics. Notably, whey is However, since the introduction of ultrafiltration techniques in the cheese industries to produce whey-concentrates, whey is more valued mainly for the nutritional benefits of the proteins recovered (Sabater et al., 2017b, Djouab and Aïder, 2019b). The whey protein isolates, or concentrates can be simply used in the food industries due to their nutritional value and inclusive functionality; while the remaining co-product of this process, WP has so far been limited applications. Generally, WP is dried to sell as powder for certain applications as feed additive. Moreover, WP and/or whey have been used as raw material to produce lactose using cold crystallization of saturated solutions after the purification from N-containing molecules and minerals. However, the methods of drying, demineralization and purification make the process costly, that lead to a low commercial value of the end-product. Besides, utilization of lactose in foods is limited because of its low solubility and intolerance.

Thus, lactose is utilized as raw material for producing several costly derivatives including lactitol, mannitol, lactosyl urea, lactosucrose, sorbitol, lactobionic acid, and so on (Yadav et al., 2015c). Seemingly, the industries are adopting various approaches to make the process economically viable, however, the commercial value of these valuable products remained low due to a complex and highly expensive process.

In recent time, the production of lactulose is of great interest due to its bifidogenic (prebiotic) functionality with various applications in the food, pharmaceutical, and nutraceuticals industries (Panesar and Kumari, 2011b, Kareb and Aïder, 2019). Notably, cheese whey can potentially be valorized into lactulose (4-O-β-d-galactopyranosyl-d-fructose) as it contains high lactose content (> 70% on a dry mater basis) (Paseephol et al., 2008b, Djouab and Aïder, 2019a). Lactulose is a disaccharide comprised with galactose and fructose, that can be created through isomerization of lactose (galactose and glucose). It is considered as a prebiotic carbohydrate, which promotes the activity of health promoting bacteria in gastrointestinal tract, e.g., *Bifidobacteria* and *Lactobacilli*. Concurrently, it can also impede the growth of pathogenic bacterial like *Salmonella*. Furthermore, it is used for the treatment of hepatic encephalopathy, constipation, maintain blood glucose and insulin level, and tumour prevention, etc. (Nooshkam and Madadlou, 2016). Raw whey or WP could be directly used as feed medium for producing lactulose by *in situ* isomerization reaction of lactose, which might be advantageous not only from nutritional but also from economic pint of view (Yadav et al., 2015c, Karim and Aïder, 2020). Thus, under bio-economic aspects, the isomerization of lactose *in situ* of whey is of a great research interest for a large-scale production of prebiotic lactulose. Traditionally, it is produced through chemical isomerization of lactose under strong alkaline conditions involving catalysts and elevated heat (Montgomery and Hudson, 1930b, Speck Jr, 1958a). This method is costly due to the low process efficacy resulted from the requirement of several purification and concentrating steps (Aider and Gimenez-Vidal, 2012, Seo et al., 2015b). To overwhelmed the limitations of chemical method, electroactivation (EA) technology has recently been revealed to transform lactose into lactulose in adequately designed reactor following water electrolysis at the solution-cathode interface (Karim and Aider, 2020c, a).

In this review, the prospect of whey as a feed medium for lactulose production is reviewed as a part of integrated solutions to valorize cheese whey. A comprehensive insight

into the process mechanism and challenges of current production practices of prebiotic lactulose is also discuss with an aim to gain directions for further improvement in future application strategies.

1.1.2 Whey characteristics and its waste burden

Whey is a yellow-green liquid by-product of casein or cheese manufacturing process,, typically comprising of water (≈93-94%), lactose (≈4-5%, w/v), soluble proteins (≈0.6-0.8%, w/v), lipids (≈0.4-0.5%, w/v), and mineral salts (≈0.8-1.0%, w/v) (Djouab and Aïder, 2019a, Schmidt et al., 2020). Mostly, it contains 5-8% (w/w) solids in which 60-80% is characterised by lactose, 10-20% by proteins, and 12-15% by minerals (Parashar et al., 2016a, Ryan and Walsh, 2016). Depending on the production process, two types of whey are generated, namely acid whey and sweet whey (**Figure 1.1**). The sweet whey is created from rennet coagulation of milk during hard and soft cheese production process, like Cheddar or Swiss cheese. In contrast, the acid whey is generated as a co-product of fresh acid coagulated milk associated with Cottage cheese and Greek style yogurt production process (Chandrapala et al., 2016). However, the sweet whey is the most widely produced whey.

Generally, the physicochemical characteristics and constituents of acid and sweet whey are quite similar (**Table 1.1**), and is composed of lactose (70%), proteins (14%), minerals and some residual fats. Depending on the acidity, mineral contents, and whey protein fractions, these two types of whey can be distinguished. Acid whey comprises of relatively superior mineral concentration compared to the sweet whey due to the colloidal calcium phosphate of casein micelles during the acidification process (Lievore et al., 2015). On contrary, the sweet whey contains glycomacropeptide fraction produced via enzymatic hydrolysis of κ-casein. Furthermore, free amino acid content in the acid whey is ten times higher than in the milk, whereas four times higher in the sweet whey (Kareb and Aïder, 2018).

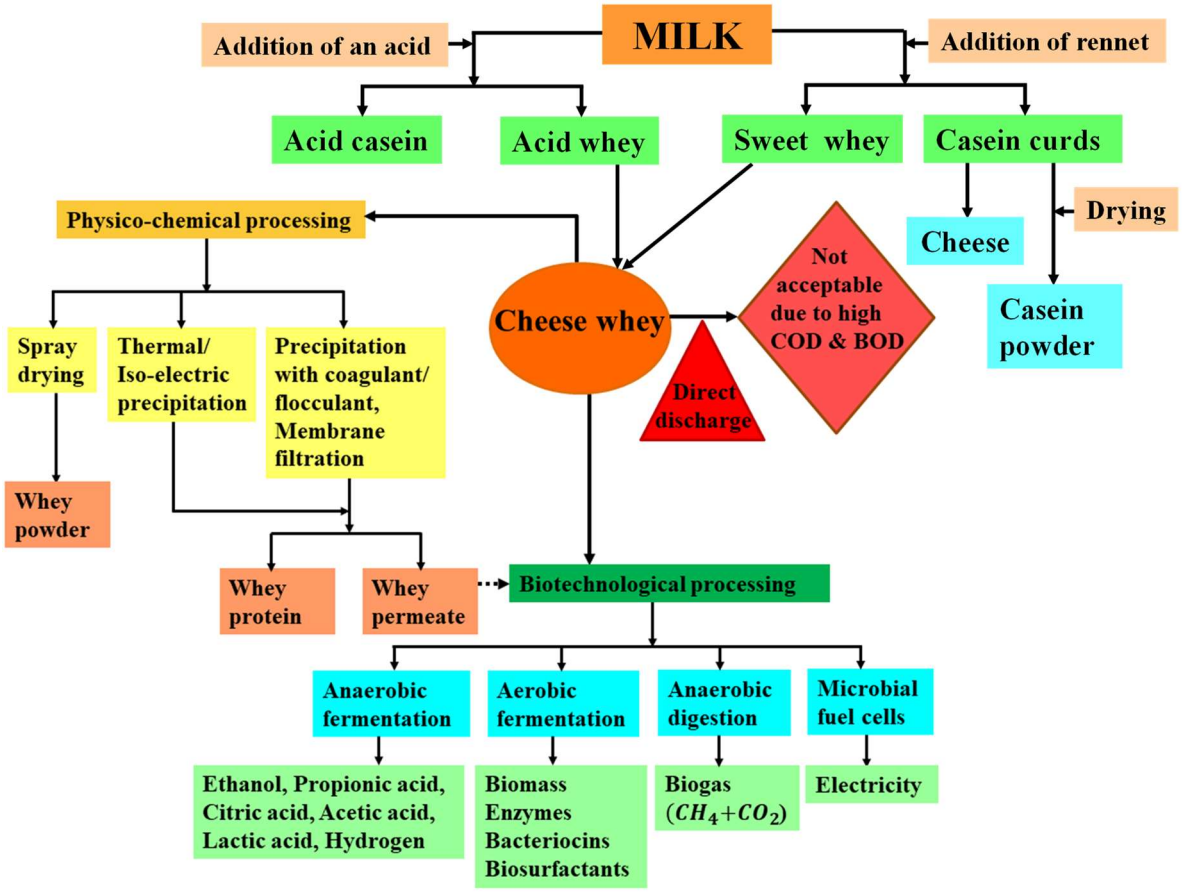

Figure 1.1: Overview of the whey generation and its processing by physico-chemical and biological approaches (Yadav et al., 2015c, Ryan and Walsh, 2016).

Table 1.1: Typical composition of acid whey and sweet whey. Adopted from (Panesar et al., 2013).

Ingredients	Acid whey (g/L)	Sweet whey (g/L)
Total solids	63.0-70.0	63.0-70.0
Lactose	44.0-46.0	46.0-52.0
Protein	6.0-8.0	6.0-10.0
Fat	0.4	5.0
Lactate	6.4	2.0
Ash	8.0	5.0
Calcium	1.2-1.6	0.4-0.6
Phosphate	2.0-4.5	1.0-3.0
Chloride	1.1	1.1

Whey comprises almost all necessary nutrients that present in the milk, including vitamins, minerals, proteins, and mainly lactose. In dairy industries, they are usually considered as the unutilized waste residue. Although a potion of whey is utilized in the animal feed and food industries, and transformed into valuable products, which is only about half of the total whey production (Carvalho et al., 2013). Classically, the proteins are collected from whey and the remaining whey comprising lactose is disposed off after separation of whey proteins. Discarding of this whey could lead to huge environmental challenges as the lactose content in the whey is largely accountable for high COD and BOD (Carvalho et al., 2013).

Dumping of whey on the arable land creates serious pollution concerns to the surrounding environment by modifying the physicochemical characteristics of soil and resulted in reduced crop yield. This is because the breaking down of milk proteins and sugars of whey causes rapid consumption of oxygen in the soil. Furthermore, land spreading of whey may lead to the construction of different compounds or salt complexes in the soil, which can damage the soil features and influence the growth of plants (Ryan and Walsh, 2016). Nearly

400-500 kg of salt per hectare could be added to the soil for each millimetre (10^3/ha) of whey, which in turn may cause soil salinity and reduce crop yield. Moreover, the low pH (≈4.5) of acid whey can also severely affect the soil (Ghaly et al., 2007a). In addition, whey can reduce the redox potential of soil, which results in the solubilization of Mn and Fe present in the soil and thus, it may potentially contaminating ground water supplies (Ryan and Walsh, 2016). Nevertheless, discharging of whey into water bodies reduces dissolved oxygen, hinders biodegradability, and causes a huge risk to aquatic life, environment and human health (Ghaly et al., 2007b). A spillage of acid whey to a water body in Ohio, US in 2008 destroyed more than 5,400 wild animals, mainly fish in the water body because of the oxygen depletion leading to eutrophication (Ryan and Walsh, 2016). Moreover, Janczukowicz et al. (2008) reported that the degradability of whey components is difficult because it contains complex components and create a key problem to any wastewater treatment plant that treats other effluents. Thus, proper management practices for treating or reusing of whey is essentially required before its disposal which would be ecofriendly and economical.

1.1.3 Valorization of whey into value-added products

Although lactose is the main component contributes to high organic load, there are other responsible factors of residual milk including nutrient proteins, lipids, and vitamins. The presence of various whey nutrients either inorganic or organic is regarded as potential resources to produce different value-added products. Thus, different approaches have been studied to address the whey management issue. Generally, there are two processes to convert whey into added value products (**Figure 1.1**). First, direct processing (e.g., physical/thermal treatment) of whey to produce whey protein isolates, whey powder, whey proteins concentrate, WP, lactose, and other fractions. Second, biotechnological processing of whey to obtain value added end products such as single cell protein (SCP)/bioproteins, animal feed, probiotics, organic acids, enzymes, exopolysaccharides, bioplastics, carotenoids, bio-preservatives, biological gums, etc. using whey as a substrate for various enzymatic or microbial processes (Panesar and Kennedy, 2012, Mollea et al., 2013). Recently, these kinds of products and the methods of production have been confirmed technically feasible because whey contains vitamins and minerals which could improve the physiological activity of different microorganisms to produce valuable food ingredients (Yadav et al., 2014a, Yadav et al., 2015c). However, more research is required to elucidate the process mechanisms and

to make the products even further economically viable. In addition, it is especially imperative to choose a suitable strain with appropriate physiological characteristics to attain a decent consumption of lactose from the whey.

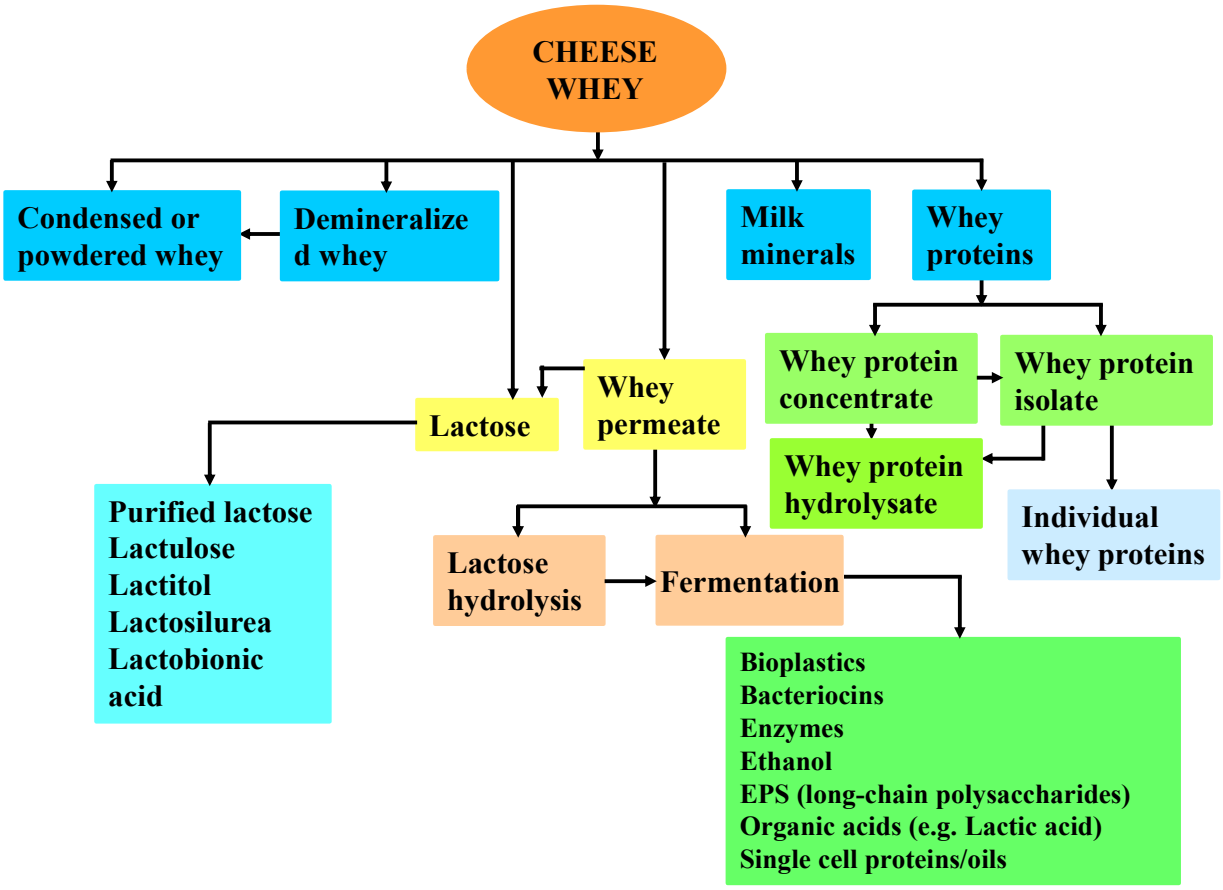

Figure 1.2: Overview of different valuable products from whey.

The concept of whey utilization has recently been switched from being a byproduct to coproducts resource for producing different added value products. Whey proteins and lactose distinguished to show diverse functional, nutritional, and physiological features, which make them suitable for versatile applications (**Table 1.2**). Whey proteins have attained great interest to valorize due to their particular properties (e.g., nutritional, digestive, etc.) and a prospective source of bioactive peptides having a broad range of health benefits (Oriach et al., 2016). Specific protein fractions could be useful to induce sleep and to control blood pressure. Moreover, it has many applications in the pharmaceutical industry (Korhonen, 2009). Therefore, the demand of whey proteins is increasing day by day because of their high

nutritional and functional values. However, the conversion of whey to the whey protein concentrates generates a huge volume of lactose fraction, commonly known WP, that needs further processing to make the whole process techno-economically feasible (Peters, 2005). Consequently, the production of whey protein concentrate as the only product is considered as nonprofitable. However, the value of whey protein isolates probably be enhanced through enzymatic hydrolysis of proteins to non-allergenic peptide fractions. Additionally, whey proteins can be converted into bioactive peptides by enzymatic or fermentation processes. Thus, an imperative step of cheese whey processing is the recovery of protein fraction and transformation of WP (lactose fraction) into various beneficial products (**Figure 1.2**) including enzymes, ethanol, biopolymer, biogas, lactic acid, kefir like whey drinks, SCP, probiotics, etc. (Koutinas et al., 2009, Panesar and Kennedy, 2012, Mollea et al., 2013).

Table 1.2: Typical constituents of various whey-derived products. Adopted from (Yadav et al., 2015c).

Products	**Major components (%)**				
	Protein	**Lactose**	**Fat**	**Ash**	**Moisture**
Acid whey powder	11.0-13.5	61.0-70.0	0.5-1.5	9.8-12.3	3.5-5.0
Sweet whey powder	11.0-14.5	63.0-75.0	1.0-1.5	8.2-8.8	3.5-5.0
Dematerialized whey	11.0-15.0	70.0-80.0	0.5-1.8	1.0-7.0	3.0-4.0
Reduced lactose whey	18.0-24.0	52.0-58.0	1.0-4.0	11.0-22.0	3.0-4.0
Whey protein isolate	90.0-92.0	0.5-1.0	0.5-1.0	2.0-3.0	~4.5
Whey protein concentrate-80	80.0-82.0	4.0-8.0	4.0-8.0	3.0-4.0	3.5-4.5
Whey protein concentrate-75	75.0-78.0	10.0-15.0	4.0-9.0	4.0-6.0	3.0-5.0
Whey protein concentrate-60	60.0-62.0	25.0-30.0	1.0-7.0	4.0-6.0	3.0-5.0
Whey protein concentrate-50	50.0-52.0	33.0-37.0	5.0-6.0	4.5-5.5	3.5-4.5
Whey protein concentrate-34	34.0-36.0	48.0-52.0	3.0-4.5	6.5-8.0	3.0-4.5

Among the others, bioconversion of WP into galacto-oligosaccharides and protein rich biomass could be a viable option (Panesar and Kennedy, 2012). Nevertheless, the bioconversion of whey (mostly lactose) into microbial biomass can be advantageous as it is also a simple wastewater treatment process (i.e., removal of COD). The final disposal of the treated whey may be more ecofriendly because the pollutant load is substantially diminished. However, the production of SCP or bioprotein can be carried out using only lactose consuming microbes, particularly, yeasts such as *Kluyveromyces* spp. and bacteria like *Lactobacillus* spp. (Panesar et al., 2013, Karim et al., 2020b). Non-lactose consuming microbes such as *Candida* spp. could be utilized to produce SCP via hydrolysis of lactose into monosaccharides (glucose and galactose).

On the other hand, the problem with direct utilization of lactose from milk or whey has been described in different literatures (Siso and Doval, 1994, Caballero et al., 1995). There are many people, even whole populations, who cannot hydrolyse lactose due to the lack of *β*-galactosidase in their digestive tract. The problem is mostly related to the older people. This fact limited the applicability of whey/lactose in food or feed. Consequently, the hydrolysis of whey lactose by the enzyme lactase to glucose and galactose could be an alternative approach to utilize whey (Siso and Doval, 1994). The hydrolysis of lactose is very useful for the improvement of production process for ice cream or/and baby food. It can be also interesting for the production of additives for human and animal food from whey (Caballero et al., 1995). Furthermore, the transformation of lactose content into several bioactive products including lactulose, lactobionic acid, lactosucrose, and other derivatives is one of the most attractive approach for whey valorization. Thus, it is apparent that industry is multiplying various methods make the process economically viable through the production of various products with relatively good commercial value. Considering the inevitability to explore other non-traditional aspects of whey valorization, the utilization of whey or WP as a whole component as feed medium for producing prebiotic lactulose through *in situ* isomerization of whey lactose might be advantageous from nutritional as well as economical point of view (Yadav et al., 2015c).

1.1.4 Lactulose production from lactose present in the whey

Lactulose is one of the best precious derivatives of lactose and retains prebiotic properties. Therefore, the demand of the lactulose production continuously increasing since

its discovery in 1930 by Montgomery and Hudson (1930b) due to its applications in several fields of food and pharmaceuticals industries (**Table 1.3**). This is a synthetic disaccharide composed of fructose and galactose moieties connected with a 1-4 β-glyosidic linkage. From chemical point of view, lactulose can be synthesis through the isomerization in an alkaline medium mainly based on the molecular re-arrangement of lactose according to Lobry de Bruyn-Alberda van Ekenstein (LA) transformation. Under the LA rearrangement, aldose (glucose) epimerised to ketose (fructose), and the ketose reacts with galactose to form lactulose in the alkaline media (Montgomery and Hudson, 1930b, Speck Jr, 1958a). The commercial lactulose is mostly produced using the chemical isomerization process up to now. Another approach is the use of enzymes like β-galactosidases through the transgalactosylation reaction with lactose and fructose as substrate (Sitanggang et al., 2014, 2016b). Although the chemical and enzymatic methods are most common methods of lactulose production, the EA has been introduced as an efficient approach in the last one decade. In the recent studies, it was recognized as a safe, reagent-less, and economical process to synthesis lactulose from lactose via sel-generating of required alkaline conditions in the reaction medium (Aider and Gimenez-Vidal, 2012, Kareb and Aïder, 2018).

Table 1.3: Application of lactulose in various field of food and pharmaceutical industries.

Area	Applications	References
Food industry	-Used as a bifidus factor or as a functional ingredient for intestinal regulation. -Applied for beneficial modification to food favour and physicochemical characteristics for making useful to consumers health. -Can be utilized as a sweetener for diabetics as a sugar alternative in confectionery goods, bakery products, beverages, yoghurts, infant milk powders, dairy desserts and in many liquid or dried food preparations, etc. -Can be employed for improving flavour improving properties, favourable browning performance, enhancing solubility in water, etc. in food products. -Can be used to enhance the survival of available probiotic strains in yoghurt.	(Strohmaier, 1998, Tabatabaie and Mortazavi, 2008, Panesar and Kumari, 2011b)
Pharmaceutical industry	-Utilized for the treatment of constipation and hepatic encephalopathy, tumour prevention and immunology, impediment of liver disease, *Salmonella* carrier, inflammatory bowel disease, anti-endotoxin effects, colon carcinogenesis, maintain blood glucose and insulin level, etc.	(Schumann, 2002, Panesar and Kumari, 2011b)

1.1.4.1 Chemical synthesis of lactulose

Production of lactulose using a chemical method is usually carried out through the LA re-arrangement in an alkaline media. In this process, a substantial amount of catalysts such as sodium hydroxide, magnesium oxide, calcium hydroxide, sodium aluminate, potassium hydroxide and carbonates, and tertiary amines have been used(Aider and de Halleux, 2007, Seo et al., 2016b). The low yield of lactulose and formation of by-products limited these processes since homogeneous catalysis is mostly associated with huge challenge of catalysts removal at the end of the process (Aider and Gimenez-Vidal, 2012). Furthermore, a significant amount of catalysts is required to achieve the high yield which is not economically feasible since it leads to an extensive separation and purification steps, subsequently, increase the production cost. In addition, the formation of side products is not desirable for applications in the food, pharmaceutical, and medical (Kareb and Aïder, 2018). On the other hand, heterogeneous catalyst such as eggshell powder, oyster shell powder, zeolites, and sepiolites have been employed to avoid the pitfalls associated with homogeneous catalysis. Although these catalysts can be easily removed by centrifugation, they are also less appealing due to the low reaction yield (Sitanggang et al., 2014). The yield of isomerization of lactose into lactulose could be increased using several complexing reagents such as borates and aluminates with least secondary reactions, however, they are considered incompatible with food grade lactulose from the industrial viewpoint due to the high toxicity even at low concentrations and complexity of eliminating the borate and aluminate at the end (Zokaee et al., 2002, Sitanggang et al., 2016b). **Table 1.4** shows the production yield of lactulose by using different chemical catalysts.

Table 1.4: Lactulose production by using different chemical and enzymatic catalysts.

Methods	Catalysts	T, (°C)	pH	Incubation time, (min)	[Substrate], (%, w/v)	Y, lactulose to lactose, (%)	Yield, (g/L/h)	Reference
Chemical catalysis	Calcium hydroxide	35	-	2160	-	~ 30	-	(Montgomery and Hudson, 1930b)
	Boric acid with triethylamine	70	11	240	-	87	-	(Hicks and Parrish, 1980)
	Sodium hydroxide and sodium sulphite	75-80	-	-	-	30	-	(Carobbi et al., 1985)
	Boric acid	70	11	Continuous production	20	~ 75	-	(Kozempel et al., 1995)
	Sodium hydroxide and boric acid	70	11	120	10	77-80	-	(Zokaee et al., 2002)
	Natural sepiolite	100	6.2 6.5	1	5	20.8	-	(Troyano et al., 1996)
	Alkaline sepiolite	90	8	60	5	20	-	(de la Fuente et al., 1999)

Methods	Catalysts	T, (°C)	pH	Incubation time, (min)	[Substrate], (%, w/v)	Y, lactulose to lactose, (%)	Yield, (g/L/h)	Reference
	Sepiolite with alkaline ions	90	-	150	-	20	-	(Villamiel et al., 2002)
	Sodium carbonate Sodium bicarbonate	60	10	3	10	18.75	-	(Moreno et al., 2003)
	Egg shell	98	-	60	-	1.18	-	(Montilla et al., 2005)
	Calcium carbonate	96	-	120	-	18-21	-	(Paseephol et al., 2008b)
	Sodium hydroxide	70	11	60	10	25.4	-	(Hashemi and Ashtiani, 2010a)
	Sodium carbonate	90	-	20.41	10	29.6	-	(Seo et al., 2015b)
	Ammonium carbonate	97	-	28.44	Cheese whey (4-4.5% lactose)	29.6	-	(Seo et al., 2016b)
	Sodium aluminate	60	12	50	35%	85.45	-	(Wang et al., 2017)

Methods	Catalysts	T, (°C)	pH	Incubation time, (min)	[Substrate], (%, w/v)	Y, lactulose to lactose, (%)	Yield, (g/L/h)	Reference
Enzymatic catalysis	*β*-galactosidase from *Saccharomyces fragilis*	37	7.2	-	$C_{Lactose}$ 12 $C_{Fructose}$ 20	7.5	-	(Vaheri and Kaupinnen, 1978)
	Permeabilized cells of *Kluyveromyces lactis*	40	7.0	180	$C_{Lactose}$ 40 $C_{Fructose}$ 20	-	6.8 g/L/h	(Lee et al., 2004)
	β-galactosidase (*Aspergillus oryzae*)	-	-	-	$C_{Lactose}$ 3.4 $C_{Fructose}$ 27	30 mmol/L (30% relative to lactose) for *A. oryzae*	16 mmol/L /h for *A. oryzae*	(Mayer et al., 2004)
	Hyperthermostable *β*-glycosidase from *Pyrococcus furiosus* (CelB)	-	-	-	-	46 mmol/L (44% relative to lactose) for *Pyrococcus furiosus* CelB	24 mmol/L /h *Pyrococcus furiosus* CelB	(Mayer et al., 2004)
	β-galactosidase (*Sulfolobus solfataricus*)	80	6.0	360	$C_{Lactose}$ 40 $C_{Fructose}$ 20	-	8.3 g/L/h	(Kim et al., 2006)

Methods	**Catalysts**	**T, (°C)**	**pH**	**Incubation time, (min)**	**[Substrate], (%, w/v)**	**Y, lactulose to lactose, (%)**	**Yield, (g/L/h)**	**Reference**
	β-galactosidase (*A. oryzae*)	40	6.5	-	$C_{Lactose}$ 20 $C_{Fructose}$ 15	-	-	(Adamczak et al., 2009)
	Immobilized thermostable *β*-glycosidase (*P. furiosus*)	75	5.0	Continuous production	-	-	52 g/L/h (Amberlite IRA-93) 15 g/L/h (E upergit® C)	(Mayer et al., 2010)
	β-galactosidase (*K. lactis*)	-	-	-	40	7.7	-	(Shen et al., 2012)
	β-galactosidase (*K. lactis*)	47	7.5	-	$C_{Lactose}$ 40 $C_{Fructose}$ 20	3.95	1.32 mg/U·h	(Song et al., 2013c)
	Commercial β-galactosidase (*K. lactis*)	40	7.5	-		3.2	2.88	(Hua et al., 2013)

Methods	**Catalysts**	**T, (°C)**	**pH**	**Incubation time, (min)**	**[Substrate], (%, w/v)**	**Y, lactulose to lactose, (%)**	**Yield, (g/L/h)**	**Reference**
	β-galactosidase from *Lactobacillus acidophilus* NRRL 4495	-	6.6	420	$C_{Lactose}$ 40 $C_{Fructose}$ 20	6.25	-	(Hashem et al., 2013)
	Commercial β-galactosidase (*K. lactis*)	40	6.7	-	$C_{Lactose}$ 12 $C_{Fructose}$ 36	8.66	4.15	(Khatami et al., 2014)
	β-galactosidase (*A. oryzae*)	40	4.5	-	50	28.2	-	(Guerrero et al., 2011)
	β-galactosidase (*K. lactis*)	-	-	Continuous production	50	5.95	-	(Sitanggang et al., 2015)
	β-galactosidase (*A. oryzae*)	-	-	Continuous production	50	5.47	-	(Sitanggang et al., 2016a)

1.1.4.2 Enzymatic synthesis of lactulose

In enzymatic method, lactulose is mainly produced via transgalactosylation of lactose using fructose as galactosyl acceptor and *β*-galactosidase or *β*-glycosidase as biocatalysts (Sitanggang et al., 2016b). Although there are two suitable classes of enzyme such as glycosyltransferses and glycosidases which are capable of catalyzing lactulose synthesis, the glycosidases (e.g., *β*-galactosidase) are mostly used because they are relatively inexpensive, commercially available, and widely employed in the food industry (Hua et al., 2010). This enzyme can be acquired from several sources including plants, animal, and microorganisms; however, the properties significantly vary based on their sources of origin. Some microbial *β*-galactosidases are of higher technological and commercial value than the enzymes of animals and plants sources because of some advantages like higher multiplication rate, easy handling, high production yield, etc. In lactulose production process, free *β*-galactosidases enzyme as well as whole microbial cell (Cardoso et al., 2017) and immobilized form could be used (Panesar and Kumari, 2011b). The performance of reactor in the enzymatic process could be effected by molar ration of feed substrate, temperature, pH, inlet concentrations of sugar substrates, enzyme loading, and feed flow rate, etc. (Ubilla et al., 2020).

In this process, the probability of using whey or WP as a feedstock is relatively higher, while the chemical process required high purity lactose to avoid the formation of by-products in order to reduce the possibility of secondary reactions (Cardelle-Cobas et al., 2016). Nevertheless, the enzymatic production of lactulose could be an appropriate alternative to defeat the drawbacks of chemical synthesis as it is usually performed under mild reaction conditions. Therefore, the possibility of the side product formation is reduced and subsequently, it simplifies the purification steps and provides high purity final products (Kareb and Aïder, 2018). Still, the key challenges are the lack of reaction selectivity and the low yield depending on the microbial source of enzyme catalysts (Shen et al., 2016). Most of the studies of the enzymatic synthesis of lactulose through transgalatosylation showed the maximum yields of 5-15% only (**Table 1.4**). Another major disadvantage of this method is the requirement of the sweet co-substrate fructose as a galactose acceptor, which is a cost and taste factor, respectively (Rentschler et al., 2015). **Figure 1.3** shows a schematic of the chemical and enzymatic processes for lactulose production.

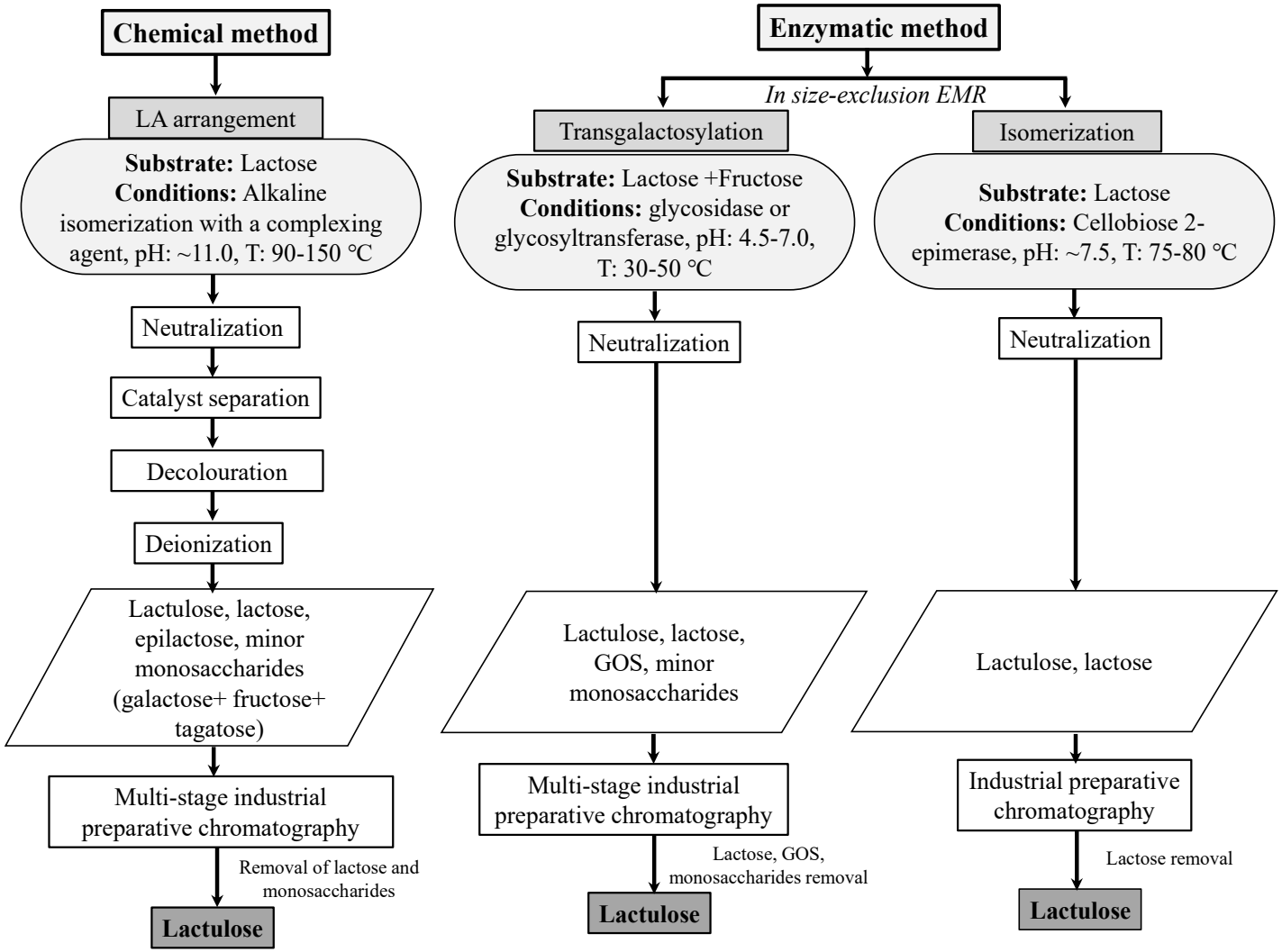

Figure 1.3: Schematic of the chemical and enzymatic processes for lactulose production. LA- Lobry de Bruyn-Alberda van Ekenstein, EMR- Enzymatic membrane reactor, GOS- Galactooligosaccharide. Adopted from Sitanggang et al. (2016b).

Besides, the transgalactosylation of lactose, the isomerization of glucose moiety in lactose into a fructose moiety to produce more economical enzyme-based lactulose was employed (Sitanggang et al., 2016b) being motivated with the fact that the enzyme cellobiose 2-epimerase was capable to isomerize the glucose moiety in cellobiose (disaccharide) into fructose or mannose (Park et al., 2011). In this method, the first isomerization lactose into lactulose achieved a maximum lactulose concentration of 408 g/L after 2 h using a feed of 700 g/L (yield=58.3%) lactose solution and *Caldicellulosiruptor saccharolyticus* cellobiose 2-epimerase (Kim and Oh, 2012). However, the formation of epilactose as a by-product is reported in lactulose synthesis via direct epimerization of lactose using cellobiose 2-epimerase (Shen et al., 2016). Therefore, some new strategies have been suggested to enhance the productivity, yield, and the specificity of lactulose production including molecular modification of enzymes (Shen et al., 2016), use of immobilized enzymes (Song et al., 2013b), and continuous mode of reaction (Ubilla et al., 2020).

1.1.4.3. Electro-activation technology for lactulose production

The EA is appealing technology to synthesis lactulose through the isomerization of pure lactose or in situ whey/WP. This technique is grounded on the electrolysis of aqueous solutions to make the solution highly reactive, which is effective in various physiochemical and biological reactions (Aider et al., 2012b). The isomerization of lactose into lactulose in the EA could be performed under autocatalytic conditions (i.e., without addition of catalysts and external heating) by following a self-created alkaline condition via water electrolysis (Djouab and Aïder, 2019a). It was reported that the synthesis of lactulose using the EA followed the LA transformation (Aider et al., 2012b). Commonly, the isomerization reaction via LA transformation needs proton acceptors, and it could be obtained by hydroxyl ions produced in a highly alkaline medium by rigorous water electrolysis at cathode-solution interface under the effect of an external electric field (Aissa and Aïder, 2013c). The basic principle of the EA is that the oxidation-reduction reactions take place at the anode and cathode of an EA reactor, respectively while an external electric field is applied. These occurrences lead to rapid changes in the solution pH whereas an acidification process happens at the anode side and a basification process occurs at the cathode interface. However, the EA solution pH and oxidation-reduction potential (ORP) can be controlled using suitable reactor configuration and appropriate ion exchange membranes (Aider et al., 2012b). It is

worthy to note that the EA technology successfully employed in various fields of food and agriculture industry (Petrushanko and Lobyshev, 2001, Aider and de Halleux, 2007).

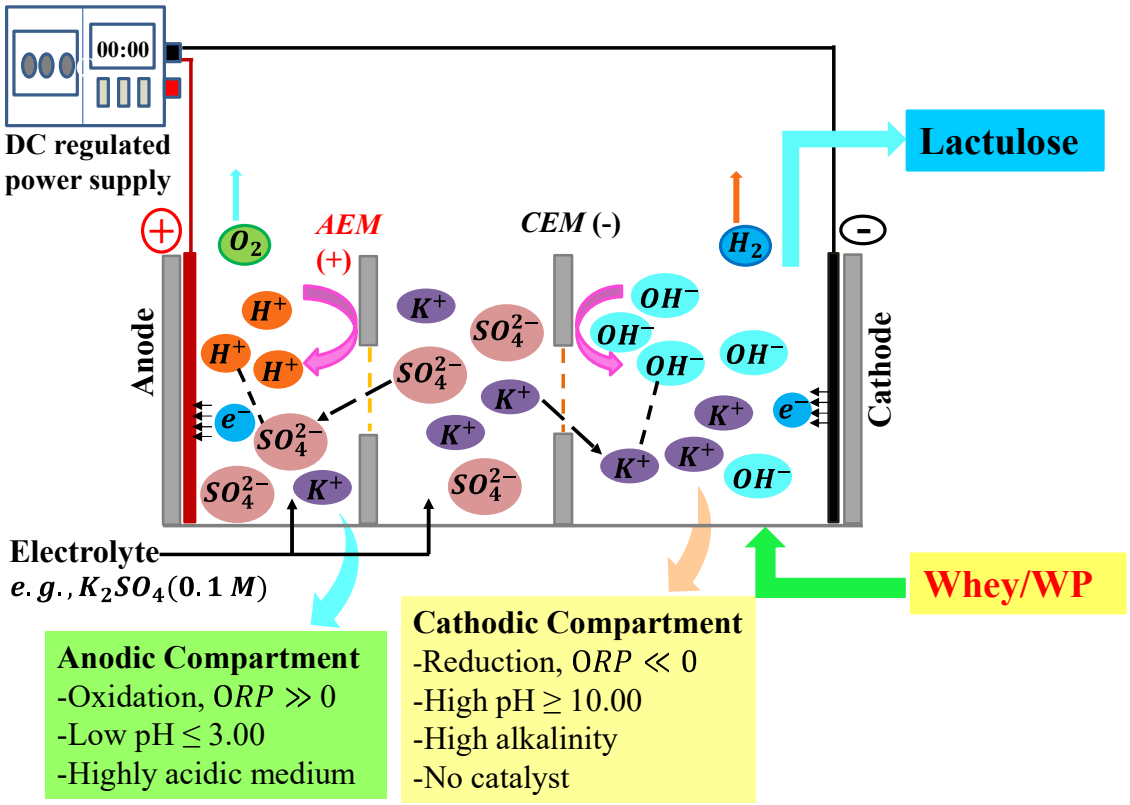

Figure 1.4: The schematic representation of an electro-activation reactor with three compartments to isomerize lactose to lactulose. CEM, cation exchange membrane; AEM, anion exchange membrane; DC, direct current; OPR, oxidation-reduction potential.

In the last decade, the EA process has successfully been used to isomerize lactose into lactulose without addition of any alkalinizing chemicals (**Table 1.5**). The EA is considered as a clean, safe, eco-friendly, and energy saving approach compared to the chemical and enzymatic processes, which are associated with various complications of purification steps as well as increased cost and energy consumption. However, EA is a comparatively innovative science, and the thermodynamics behind this technique is still unrevealed (Shirahata et al., 2012, Aissa and Aïder, 2013a). Generally, the charged species migrate/drift toward the electrode of reverse charge if an aqueous solution is exposed to an external electric field. Two phenomena, namely oxidation and reduction are usually occurred in the electrolysis of water or any aqueous solution (**Figure 1.4**). First, a reduction reaction

happens at the cathode (negatively charged electrode) and electrons (e^-) are donated from cathode to the positively charged ions, such as hydrogen cations to create hydrogen gas (H_2).

Cathode:

Water electrolysis: $4H_2O + 4e^- \rightarrow 2H_2(g) + 4OH^-$

Reduction reaction: $2H^+ + 2e^- \rightarrow H_2(g)$

Reduction reaction: $2H_2O(l) + 2e^- \rightarrow H_2(g) + 2OH^-(aq)$

Second, an oxidation reaction take place at the anode (positively charged electrode) and free electrons (e^-) transferred to the anode, such as negatively charged oxygen migrate to the anode and produces oxygen gas (O_2) by transmitting electrons (e^-) to the anode to achieve the following reactions:

Anode:

Water electrolysis: $6H_2O \rightarrow O_2(g) + 4H_3O^+ + 4e^-$

Oxidation reaction: $2H_2O \rightarrow O_2(g) + 4H^+ + 4e^-$

Oxidation reaction: $4OH^-(aq) \rightarrow O_2(g) + 2H_2O(l) + 4e^-$

Table 1.5: Electro-isomerization of lactose into lactulose using electro-activation technology.

Electro-activation conditions	**Temperature (°C)**	**pH**	**Reaction time, (min)**	**Substrate (%, w/v)**	**Lactulose yield (%)**	**Reference**
Electrolyte = 0.05 M NaCl, current = 200 mA	23	~12	60	10% lactose	25.47	(Aider and Gimenez-Vidal, 2012)
Electrolyte = 0.5 M Na_2SO_4, current = 200 mA	26	10.79	20	5% lactose	~30	(Aissa and Aïder, 2013c)
Electrolyte = 0.3 M Na_2SO_4, current = 200 mA, addition of 0.05 M	10	11.00	30	10% lactose	30.19	(Aissa and Aïder, 2014b)

Electro-activation conditions	**Temperature (°C)**	**pH**	**Reaction time, (min)**	**Substrate (%, w/v)**	**Lactulose yield (%)**	**Reference**
$CaCl_2$ into the lactose solution						
Electrolyte = 0.3 M Na_2SO_4, current = 300 mA, addition of 0.05 M $CaCl_2$ into the lactose solution	10	10.50	30	5% lactose	32.50	(Aït-Aissa and Aïder, 2014)
Electrolyte = 0.5 M Na_2SO_4, current = 400 mA	10	10.65	40	7% whey	34.57	(Kareb et al., 2016b)
Electrolyte = 0.5 M Na_2SO_4 (anodic compartment), 0.25 M KCl (central compartment), current = 330 mA	-	11.51	21	6% whey permeate	35.1	(Djouab and Aïder, 2019b)
Electrolyte = 0.5 M Na_2SO_4 (anodic compartment), 0.25 M KCl (central compartment), current = 330 mA	-	11.59	14	5% lactose	38.66	(Djouab and Aïder, 2019b)

Electro-activation conditions	Temperature (°C)	pH	Reaction time, (min)	Substrate (%, w/v)	Lactulose yield (%)	Reference
Electrolyte = 0.5 M Na_2SO_4 (anodic compartment), 6% whey permeate (central compartment), current = 330 mA	-	11.62	35	6% whey permeate	39.78	(Djouab and Aïder, 2019b)

1.1.5 Electro-activation technology vs chemical isomerization process

In chemical isomerization process, the efficiency of lactose isomerization reaction mainly effected by catalyst concentration (or pH), temperature, and reaction time. A higher catalyst concentration (pH≈11.0) and elevated temperature (70-130 °C) are usually needed to attain a higher lactulose yield (≈ 25%) in an acceptable reaction time (60-120 min) (Hashemi and Ashtiani, 2010a, Seo et al., 2015b). However, high temperature lead to the development of a brownish color, and subsequent by-products including epilactose, glucose, galactose, isosaccharinic acid (**Figure 1.5a**) because of the higher degradation of lactose and lactulose (Song et al., 2013b, Sakkas et al., 2014b). It worth mentioning that the transformation of lactose into lactulose is followed by a rapid degradation of the latter into isosaccharinic acids and galactose gradually decayed into acidic compounds during chemical isomerization (Corbett and Kenner, 1953b, Hashemi and Ashtiani, 2010a).

In comparison with chemical isomerization process, the EA technique operated under a low temperature (0-30 °C) and a lactulose yield of ≈35% with a purity of ≈95% was obtained in a short period (20 min) without using any base catalysts (Aissa and Aïder, 2013c). Moreover, optimization of different process parameters including reaction time, current intensity, electrolyte concentration, and reactor configuration ensued in an enhanced yield of lactulose up to 45% (Aissa and Aïder, 2014b).

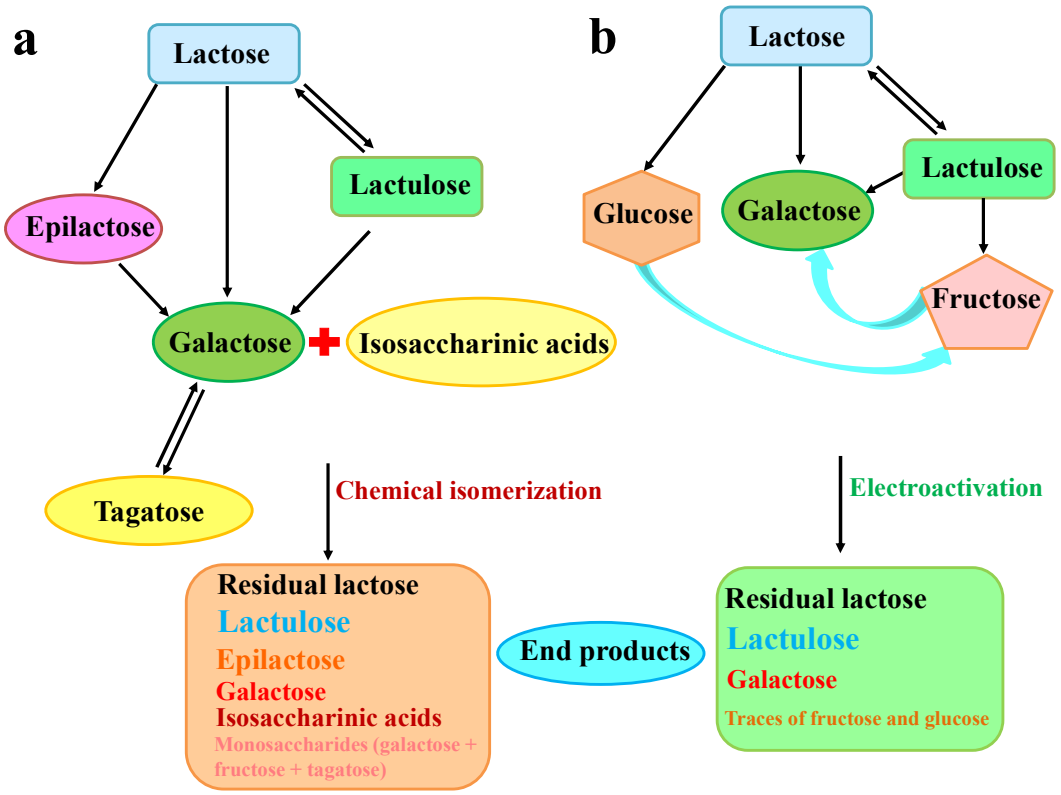

Figure 1.5: Schematic of possible pathway and end products formation of lactose into lactulose; (a) chemical isomerization method (Hashemi and Ashtiani, 2010a) and (b) electro-isomerisation technique (Djouab and Aïder, 2019b).

Nevertheless, no other acidic compounds but only galactose (**Figure 1.5b**) were found during the electro-isomerization of lactose, WP or whey (Kareb et al., 2016b, Djouab and Aïder, 2019b). As a result, the purification step would be simplified by way of avoiding by-product formation (**Figure 1.6**), that is an environmental and economical benefit for industrial application (Aider and Gimenez-Vidal, 2012, Djouab and Aïder, 2019b). Hence, it can be argued that the EA process is more selective compared to the chemical method. Furthermore, it may probably decrease the consumption of energy by eluding utilization of alkalinizing chemicals and heating. Apart from this, a higher yield of around 35-45% with a purity of 95-96% could be attained in a short reaction period using electro-isomerization, which is comparable to the requirements of Pharmakopoeia for lactulose powder (Aider and Gimenez-Vidal, 2012, Aissa and Aïder, 2013c, Kareb et al., 2016b). It contrast, they yield always remained < 30% in the chemical isomerization process (Hashemi and Ashtiani, 2010a, Seo et al., 2016b).

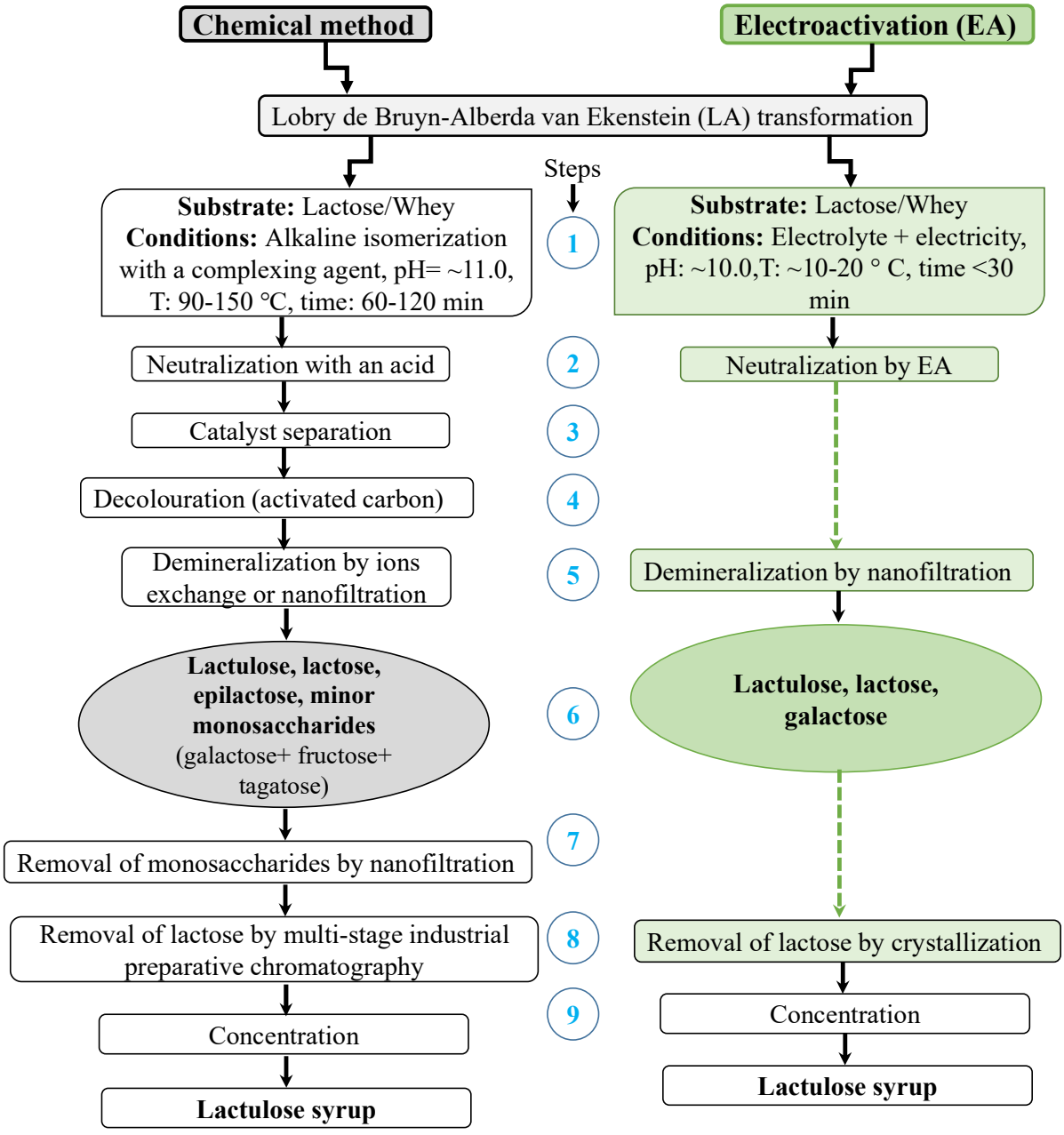

Figure 1.6: Schematic of the chemical isomerization and electro-isomerization processes for lactulose production.

1.1.6 Influencing factors of electro-isomerization process

The efficiency of electro-isomerization using the EA technology is influenced by various operational and experimental conditions. In the last decade, several studies have been conducted to explore different parameters, which significantly effect the performance of

electro-isomerization process in the EA reactor. Aider and Gimenez-Vidal (2012) studied the influence of substrate (i.e., 5 and 10% lactose, whey) concentration and electric current density (i.e., 100 and 200 mA) during lactulose synthesis by reagent-less EA process. Moreover, the influence of reaction time on lactulose production yield, formation of by-product, and global electric resistance of the EA reactor was also studied. They observed that the electric current intensity has a strong effect ($p<0.001$) on the isomerization rate/lactulose formation yield for all substrate used. Better isomerization yields were achieved when higher current (i.e., 200 mA) intensity was applied. Regarding the substrate, no significant variation was detected between the yields of 5% lactose versus 10% lactose solutions, however, type of the feed solutions (lactose vs whey) was a significant factor. Furthermore, the EA-time demonstrated distinct tendencies based on the reaction conditions used. The increased EA-time led to a decreased in the global electric resistance of the EA reactor suggesting a high energetic efficacy of the electro-isomerization process (Aider and Gimenez-Vidal, 2012). In another study, the influence of temperature, feed concentration, and Froude number (Fr) on current density, conductivity, pH, and lactulose yields were investigated for electro-isomerization of lactose into lactulose (Aissa and Aïder, 2013a). They obtained a maximum of ≈25% lactulose yield with a purity of ≈95% at Fr = 2.05×10^{-2}, temperature = 0-10 °C, 10% lactose, time = 2.00 min, pH = 10.30, conductivity = 4.64 mS/cm, current density = 4.86 mA/cm^2. It is worth noting that the application of EA at the low temperature range offers a clear prospect of producing highly pure lactulose with a minimal possibility of by-products formation (Aissa and Aïder, 2013a).

In another study, Aissa and Aïder (2013c) studied electro-catalytic production of lactulose via isomerization of lactose in an EA reactor with several electrical potential difference, central cell compartment electrolyte concentration, and EA-time at an ambient temperature (22 ± 1 °C) using 5% lactose solution. Thereafter, they correlated the results with pH, temperature, electric current density, system global electric resistance, and Joule heating effect. A maximum of 30% lactulose yield was achieved during the lactose isomerization at the voltage of 28 V, electrolyte concentration of 0.5 M Na_2SO_4, temperature of 26 °C, pH of 10.79, and running time of 20 min. In a subsequent study, they optimized the reactor configuration, temperature, and electric current using 10% lactose feed solution (Aissa and Aïder, 2014b). Based on the optimized conditions, they observed to produce 30.19±1.37%

lactulose after 30 min of EA process at 10 and 0 ℃. The formation of galactose did not exceed even 6% of the total product and it was decreased when temperature was lowered to 0 ℃. Nevertheless, the influence of active membrane surface area to electrode surface area ratio, electrode material, inter electrode-membrane distance of an EA reactor was also studied by Aït-Aissa and Aïder (2014) using 5% lactose solution and 300 mA current for 30 min at 10 ℃. They obtained a maximum of ≈32.50% lactulose production using a copper electrode (titanium, stainless steel, and copper were considered), an inter electrode-membrane distance of 0.36 (0.36, 0.68, and 1 were used), and a ration of membrane: electrode surface area of 0.23 (0.23, 0.06, and 0.015 were studied). Therefore, an appropriate reactor configuration with suitable ion exchange membranes and electrodes is crucial to regulate the chemical composition of the desired medium to control the lactulose synthesis under the EA conditions (Aissa and Aïder, 2013c).

Nevertheless, the synthesis of lactulose using electro-isomerization of lactose *in situ* of whey and the effect of EA on the whey proteins were studied in a study of Kareb et al. (2016b). They investigated the influence of working temperatures (25, 10, and 0 °C), current intensities (800, 600, and 400 mA), feed volume (300, 200, and 100 mL), and feed concentrations (7, 14, and 28 %, w/v) on lactulose formation yield in the EA reactor. They achieved the highest lactulose yield of 35% at the temperature of 10 °C and reaction time of 40 min lactulose under an electric current of 400 mA using a 100 mL of 7% whey solution as feed medium. The investigation of structural attributes of whey proteins and antioxidant functionality showed a significant increase in antioxidant capacity of EA whey compared with the untreated samples. Recently, Djouab and Aïder (2019b) investigated the potential of WP to be transformed into lactulose through *in situ* isomerization of lactose in an EA-reactor, which was modulated by cation and anion exchange membranes. They studied the influence electric current (110, 220, and 330 mA) and type of salt ($MgCl_2$, $CaCl_2$, and KCl) on the lactose isomerization rate using 5% lactose and 6% WP solutions. The conversion rate of lactose was 35.1% at 330 mA current after 21 min of EA using KCl in the central compartment and WP as a feed in the cathode. The rate of lactose conversion was 38.66% at 330 mA after 14 min while KCl and lactose solution were used in the central and cathodic compartments, respectively. The highest yield of 39.78% lactulose was achieved for 330 mA

current after 35 min of EA using WP in both cathodic and central compartments without adding any salt.

1.1.7 Effect of electro-activation in whey/WP

Lactose content of whey or WP could be converted into lactulose, glucose and fructose; subsequently, glucose and fructose converted to galactose via *in situ* electro-isomerization of lactose using the EA technology (**Figure 1.5b**) (Djouab and Aïder, 2019b). Thus, the EA process increases the availability of monosaccharides and lactulose (a prebiotic) in electro-activated whey (EA-whey) or electro-activated whey permeate (EA-WP), which may potentially be able to provide an optimum growth condition for microorganism. The EA-whey/WP also contain other compounds like free amino acids, small peptides, vitamins, and minerals, that can stimulate the growth of microbes (Kareb et al., 2018b). Moreover, the improved buffering capacity and the potential redox could be also interesting characteristics of EA-whey/WP. Kareb et al. (2016b) reported that the EA-whey is a versatile prebiotic with improved antioxidant functionalities and have an excessive potential to stimulate the growth of probiotic bacteria. This might be due to the synergistic effect of whey proteins hydrolysis, and glycation of amino acids with different peptides and reducing sugars under the EA conditions, which are favourable to promote the formation of Maillard Reaction Products (MRPs). Indeed, MRPs enhance the antioxidant capacity of EA-whey (Kareb et al., 2017c). Notably, the MRPs are often used to enhance the biological and technological characteristics of foods, and would be especially interesting as potential prebiotic because they positively influence the growth of health promoting bacteria in the gastrointestinal tract as reported in several studies (Corzo-Martínez et al., 2012, Seiquer et al., 2014). It was also postulated that the generation of antioxidant compounds are able to scavenge free radicals (Kareb et al., 2016b, Kareb et al., 2017c). Therefore, EA-whey can be considered as a complex mixture of various bioactive compounds, in which diverse conjugates and several antioxidant mechanisms are probably acting.

Recently, Kareb et al. (2018b) demonstrated that the EA-whey enhance the growth of *Bifidobacterium* (*B. animalis* subsp. *lactis* Bb12, *B. longum* subsp. *bifidum* R0175), *Lactobacillus* (*L. bulgaricus* R5083, *L. rhamnosus* GG), and *Streptococcus* (*S. thermophilus* R0292) strains. Moreover, the evolution of *L. johnsonii* La-1 was significantly increased under aerobic conditions using EA-whey as a supplement in the growth medium. They

correlated this growth stimulating impact to the fact that the EA-whey could have the ability to scavenge the accumulation of hydrogen peroxide and to its high antioxidant capacity and prebiotic lactulose content. In a study, it is also reported that the EA-whey could have a better bifidogenic effect compared to sole lactulose (López-Sanz et al., 2015). In another recent study, Djouab and Aïder (2019) showed that the EA enhanced the antioxidant activity of WP due to the intermediate MRPs and the drying temperature significantly influenced the antioxidant activity of EA-WP.

1.1.8 Conclusions and future directions

Cheese whey is gradually known as a resource of value-added products rather than just a waste stream with an extreme pollution load. Particularly, the demand of whey proteins and bioactive peptides are continuously increasing for many applications in food and pharmaceutical industries. The lactose-rich stream, which persists after separating proteins or other bioactive compounds is remained a main concern from environmental point of view, thus, industries and researchers are still looking for different processes to obtain products with good commercial value. As the present market is saturated with a huge surplus of lactose, its transformation to bulk commodities including bioethanol and different fermented products or other costly derivatives of lactose such as lactitol, mannitol, sorbitol, lactosucrose, lactosyl urea, lactobionic acid, etc. could be considered as a potential solution for its valorization. Recently, there is an increasing interest of producing the prebiotic lactulose via isomerization of lactose that is present in the whey or WP. Specifically, the chemical and enzymatic methods have traditionally been used, however, they are associated with several complications of purification steps that lead to an increase in cost and energy consumption. Even though there has been a prolonged research and development effort in this arena, there is still a need to develop an efficient and eco-friendly technique to produce lactulose in an eco-friendly and energy saving approach.

In recent years, the EA technology demonstrated promising results for producing lactulose through electro-isomerization of lactose. It can also be considered as a safe, clean, economic, and environmentally friendly method. Currently, it needs to be scaled-up to deliver evidence of industrial significance. Moreover, the EA is a comparatively new technology, and the thermodynamics and mechanism of action that is involved with EA technique to obtain the required solution alkalinity, particularly, involving its action on the reaction

activation energy behind this process is remain unknown. Such state-of-the-art process mechanism is crucial to exploit this technology in large-scale. Besides, further research is still required regarding the influence of several parameters such as alkalinity, real time temperature increase due to system global resistance, oxidation-reduction potential, etc. Despite some evocative accomplishments of the studies on EA parameters and their correlations, a standard optimized condition should be established, which is more economical in terms of the configuration and geometry of the reactor considering the concentration and type feed solutions. Nevertheless, the neutralization of a highly reactive and alkaline solution using the EA technology without any external chemicals is yet to be investigated. It is also essential to carry out some comparison studies such as EA vs chemical or EA vs enzymatic methods using an equivalent process input to decide if the EA is really achieved a superior yield. Furthermore, the suitability of bioprocesses using EA solutions for producing different fermented bioproducts could be studied because the EA solutions possess high antioxidant capacity, prebiotic lactulose, and availability of other simple sugars.

1.2 Bioconversion of whey into valuable fermented products: A potential way for sustainable whey management

Whey is an outstanding resource of functional peptides and proteins, vitamins, lipids, minerals, and lactose, which could be used by the biotechnology, agri-food, medical, and related industries. In last few decades, considerable efforts have been undertaken globally to discover different techniques to convert surplus whey to a feed stock for bioconversion towards many valuable products such as bioethanol, vinegar, antibiotics (e.g., bacteriocin nisin), yeasts for yeast extract production, surfactants (e.g., sophorolipids), single-cell protein, polyhydroxyalkanoates, lactic acid, polylactic acid (Ryan and Walsh, 2016). Lactose, as the main carbohydrate component of whey acts as a carbon source for growth and product formation in various biotechnological processes (**Table 1.6**). Thus, whey/WP is directly used for fermentation using lactose consuming microbes for its biotransformation. However, hydrolysis of lactose is performed before fermentation in some cases, while microorganisms are not able to hydrolyze lactose, to increase the usefulness of whey. Hydrolysis of lactose into monosaccharides (glucose and galactose) is carried out either with the enzyme β-galactosidase or by acid hydrolysis (Yadav et al., 2015c).

Table 1.6: Bio-utilization of whey to produce various value-added products. Adopted from Kaur et al. (2020).

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
Single cell protein	*Kluyveromyces marxianus* and *Candida krusei*	Whey	Stirred tank bioreactor	Batch	15 L	3.5	40	22 h	10.1 g/L	(Yadav et al., 2014a)
	K. marxianus	Whey	Erlenmeyer flask	Batch	20 mL	5.5	35	30 h	0.27 g/g lactose consumed	(Yadav et al., 2016)
	K. marxianus and *Saccharomyces cerevisiae*					6.5	30	30 h	0.31 g/g lactose consumed	
	K. marxianus CBS 6556	Sweet whey	Stirred bioreactor	Batch	15 L	5.8	30	–	50 g/L	(Schultz et al., 2006)
		Sour whey			30 L	4.8	30	–	65 g/L	
	Kefir microflora	Whey	Bioreactor	Fed batch	4 L	5.5	30	9 h	-	(Paraskevopoulou et al., 2003)
Bio pigments	*Rhodotorula glutinis* NRRL YB-252 and *L.*	Whey	Erlenmeyer flask	Batch	100 mL	3.5–4.5	30	6 days	474.76 µg/g	(Chandi et al., 2010)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
	casei subsp. *casei* NR RL B-441									
	Penicilium aculeatum	Whey	Erlenmeyer flask	Batch	100 mL	6.5	30	240 h	1.38 g/L	(Afshari et al., 2015)
	Rhodotorula glutinis	Salted cheese whey	Erlenmeyer flask	Batch	100 mL	6.6	30	120 h	6.54 mg /L	(Kanzy et al., 2015)
	Monascus purpureus	Corn meal, molasses, and whey	Polypropylene plastic bags		5 g	–	30	14 days	68.89 mg/gds	(Nimnoi et al., 2015)
Bioethanol	*Candida tropicalis* and *Blastschizomyces capitatus*	Mozzarella cheese whey and sugarcane molasses	–	–	–	–	25–27	24 h	8.49%	(Balia et al., 2018)
	S. cerevisae and *Gluconobacter oxydans*	Cheese whey powder	Erlenmeyer shake flask		100 mL	–	30	24 h	110 g/kg cheese whey powder	(Zhou et al., 2019)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
Biobutanol	*Clostridium acetobutylicum* DSM 792	Cheese whey	Fermentor	Batch	3 L	7	37	75 h	0.71±0.06 g/L	(Foda et al., 2010)
	C. acetobutylicum DSM 792	Cheese whey powder	Packed bed biofilm reactors	Continuous	–	4.7		22 days	4.93 g/L	(Raganati et al., 2013)
	Clostridium saccharobutylicum P2 62	Whey permeate	Bioreactor	Batch	–	5	35	92 h	9.26 g/L	(Qureshi et al., 2014)
	Clostridium beijerinckii CECT 508	Nanofiltered sheep cheese whey	Rubber-capped bottles	Batch	100 mL	6	35	92 h	8.91 g/L	(Díez-Antolínez et al., 2016)
Biodiesel	*Scenedesmus obliquus*	Lake water and whey permeate	Erlenmeyer shake flask	Batch	80 mL	–	22.5	13 days	105±25 mg/g dry biomass	(Girard et al., 2014)
	Cryptococcus curvatus	Ricotta cheese whey	Stirred tank reactor	Batch	2 L	–	30	5 days	32.6%	(Carota et al., 2017b)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
	Crypthecodinium cohnii	Cheese whey and corn steep liquor	Shake level	Batch	–	–	–	–	28.7%	(Isleten-Hosoglu and Elibol, 2017)
Bacteriocin	*Enterococcus faecium*	Cheese whey	Shake level	Batch	10%	6	37	24 h	25,500 AU/mL	(Schirru et al., 2014)
	Leuconostoc mesenteroides A11	Cheese whey	Shake level	Batch	50 mL	–	25	24 h	–	(Okuda et al., 2018)
	L. helveticus	Cheese whey					37	24 h	–	(Hati et al., 2018)
Lactic acid	*L. plantarum* TISTR 2265	Cheese whey	Cylindrical reactor in a sequencing batch reactor	Batch	–	4.5	–	23 h	12.58 g/L	(Monkoondee et al., 2016)
	Pediococcus sp.	Hydrolyzed whey	Shake level	Batch	100 mL	4.4–4.5	37	48 h	47–51.2 g/L	(Juodeikiene et al., 2016)
	Engineered *S. cerevisae*	Whey	Shake level	Batch	25 mL	–	30	–	0.358 g/g lactose	(Turner et al., 2017)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
	L. casei and *Pseudomonas taetrolens*	Whey	Shake level	Batch	400 mL	–	30	48 h	0.95 g/g lactose	(García et al., 2017)
Galactooligosaccharides	*Streptococcus thermophilus*	Whey permeate	–	Batch	–	6.5	50	–	50%	(Geiger et al., 2016)
	K. lactis	Demineralized whey powder	Screw cup flasks	Batch	400 mL	–	–	90 min	18.02%	(Vénica et al., 2017)
	A. oryzae	Acid whey	Shake flask	Batch	–	4.5	55	–	11.32±0.59%	(Fischer and Kleinschmidt, 2015)
	K. lactis					6.5	45	–	10.56±0.41%	
	A. oryzae	Whey permeate	Packed bed reactor	Continuous	–	5.2	60	–	39.3%	(Eskandarloo and Abbaspourrad, 2018)
	K. lactis	Whey	Flask	Batch	–	–	35	12 h	25 g GOS/100 g lactose	(Mano et al., 2019)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
β-Galactosidase	*K. marxianus*	Whey	Shake flask	Batch	–	5.5	30	28 h	1710 IU /g DW	(Kumari et al., 2011)
	A. tubingenesis GR1	Wheat bran and whey	Flask	Batch		–	28	8 days	15, 936 U/g ds	(Raol et al., 2015)
	Aureobasidium pullulans	Whey	Flask	Batch		4.5	28	168 h	1700 IU /L	(Kaur et al., 2015)
	K. marxianus	Whey				5.2	30	20 h	2886 IU /g DW	
	Lactobacillus sp. KLSA 22	Whey	Erlenmeyer flask	–	–	6.5	35	72 h	97.99±2 .38 IU	(Ahmed et al., 2016)
	K. lactis	Whey powder	Bioreactor	Fed-batch	4 L	–	27.6	20 h	119.30 I U/mL	(You et al., 2017)
	Enterobacter ludwigii	Whey	Erlenmeyer flask		50 mL	9	20	48 h	35.32 U/ mL	(Alikunju et al., 2018)
	A. oryzae CCT 0977	Cheese whey	Shake flask	Batch	–	–	28	5 days	0.44 U/ mL	(Viana et al., 2018)
	Rhizomucor pusillus	Wheat bran and whey	Erlenmeyer flask	Batch		5.5	50	7 days	101.89 I U/gds	(Kaur et al., 2018)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
Protease	*Bacillus* sp. SMIA-2	Cheese whey and passion fruit rind	Erlenmeyer flask	Batch	50 mL	7.5–8.0	50	48 h	11.6 U/mL	(Barbosa et al., 2014)
	Rhizopus oryzae	Whey	Flask	Batch	100 mL	6	35	168 h	36.5 U/g biomass	(Mushtaq et al., 2014)
Amylase	*Bacillus amyloliquefaciens*	Wheat bran, whey, peptone				–	–	–	11,196 U/mL	(Selen and Saban Tanyildizi, 2017)
	Anoxybacillus beppuensis	Whey	Erlenmeyer flask	Batch	2%	–	60	5 days	0.88 U/mL	(Jabeen et al., 2019)
	Bacillus amyloliquefaciens	Orange peel and whey	–	–	–	–	33	92 h	220 U/mL	(Uygut and Tanyildizi, 2018)
Polysaccharide	*Xanthomonas campestris*	Whey, yeast extract, and tryptone	Erlenmeyer flask	Batch	80 mL	7.2	28	3 days	25 g/L	(Silva et al., 2009)

Products	Microorganisms	Substrate	Type of equipment	Mode of operation	Volume of fermentation broth	Process conditions			Yield	References
						pH	Temp (°C)	Time		
	Rhizobium radiobacter S10	Whey	Bioreactor		4.5 L	–	30	96 h	2834.2 mg/L	(Zhou et al., 2014a)
	Streptococcus zooepidemicus	Whey, yeast extract, and tryptone	Bioreactor	Batch	4.5 L	6.7	37	–	4.02 g/L	(Amado et al., 2016)
	L. kefiranofaciens	Whey and spent yeast cell hydrolysate	Bioreactor	Fed-batch	0.7 L	5.5	30	–	2514±9 3 mg/L	(Cheirsilp et al., 2018a)
	K. marxianus	Whey	Bioreactor	Fed batch	5 L	4.5	30	20 h	28.13 g/L biomass	(Belem and Lee, 1999)

1.3 Kefir culture: A symbiotic consortium to produce various food grade metabolites

1.3.1 Why a mixed culture, originated from kefir?

Different microorganisms including pure cultures and mixed culture consortia were used in the fermentation process to produce various value-added products using whey-based substrates (Yadav et al., 2015a, Carota et al., 2017a, Kareb et al., 2018a). The mixed cultures are regarded robust inocula because they are able to utilize wide range of substrates, more tolerant to the environmental fluctuations, easy to obtain in nature, applicable in large scale, and less susceptible to contamination (Karim et al., 2018a, Islam et al., 2020). Kefir grain, a mixed microbial consortium, produces a complex microflora that is containing more than hundred of yeasts and bacteria securely embedded (Cheirsilp and Radchabut, 2011). This mixed-microbiota is a decent example of a mutually beneficial community where lactic acid bacteria (LAB) including *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* are the main population (10^8-10^9 CFU/gram of grain) followed by yeast such as *Kluyveromyces*, *Candida*, and *Saccharomyces* (10^7-10^8 CFU/gram of grain) and acetic acid bacteria (AAB) like *Acetobacter* and *Gluconobacter* (10^5-10^6 CFU/gram of grain) (Dong et al., 2018, Bengoa et al., 2019b). **Figure 1.7** illustrates a scanning electron microscope (SEM) micrograph of Argentine kefir grains described by Bengoa et al. (2019a).

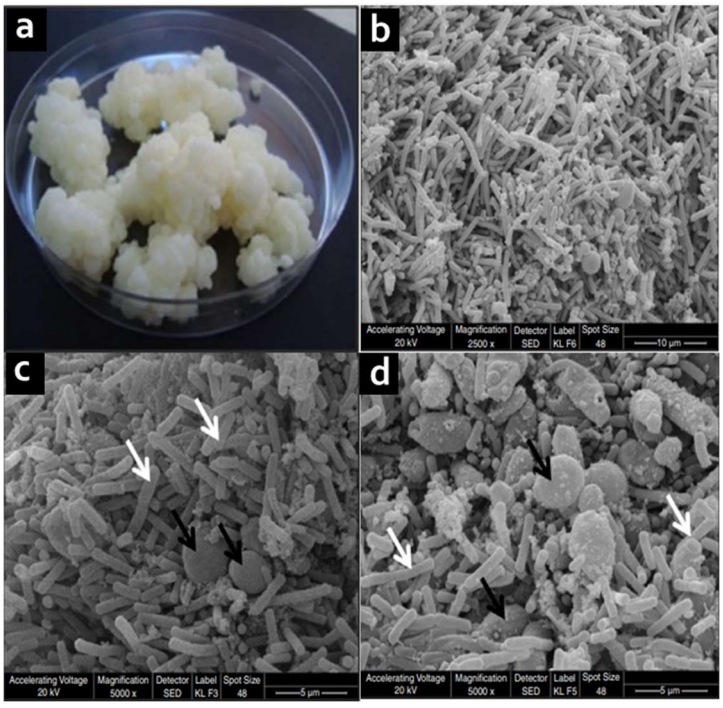

Figure 1.7: Macroscopic (a) and microscopic aspect (b-d) of kefir grains from Argentina and list of the main bacteria and yeasts genera described in kefir grains from different sources. The presence of bacteria (white arrows) and yeasts (black arrows) is indicated in microphotographs c and d. Adopted from Bengoa et al. (2019a).

1.3.2 Microbial diversity and metabolites production capacity

Due to its' excellent microbial diversity composition, a whole kefir culture is capable of adapting to various food/waste substrates, which can henceforth be used to manufacture innovative beverages with probiotic function (Tu et al., 2019). For instance, the LAB present in the consortium improve the conservation period of the products by producing acetic acid, lactic acid or other antimicrobial compounds (John and Deeseenthum, 2015). They also support to enhance organoleptic properties through the formation of numerous volatile compounds (e.g., ethyl acetate, acetaldehyde, isobutyl alcohol, etc.), functional exopolysaccharides (e.g., kefiran) or free amino acids (Dertli and Çon, 2017, Bengoa et al., 2019b). Moreover, the yeasts produce carbon dioxide and alcohol during the fermentation, which could increase mouthfeel and taste of the fermented products (Rosa et al., 2017). The microbial composition of kefir can vary depending on several factors such as the origin and storage of the kefir grains, the processing conditions of product (e.g., grain/milk ratio, temperature), the type of the milk used, etc. (Nielsen et al., 2014). The microbial composition of kefir microbiota from different origins as well as the methods employed to study are listed in **Table 1.7**.

Table 1.7: Microbial composition of kefir grains and beverages from different sources of origin. Adopted from Bengoa et al. (2019a).

Origin	Microorganisms	Methods employed to study kefir microbiota	References
Argentine kefir grains	*Lactobacillus kefiri* (*L. kefiri*), *L. parakefiri*, *L. paracasei*, *L. kefiranofaciens* sp. *kefiranofaciens*, *L. kefiranofaciens* sp. *kefirgranum*, *L. plantarum*, *Lactococcus lactis* sp. *lactis* (*Lc. lactis*), *Lc. lactis* sp. *lactis* biovar *diacetylactis*, *Leuconostoc mesenteroides*, *Acetobacter* sp., *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *S. unisporus*	Identification of isolates by biochemical test, whole cell protein pattern, FTIR, RAPD-PCR, Rep-PCR fingerprinting (GTG) 5, phenylalanyl-tRNA synthase (*pheS*) gene sequencing, ITS region polymorphism. PCR amplification of 16S and 26S rDNA sequences-DGGE and identification of DGGE bands	(Garrote et al., 2001) (Golowczyc et al., 2008) (Londero et al., 2012) (Hamet et al., 2013) (Diosma et al., 2014)
Belgium kefir grains and their products	*L. kefiri*, *L. kefiranofaciens*, *Lc. lactis* ssp. *cremoris*, *Leuconostoc mesenteroides*, *Gluconobacter frateurii*, *A. orientalis*, *A. lovaniensis, Naumovozyma* sp., *K. marxianus, Kazachastania kefir*	Metagenetic analysis targeting the 16S and 26S ribosomal DNA fragments by pyrosequencing	(Korsak et al., 2015)
Brazilian kefir grains and beverage	*L. kefiranofaciens*, *L. parakefiri*, *L. kefiri*, *L. amylovorus*, *L. buchneri*, *L. crispatus*, *L. paracasei*, *L. helveticus*, *L. uvarum*, *Lc.*	Identification of isolated micro-organism by phenotypic and genotypic methods.	(Miguel et al., 2010) (Magalhães et al., 2011b)

Origin	Microorganisms	Methods employed to study kefir microbiota	References
	lactis, *Leuconostoc mesenteroides*, *G. japonicus*, *A. syzygii*, *S. cerevisiae*	PCR amplification of 16S and 26S rDNA sequences-DGGE and pyrosequencing	(Leite et al., 2012) (Zanirati et al., 2015)
Irish kefir grains and beverage	*L. kefiranofaciens*, *L. kefiri*, *L. helveticus*, *L. parabuchneri*, *L. acidophilus*, *L. parakefiri*, *Leucoconstoc* sp.	16S compositional sequencing analysis.	Dobson *et al*. (2011)
Italian kefir grains	*L. kefiranofaciens*, *Lc. lactis*, *Streptococcus thermophilus*, *Enterococcus* sp., *Bacillus* sp., A. *fabarum, A. lovaniensis*, *A. orientalis*, *Dekkera anomala*	PCR-DGGE of kefir grains and identification of DGGE bands Analysis of bacterial and yeast diversity by rRNA gene pyrosequencing	(Garofalo et al., 2015)
South African kefir grains	*L. plantarum*, *L. delbrueckii* sp. *delbrueckii*, *L. brevis*, *L. delbrueckii* sp. *lactis*, *L. curvatus*, *L. fermentum*, *Lc. lactis* sp. *lactis*, *Leuconostoc mesenteroides* sp. *cremoris*, *Leuconostoc mesenteroides* sp. *mesenteroides/dextranicum*, *Candida lipolytica*, *C. lambica*, *C. krusei*, *C. kefyr*, *C. holmii*, *S.*	Isolation in selective growth media and identification by using morphological and biochemical characteristics PCR-DGGE of kefir grains and identification of DGGE bands	(Witthuhn et al., 2005) (Garbers et al., 2004)

Origin	Microorganisms	Methods employed to study kefir microbiota	References
	cerevisiae, *Zygosaccharomyces* sp., *Cryptococcus humicolus*, *Geotrichum candidum*		
Taiwanese kefir grains	*L. kefiranofaciens*, *L. kefiri*, *Lc. lactis*, *Leuconostoc Mesenteroides*	PCR-DGGE of isolates and DNA sequencing techniques PCR-DGGE of kefir grains and identification of bands	(Chen et al., 2008)
Tibetan kefir grains	*L. kefiranofacien*, *L. kefiri*, *L. casei*, *L. paracasei*, *L. helveticus*, *Lc. lactis*, *Leuconostoc mesenteroides*, *Streptococcus thermophilus*, *K. marxianus*, *S. cerevisiae*, *Kazachstania exigua*, *Kazachstania unispora*	DGGE of partially amplified 16S rRNA or 26S rRNA followed by sequencing of the bands Isolation of micro-organisms and typing by 16S rDNA and 26S rDNA-D1/D2 gene sequencing technology, (GTG)5-Rep-PCR genomic fingerprinting	(Zhou et al., 2009) (Gao and Zhang, 2018)
Turkish kefir grains	*L. kefiri*, *L. kefiranofaciens*, *L. casei*, *L. paracasei*, *L. parakefiri*, *L. plantarum*, *L. acidophilus*, *L. amylovorus*, *L. brevis*, *L. buchneri*, *L. crispatus*, *L. delbrueckii*, *L.diolivorans*, *L. gallinarum*, *L. gasseri*, *L. helveticus*, *L. johnsonii*, *L.*	16S RNA pyrosequencing Whole genome shotgun pyrosequencing	(Nalbantoglu et al., 2014)

Origin	Microorganisms	Methods employed to study kefir microbiota	References
	otakiensis, *L. parabuchneri*, *L. reuteri*, *L. rhamnosus*, *L. rossiae*, *L. sakei*, *L. salivarius*, *L. sunkii*, *Lc. garvieae*, *Lc. lactis*, *Leuconostoc mesenteroides, Oenococcus oeni*, *Pediococcus* sp., *Tetragenococcus halophilus*		
Russian kefir grains	*L. casei*, *L. paracasei*, *L. kefiri*, *L. kefiranofaciens* sp. *kefirgranum*, *Lc. lactis* sp. *cremoris/lactis*, *Leuconostoc pseudomesenteroides*, *S. cerevisiae*, *Kazachstania unispora*	Classical microbiological analysis and DGGE-PCR method	(Kotova et al., 2016)

1.3.3 Health promoting properties of kefir microorganisms

The functional and probiotic properties of kefir have been described in several studies (Bourrie et al., 2016, Sharifi et al., 2017). Health benefits comprise antimicrobial activity, tumour suppression, wound healing properties, immunomodulation, anti-inflammatory, anti-obesity, cholesterol lowering and antioxidant effects, improvement in lactose tolerance, alleviation of fatty liver and enhancement of intestinal bacterial flora. These beneficial health properties might be ascribed both to the presence of probiotic microorganisms, as well as to the metabolic products that appear in the fermented milk (Bengoa et al., 2019a). The probiotic properties of *Lactobacilli* isolated from kefir could be stated including the ability of *Lactobacillus plantarum* CIDCA 83114 to prevent the detachment of Hep-2 cells incubated with *Escherichia coli* enterohaemorrhagic (EHEC) to Hep-2 cells (Hugo et al., 2008) and antagonize the cytotoxic effects of EHEC Shiga 2 toxin (Kakisu et al., 2013). Furthermore *L. kefiri* strains can inhibit the adhesion and invasion of *Salmonella enterica* serovar. Typhimurium to Caco-2/TC-7 cells (Golowczyc et al., 2007).

In relation to yeasts, strains belonging to species *S. cerevisiae*, *S. unisporus*, *I. occidentalis* and *K. marxianus* were studied by our group, determining their resistance to gastrointestinal conditions both *in vitro* and *in vivo*. Additionally, their capacity to adhere to Caco-2 cells was studied (Diosma et al., 2014). The *in vitro* modulation of the epithelial innate immune response was studied, detecting that kefir yeasts modulate the proinflammatory response of flagellin-induced in Caco-2:CCL20 luc cells (Romanin et al., 2010). The multiplicity of interaction (relation micro-organisms/epithelial cells) and the incubation time showed to be factors that influence the modulatory effect. Furthermore, the response triggered by other proinflammatory agonists such as IL-1β, TNF-α and LPS was also modulated by the yeasts. Romanin et al. (2010) showed that the modulation of gene expression is specific for proinflammatory genes with no alterations in the expression of nonimmunological genes.

The potential use of *K. marxianus* as a probiotic has been suggested in several reports (Maccaferri et al., 2012b). Romanin et al. (2016) strengthened the study of the anti-inflammatory capacity of the kefir yeast *K. marxianus* CIDCA 8154 in different models. They showed *in vitro* that the pretreatment of the epithelial cells with yeast decreases the intracellular levels of reactive oxygen species (ROS), presuming that the modulation of the

intestinal inflammatory response occurs through a mechanism independent of ROS generation. Furthermore, it was revealed in a model of *Caenorhabditis elegans* that the yeast was capable to protect from oxidative stress. Similarly, mice treated orally with *K. marxianus* CIDCA 8154 presented a less histopathological damage and lower levels of circulating IL-6 in a TNBS-induced colitis model (Romanin et al., 2016).

Some studies have also explored the probiotic potential of kefir yeasts. de Lima et al. (2017) observed that *S. cerevisiae* strains isolated from Brazilian kefir offered interesting *in vitro* probiotic properties. However, Cassanego et al. (2017) noticed that *S. cerevisiae, Hanseniospora uvarum* and *K. unispora* isolated from other Brazilian kefir were not able to tolerate the passage through the simulated gastrointestinal tract. Xie et al. (2012) studied the positive effect of the kefir yeasts on *Lactobacillus* probiotic potentials and Cho et al. (2018) recently found that a combination of kefir-derived *Kluyveromyces* KU140723-02 and polyphenol-rich grape seed flour or its extract has an incremented antioxidant activity.

1.3.4 Metabolites produced by kefir microorganism

Since several health promoting properties of kefir were attributed to its nonmicrobial fraction, it is relevant to gain a better understanding of the metabolites and major changes generated in the milk. Microbes ferment lactose, hydrolyse proteins, produce exopolysaccharides (EPS) and other metabolites such as organic acids, vitamins, ethanol, acetaldehyde, diacetyl, carbon dioxide and bacteriocins. One activity associated to this fraction was the antimicrobial capacity accredited to the presence of organic acids sometimes accompanied by other inhibitory compounds like bacteriocins (Iraporda et al., 2017). Lactic acid level in kefir varies between 0.078 and 0.255 mol/L (Leite et al., 2013) and acetic acid concentration range between 0.015 and 0.038 mol/L depending on the diversity of microbial population in the kefir grains and the fermentation conditions (Iraporda et al., 2014). The inhibitory activity of nonmicrobial fraction of kefir as well as cell free supernatant of fermented milks with microorganisms isolated from kefir was demonstrated against several pathogenic bacteria (Iraporda et al., 2017, Bengoa et al., 2019a). The inhibitory effect of kefir against *Salmonella* is lost by neutralizing the nonmicrobial fraction even when concentrated five times, indicating that the organic acids in their non dissociated form would be responsible for this effect (Iraporda et al., 2017). Otherwise, *in vitro* studies indicate that incubation of *Salmonella enterica* serovar Enteritidis with the neutralized nonmicrobial

fraction of kefir did not affect pathogens viability but decrease their invasive capacity to intestinal epithelial cells in culture (Iraporda et al., 2017, Bengoa et al., 2019a).

Another health benefit ascribed to the non-microbial fraction of kefir is its ability to modulate the immune response (Iraporda et al., 2014). In this circumstance, de LeBlanc et al. (2006) showed that the non-microbial fraction of kefir delayed breast tumour development inducing an adequately balanced local immune response in the mammary glands. Lactate and other organic acid such as acetate, propionate and butyrate also down regulate pro-inflammatory responses in intestinal epithelial and myeloid cells (Iraporda et al., 2014). Furthermore, lactate can be used by the gut microbiota to produce acetate, propionate and butyrate, short chain fatty acids highly associated to gut's health.

Kefiran, a water-soluble heteropolysaccharide composed by equal amounts of D-glucose and D-galactose, is the main polysaccharide present in kefir reaching values of about 218 mg/L (Zajšek et al., 2011). This polymer is an interesting additive for the food industry since it significantly improves the viscosity and viscoelastic properties of acid milk gels and is capable of forming translucent cryogels and edible films (Piermaria et al., 2015). Kefiran is a nondigestible polysaccharide that can reach the large intestine where it can exert antimicrobial, anti-inflammatory, and antiallergenic effects (Bengoa et al., 2019a). Kefiran administration in drinking water increase the number of bifidobacteria population in the colon (Hamet et al., 2016) and also produces an increase in the number of mucus-producing cells of the gut (Medrano et al., 2011). The biological activity of kefiran could be ascribed to the ability of this polysaccharide to interact with the enterocytes or indirectly mediated by the demonstrated bifidogenic effect. Additionally, this polymer can antagonize pathogens virulence factors *in vitro* and reduce blood pressure and serum cholesterol levels.

Many authors have isolated and studied the EPS synthetized by different *L. kefiranofaciens* ssp. *kefiranofaciens* strains from kefir grains in single cultures or in co-culture with yeasts, evidencing the same structure and composition as kefiran (Maeda et al., 2004, Wang and Bi, 2008). Hamet et al. (2013) have isolated nine EPS-producing *L. kefiranofaciens* sp. *kefiranofaciens* strains from different kefir grains and observed that the degree of polymerization of the EPS produced in milk was strain dependent. However, none of them yielded fractions of a molecular weight higher than 10^5 Da. Jeong et al. (2017) showed that *L. kefiranofaciens* DN1 produces a different EPS from kefiran composed of

mannose, arabinose, glucose, galactose and rhamnose when it grows in glucose. Otherwise, *L. kefiranofaciens* 1P3 isolated from Brazil kefir grains was able to produce an α-glucan in the presence of sucrose; however, they did not report if the same strains is able to produce EPS from lactose (de Paiva et al., 2016).

It was depicted that the existence of peptides in kefir samples with biological activity such as antihypertensive, antimicrobial, immunomodulatory, opioid and antioxidative functions (Ebner et al., 2015, Dallas et al., 2016). Several recent studies revealed that the administration of kefir or commercial peptides from kefir reduced weight gain in obese mice (Bourrie et al., 2018, Tung et al., 2018). Santanna et al. (2017) demonstrated that the administration of nonmicrobial fraction resulted in a considerable decrease in vascular lipid deposition. Likewise, Brasil et al. (2018) showed that the nonmicrobial fraction of kefir hinders angiotensin-converting enzyme and decreases hypertension, this effect might be ascribed to the bioactive peptides release from milk proteins by kefir microorganisms.

1.4 *Kluyveromyces marxianus*: An emerging yeast cell factory for applications in food and biotechnology

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RÉSUMÉ

Plusieurs levures, qui sont des micro-organismes eucaryotes, sont utilisées depuis longtemps dans différentes industries en raison de leurs applications potentielles, à la fois pour la fermentation et pour la production de métabolites spécifiques. *Kluyveromyces marxianus* est l'une des levures non conventionnelles les plus propices, généralement isolée de vastes habitats naturels tels que les produits laitiers traditionnels fermentés, les grains de kéfir, les eaux usées des industries sucrières, les feuilles de sisal et les plantes. Il s'agit d'une levure de qualité alimentaire avec divers traits bénéfiques, tels qu'un taux de croissance rapide et une thermotolérance qui la rendent attrayante pour différentes applications alimentaires, industrielles et biotechnologiques. *K. marxianus* est une levure respiro-fermentative susceptible de produire de l'énergie par voie respiratoire ou fermentaire. Elle génère un métabolite spécifique de grande importance et pourrait contribuer à une variété d'utilisation dans des industries alimentaires et biotechnologiques. Bien que *Saccharomyces cerevisiae* soit le représentant dominant le plus largement utilisé dans tous les aspects liés à l'alimentation, de nombreuses applications de *K. marxianus* dans la biotechnologie, l'alimentation humaine et l'environnement ne font que commencer à émerger de nos jours. Ainsi, certaines des applications les plus prometteuses de cette levure sont examinées dans la présente revue. La physiologie générale de *K. marxianus* est tout d'abord présentée, puis les différentes applications sont discutées. D'abord, les applications de *K. marxianus* en biotechnologie, puis les avancées récentes et les applications possibles dans les industries alimentaires et les applications environnementales sont présentées. Enfin, cette revue propose une discussion des principaux défis et quelques perspectives pour des applications ciblées de *K. marxianus* dans le domaine alimentaire moderne et la biotechnologie appliquée afin d'exploiter tout le potentiel de cette levure qui peut être utilisée comme une usine cellulaire avec une grande efficacité.

Mots clés: *Kluyveromyces marxianus*; Enzymes; métabolites; Biotechnologie; Nourriture.

ABSTRACT

Several yeasts, which are eukaryotic microorganisms, have long been used in different industries due to their potential applications, both for fermentation and for the production of specific metabolites. *Kluyveromyces marxianus* is one of the most auspicious nonconventional yeasts, generally isolated from wide-ranging natural habitats such as fermented traditional dairy products, kefir grain, sewage from sugar industries, sisal leaves, and plants. This is a food-grade yeast with various beneficial traits, such as rapid growth rate and thermotolerance that make it appealing for different industrial food and biotechnological applications. *K. marxianus* is a respiro-fermentative yeast likely to produce energy by either respiration or fermentation pathways. It generates a wide-ranging specific metabolite and could contribute to a variety of different food and biotechnological industries. Although *Saccharomyces cerevisiae* is the most widely used dominant representative in all aspects, many applications of *K. marxianus* in biotechnology, food and environment have only started to emerge nowadays; some of the most promising applications are reviewed here. The general physiology of *K. marxianus* is outlined, and then the different applications are discussed: first, the applications of *K. marxianus* in biotechnology, and then the recent advances and possible applications in food, feed, and environmental industries. Finally, this review provides a discussion of the main challenges and some perspectives for targeted applications of *K. marxianus* in the modern food technology and applied biotechnology in order to exploit the full potential of this yeast which can be used as a cell factory with great efficiency.

Keywords: *Kluyveromyces marxianus*; Enzymes; Metabolites; Biotechnology; Food.

1.4.1 Introduction

Yeasts, a heterogeneous group of eukaryotic fungi that mostly exists as a unicellular organism, has had diversified applications and spectacular impact on industrial, biotechnological, medial and environmental arena from the rise of ancient civilizations until today. Yeasts seem to have many advantageous phenotypes, such as high secretion capacity, high growth rate on a wide variety of carbon sources, potential to compartmentalize reactions in organelles, ability to carry out many post-translational modifications, easy to cultivate in small vessels or large bioreactors, easy product purification, and an absence of susceptibility to infectious agents like bacteriophages. Those attractive traits make them specially suitable for extensive applications in different sectors (Wagner and Alper, 2016). Consequently, the number of the described yeast species is increasing every year; however, biotechnological or industrial applications are limited to a small number of species, mostly belonging to *Saccharomyces cerevisiae* (*S. cerevisiae*) and its associated synonymous species (e.g., *S. uvarum*, and *S. carlsbergensis*), *Candida utilis* (*C. utilis*) and its sexual form (*Hansenula jadinii*), *Kluyveromyces marxianus* (*K. marxianus*) including its asexual forms (e.g., *K. fragilis*, *K. bulgaricus*, *C. pseudotropicalis*, and *C. Kefyr*), *Yarrowia lipolytica* (*Y. lipolytica*), and *Pichia pastoris* (Lane and Morrissey, 2010, Wagner and Alper, 2016). Among them, *S. cerevisiae* has a dominating position, as it is a widely employed and mostly used microbe in the field of biotechnology, due to its availability, genetic tractability, well-annotated genome, well-known physiology, and over-all ease of use. Furthermore, this model yeast has traditionally occupied the field of modified yeasts for industrial processing (Wagner and Alper, 2016). Other than that of conventional yeast, there are various nonconventional yeast species with beneficial characteristics for industrial bioprocesses application, some of which are also getting importance, which includes *Y. lipolytica*, *P. pastoris*, *K. lactis*, and *H. polymorpha* (Gellissen et al., 2005, Wagner and Alper, 2016). Among the nonconventional yeasts, the species of the genus *Kluyveromyces* were observed to have potential for many industrial applications. Particularly, *K. lactis* was the first species after *S. cerevisiae*, to be given Generally Regarded As Safe (GRAS) status (Bonekamp and Oosterom, 1994), a crucial precondition to be used for biotechnical applications.

Recently, the yeast *K. marxianus*, a sister species of *K. lactis*, has piqued particular research interest, due to its beneficial traits that render it exceptionally suitable industrial

application. These features mainly include the ability to utilize a broad range of sugars including lactose and inulin, thermotolerance, secretion of lytic enzymes, the highest growth rate than other eukaryotes, and the production of fuel ethanol by fermentation (Lane and Morrissey, 2010, Varela et al., 2017). *K. lactis* has been the prevalent research species within the general of *Kluyveromyces*, primarily for the studies on lactose metabolism, but later as a model for nonconventional yeasts (Fukuhara, 2006, Spohner et al., 2016). However, in contrast to *K. lactis*, *K. marxianus* has widely been adopted by industries, mainly because it owns some special features of industrial interest that are not present in *K. lactis* (Lane and Morrissey, 2010, Lane et al., 2011) such as its ability to produce inulinase enzyme to hydrolyze complex plant fructans (Arrizon et al., 2011). Moreover, it has a rapid growth compared to *K. lactis*, even at high temperatures (> 40 ℃) (Rouwenhorst et al., 1988); however, the underlying reasons for the faster growth, remained to be determined (Blank et al., 2005). In addition, it has also achieved Qualified Presumption of Safely (QPS) and GRAS status in European Union and United States respectively, due to its long history in safe association and use with regular dairy products, which makes it particularly suitable to produce pharmaceuticals and food-grade proteins.

In contrast to *S. cerevisiae,* most of the strains of *K. marxianus* are apparently Crabtree-negative or an aerobic-respiring characteristic and as such, do not undergo aerobic alcoholic fermentation. This can be a beneficial phenotype for industrial production of those compounds whose titer are linked to biomass formation (i.e., biomass-directed applications) since ethanol formation as a toxic or unintended byproducts under aerobic condition could be avoided (Wagner and Alper, 2016). Moreover, *K. marxianus* can metabolize variety of low-cost substrates including cheese whey or other dairy wastes due to its unique physiological characteristics and capability of producing heterologous proteins as a diligent host. This capacity makes it an indispensable candidate for commercial applications in the pharmaceutical, food, and feed industries (Löser et al., 2013a, Morrissey et al., 2015). However, *K. marxianus* has not been studied at the same extent as *K. lactis*, in spite of its extensive biotechnological applications including the bioethanol production from cheese whey, biomass accumulation, valuable enzyme production such as inulinase and *β*-galactosidase, and so on (Lane and Morrissey, 2010).

Increasing consumer demand for biologically synthesized molecules to be used in the foods and other products, creates a unique opportunity of exploiting the potential of *K. marxianus* in the food and environmental biotechnology applications. The main setback to its advancement has been limited fundamental knowledge of its physiology and genetics, however, this scenario is changing quickly (Morrissey et al., 2015). Most of the improvements have centered on the optimization of growth conditions and the fermentation processes. Further development of new strains by applying evolutionary or rational engineering approaches is still required. Consequently, the possibility of exploiting *K. marxianus* for diversified applications and the necessity for further development of its traits for biotechnological production of different biologically synthesized products has motivated researchers to study its physiology, metabolism mechanism and genomic sequences in more details.

In this review, we set out to explore the possible applications and future potential of *K. marxianus* in biotechnology, food industry and environmental engineering. We begin our review with a short discussion of the physiology, followed by an outline on current applications including its future application strategies and challenges. Thus, it will provide a broad insight into mechanism and limitations of current applications, and the challenges for further development in future application strategies. After all, the comprehensive understanding of its diversified applications will underpin future developments in physiology, genetics, evolutionary engineering strategies, and other important molecular tools of *K. marxianus*.

1.4.2 Taxonomy and physiology of *K. marxianus*

K. marxianus was first identified as yeast belonging to the genus *Saccharomyces* and was named as *S. marxianus*. This microorganism was isolated from beer wort and from grape. The species was then transferred to the genus *Kluyveromyces* and since then, 45 species have been classified to this genus. The closest relative of *K. marxianus* is the yeast *Kluyveromyces lactis*, which is used in the dairy industry for the production of fermented milk such as kefir and kumis.

Moreover, both *Kluyveromyces* and *Saccharomyces* are considered as a part of the *Sacchromyces* complex, subclade of the Saccharomycetes. By using the 18S rRNA gene sequencing technique, it was suggested that *K. marxianus*, *K. aestuarii*, *K. dobzhanskii*, *K.*

lactic, *K. wickerhamii*, *K. blattae*, *K. thermotolerans*, and *K. waltii* collectively constituted a distinct clade of separate ancestry from the central clade in the genus *Kluyveromyces*. Within this complex, two categories are defined based on the presence in certain taxa of a whole-genome duplication event: the two clades are referred to as pre-Whole Genome Duplication (WGD) and post-WGD. *Kluyveromyces* species are affiliated with the first of this clades while species of *Saccharomyces* belong to the latter. Separation of these clades based on the presence of the WGD event explains why, even though the two species are closely related, fundamental differences exist between them (Lane and Morrissey, 2010, Lane et al., 2011).

K. marxianus is well known due to the frequent connection with traditional dairy products like fermented milk, kefir, yoghurt, cheese and so on (Coloretti et al., 2017). It is a dairy yeast that has also been previously described by synonyms in scientific literature, notably *K. fragilis* and *S. keyfr* (Reed, 2012, Morrissey et al., 2015). *Kluyveromyces* is a genus within hemiascomycetous yeasts and contains six described species abide by the reclassification into monophyletic genera based on 26S rDNA sequence, from which two species, *K. lactis* and *K. marxianus* are very prominent (Lachance, 2007). The main popular traits of *K. marxianus* and *K. lactis* is their ability to assimilate lactose and to utilize this sugar as carbon source. This is one of the most important features that distinguish them from *S. cerevisiae*, which lacks this phenotype (Lane and Morrissey, 2010). As a result, they are considered as lactose positive yeast and they do carry *LAC12-LAC4* gene pair that is accountable for uptake and subsequent cleavage of lactose into galactose and glucose (Vigneault et al., 2007). Although lactose utilization is one of the long considered distinguishing traits of *K. marxianus*; however, it was interesting that most, but not all, stains of *K. marxianus* were reported to consume lactose efficiently. This is due to the polymorphisms in the *LAC12* gene, that is responsible for encoding a permease to transport lactose molecules into the cell (Varela et al., 2017). Generally, the *LAC12* encodes a lactose permease which is essential to uptake lactose into cell, while *LAC4* encodes a β-galactosidase, which is responsible for hydrolyzing lactose to the monomers, galactose and glucose. Although the evolutionary history of *LAC12-LAC4* gene pair is not clear enough, their regulation is well-incorporated with the *Gal4p/Gal80p* system, which is comprehensively explored in *S. cerevisiae* (Lane and Morrissey, 2010). The important

features of *K. marxianus* have been compared with *K. lactis* and *S. cerevisiae* as presented in **Table 1.8**.

K. marxianus is a yeast whose metabolism is of the respiratory-fermentative type, similar to that of *S. cerevisiae* which is widely used in brewery and bread making. Thus, *K. marxianus* produces energy by the TCA cycle following oxidative phosphorylation, as well as by the fermentation of sugars into ethanol. In reference to *S. cerevisiae*, it strongly follows the pathway towards fermentation in high sugar concentration even under aerobic conditions, implying that the cell preferentially directs pyruvate to the production of ethanol. This phenomenon is called Crabtree effect. Because of the strong 'Crabtree-positive' characteristics of *S. cerevisiae*, it usually needs a regulated supply of the carbon source to evade fermentative metabolism, which is extremely unwelcomed in biomass-directed applications. By contrast, *K. marxianus* and *K. lactis* are categorized as Crabtree-negative by tradition, meaning an incompetence to efficiently ferment sugars to ethanol (Fonseca et al., 2008, Lane et al., 2011). In this context, it is worth mentioning that that there are some contradictory reports in the literature of the 'Crabtree status' of *K. marxianus* and *K. lactis* (Merico et al., 2009). The prevalent reports of application in ethanol production suggest that both species do carry the genes required for ethanol production and could adopt the fermentation life style under certain conditions (Merico et al., 2007). However, it has been demonstrated that *K. marxianus* exhibits a strong 'Crabtree-negative' property and this was supported by the fact that it does not produce ethanol, contrary to what is largely observed in the case of *S. cerevisiae* known to be a big ethanol producer or *K. lactis* to a lesser extent (Merico et al., 2007, Merico et al., 2009). This apparent conflicting finding is probably due to the strain variability, as most of the yeast comparative studies utilized only one representative strain of each species and the level of physiological variation within a species was not evaluated. It can be concluded that a high degree of intra-species disparity exists for this yeast, not only in terms of its genetics, but also of its physiology (Lane et al., 2011).

Table 1.8: The important physiological characteristics of *K. marxianus* in comparison with *K. lactis* and *S. cerevisiae* as model yeasts.

Features	***S. cerevisiae***	***K. lactis***	***K. marxianus***	**References**
Energy metabolism	Alcoholic fermentation (Crabtree-positive)	Respiration (Crabtree-negative)	Respiro-fermentative, Respiration [a] & fermentation [b] (Crabtree-negative)	(Lane and Morrissey, 2010, Rodrussamee et al., 2011, Madeira-Jr and Gombert, 2018)
Generation time [c]	80 min	80 min	70 min	(Rodicio and Heinisch, 2013)
Glucose utilization route	Mostly glycolysis	Pentose phosphate (PP) pathway and glycolysis	-Embden–Meyerhof–Parnas pathway (EMP) -Preferentially, energy generation either *via* direct metabolism towards TCA cycle by oxidative phosphorylation or by fermentation to ethanol -Capable of carrying out simultaneous fermentation and respiration	(Blank et al., 2005, Lane and Morrissey, 2010, Rodicio and Heinisch, 2013)
Sensibility to glucose repression	High sensibility	Low sensibility	Low sensibility, since the fermentative and oxidative metabolism can occur	(Silveira et al., 2005, Lane et al., 2011)

			simultaneously. This trait is variable across *K. marxianus* strains.	
Genome structure and Ploidy	Haploid, diploid or/even polyploid	Mainly haploid and can mate to form an unstable diploid which reverts to the haplo-phase immediately	Less clear yet but both haploid and diploid lifestyle have been reported. Triploidy has been identified in *K. marxianus* .	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013, Ortiz-Merino et al., 2018)
Ploidy (Haploid lifestyle)	Stable and readily mate to form diploid (*MAT***a**/*MATα*)	Stable (*MAT***a** or *MATα*)	Stable (*MAT***a** or *MATα*)	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013)
Ploidy (Diploid lifestyle)	Stable and easily be induced to undergo meiosis to reform haploid progeny	Semi-stable	Stable	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013)
Mendelian genetics (crossing and tetrad analysis)	Yes	Yes	Yes	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013, Yarimizu et al., 2013, Cernak et al., 2018)

Vectors, selection and transformation classification	Available	Available	Available	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013, Nurcholis et al., 2020)
Homologous recombination	Excellent	Good [d]	Generally considered as poor. Some studies referred as good.	(Lane and Morrissey, 2010, Rodicio and Heinisch, 2013, Choo et al., 2014)
Genome sequence	Available (post-WGD)	Available (no-WGD) [e]	Available (pre-WGD)	(Lane and Morrissey, 2010, Jeong et al., 2012, Rodicio and Heinisch, 2013, Inokuma et al., 2015, Lertwattanasakul et al., 2015)
Deletions of ORE	Completely collected by Berkeley, EUROSCARE	Partial and scattered [f]	Partial, scattered, though some heterogenous protein generation systems become available	(Lane and Morrissey, 2010, Rodrussamee et al., 2011, Rodicio and Heinisch, 2013)
Beneficial Phenotype	-High bioethanol production	-Ability to grow on lactose	-Ability to grow on wide-range of sugars	(Löbs et al., 2017, Varela et al., 2017)

-High homogenous recombination capacity -Established physiology and genomics -Advanced synthetic biology tools	-Capacity of high protein secretion	-Faster growth capability -Thermotolerance -High ethyl acetate production -Capacity of protein secretion

[a] Energy release (respiration): $C_{12}H_{22}O_{11} + H_2O + 12O_2 \xrightarrow{Yeast} 12CO_2 + 12H_2O + \Delta E_1$

[b] Energy release (fermentation): $C_{12}H_{22}O_{11} + H_2O \xrightarrow{Yeast} 4C_2H_5OH + 4CO_2 + \Delta E_2$

[c] Approximate generation times for laboratory with a standard deviation of ± 10 min at 30 °C.

[d] Homologous recombination could be upgraded using a *Klku80* deletion background.

[e] Whole Genome Duplication (WGD) event could not happen in *K. lactis* resulting in less redundancy of genes encoding iso-enzymes.

[f] Very limited collection of deletion mutants and usually each group working on a special pathway disposes of its own.

1.4.3 Applications in biotechnology

1.4.3.1 Native enzymes production

K. marxianus is considered as a favorable host for producing extracellular proteins due to its capacity to grow on various low-cost substates such as spent sulphite liquor, molasses, and whey (Hensing et al., 1994, Fonseca et al., 2008). Moreover, *K. marxianus* is capable to grow on several polysaccharides including pectin, and inulin (Hensing et al., 1994). *K. marxianus* possess natural ability to secrete enzymes,, since all these aforementioned complex materials are hydrolyzed extracellularly to monomers (Chi et al., 2011). The growth of *K. marxianus* on feedstocks such as sucrose and inulin proceeds through the activity of extracellular enzymes, particularly inulinase (*β-1,2-D*-fructan fructanohydrolase) which is excreted in the culture medium but also retained in the cell wall (Rouwenhorst et al., 1988). The enzyme that retained in the cell wall is a tetramer whereas the secreted enzyme in the culture fluid is a dimer (Hensing et al., 1994). The latter portion comprises more than 60% of total excreted proteins in the culture fluid for certain *K. marxianus* strains, indicating that the inulinase is expressed from a strong promoter and its secretion is instigated by an effective signal sequence (Zhou et al., 2018). Furthermore, lactose degradation by excreting *β*-galactosidases is very promising due to the simultaneous production of bioingredients, biomass, and enzymes of industrial interest (Fonseca et al., 2008, Chi et al., 2011). The schematic representations in **Figure 1.8** show the uptake mechanism and metabolism in the yeast cells. The enzyme expressing efficiency of different *K. marxianus* strains is summarized in **Table 1.9** and described below.

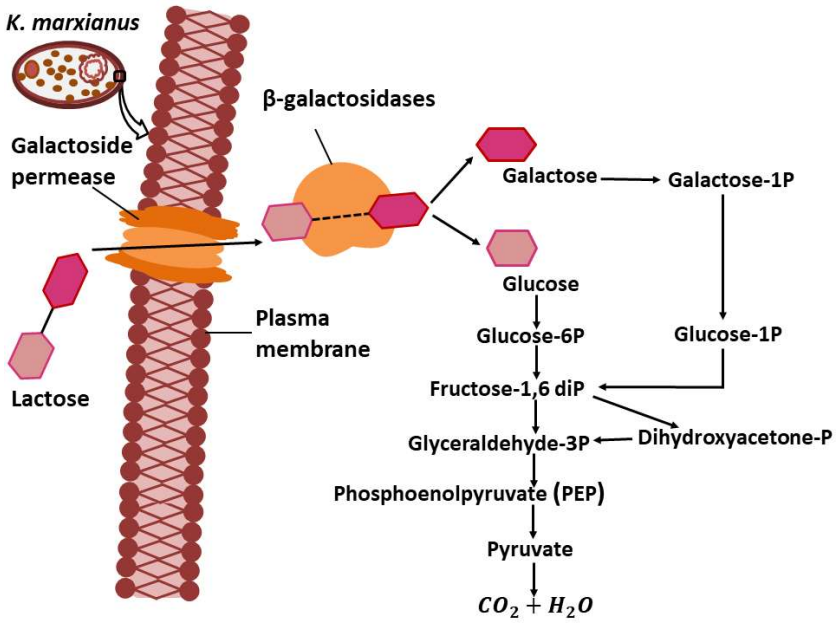

Figure 1.8: Schematic diagram of the catabolic pathway of lactose and galactose by *K. marxianus*.

1.4.3.1.1 Inulinase production

Inulinase has gained intertest in recent time, as it is widely used to hydrolyze inulin (Jerusalem artichoke, chicory roots, dahlia tubers) to produce bioethanol, fructose, and fructo-oligosaccharides, all of which are important ingredients in food and pharmaceutical industry (Gao et al., 2009, Hoshida et al., 2018). The enzymes, inulinases (EC 3.2.1.7) have been produced from different microbes such as yeast *Cryptococcus aureus* (Sheng et al., 2007), *K. marxianus* (Selvakumar and Pandey, 1999), filamentous fungi *Aspergillus niger*, *Aspergillus fumigatus* (Kango, 2008), *Penicillium* sp. (Moriyama and Ohta, 2007), *Rhizopus* sp. (Ohta et al., 2002), and bacteria *Streptomyces* sp. , *Bacillus* sp. (Gao et al., 2009), *Staphylococcus* sp. (Selvakumar and Pandey, 1999), *Xanthomonas*, and *Pseudomonas* (Kalil et al., 2005). However, the strains of *A. niger* and *K. marxianus*, have been described as the most promising microbes among the diverse kinds of microbial strains for inulinase production (Zhang et al., 2012).

Nevertheless, *K. marxianus* is considered as the most potential to produce inulinase enzyme at a commercially acceptable yields (Kalil et al., 2005, Zhou et al., 2014b). A study with fourteen yeast strains of some genera like *Kluyveromyces*, *Debaryomyces*, *Candida*, and *Schizosaccharomyces* revealed that the strain *K. marxianus* ATCC 36907 possess high

potential to produce inulinase. The enzymes produced by this microbe were shown to be stable for 60 min at low pH (4.0) and high temperature (45 ºC) (Passador-Gurgel et al., 1996). In another study, *K. marxianus* CBS 6556 exhibited superior properties than other strains in terms of high temperature, substrate specificity, and inulinase production (Rouwenhorst et al., 1988). The highest enzyme production was encountered at temperatures between 37 and 42 °C, corresponded to the optimal temperature for growth of *K. marxianus*. The high temperature for the optimum growth is particularly interesting for commercial application as this enables cooling during large-scale fermentation process where heat transfer is a limiting factor. In a recent study, higher inulinase production on xylose medium was observed with higher agitation rate in the culture media using *K. marxianus* DMKU3-1042, indicating that the oxygen supply affects the inulinase production (Hoshida et al., 2018). However, this effect of higher agitation on inulinase production probably specific for xylose medium because the inulinase production by *K. marxianus* var. *bulgaricus* ATCC16045 did not enhance by increased oxygen supply in sucrose medium (Silva-Santisteban et al., 2009). Nevertheless, Singh and Bhermi (2008a) observed that a higher agitation and aeration decreased the inulinase production by *K. marxianus* YS-1 using inulin as carbon source (Singh and Bhermi, 2008b).

Table 1.9: The efficacy of different *K. marxianus* strains with enzyme expressing activity.

Native enzymes	Strains	Cultivation medium	Specific enzyme activity [a]	References
Inulinase	*K. marxianus* YS-1	Liquid culture	55.40	(Singh et al., 2007a)
	K. marxianus ATCC 16045	Liquid culture	121	(Silva-Santisteban and Maugeri Filho, 2005)
	Kluyveromyces sp. Y-85	Liquid culture	59.50	(Xiong et al., 2007)
	K. marxianus var. *bulgaricus* ATCC 16045	Continuous cultivation	107	(Kushi et al., 2000)
	K. marxianus NRRL Y-7571	Solid-state fermentation	391.9 U g^{-1} of dry fermented bagasse	(Bender et al., 2006)
	K. marxianus NRRL Y-7571	Liquid culture	8.87 U g_{ids}^{-1} h^{-1}	(Mazutti et al., 2006)
	K. marxianus S120	Solid-state fermentation	409.8 U g_{ids}^{-1}	(Xiong et al., 2007)
	K. marxianus NRRL Y-7571	Solid-sate fermentation	436.7 ± 36.3 U g_{ids}^{-1}	(Mazutti et al., 2010)
	K. marxianus CBS 6556	Liquid culture	52 U mg^{-1} cell dry weight	(Rouwenhorst et al., 1988)
	K. marxianus ATCC 52466	Solid-sate fermentation	122.88 U g^{-1} of dry fermented substrate	(Selvakumar and Pandey, 1999)

	K. marxianus CBS 6556	Liquid culture (Fed-batch)	>2 g L^{-1}	(Hensing et al., 1994)
	K. marxianus NRRL Y-7571	Solid-sate fermentation (Fixed-bed bioreactor)	219 U/mg	(Golunski et al., 2011)
	K. marxianus CBS 6556	Liquid culture	2714 U/mg	(Zhang et al., 2012)
	K. marxianus KM-526	Liquid culture	133.5	(Zhou et al., 2014b)
	K. marxianus DMKU 3-1042	Liquid culture	330	(Hoshida et al., 2018)
	K. marxianus YS-1	Liquid culture	420 IU/mg	(Singh et al., 2017a)
β- Galactosidase	*K. marxianus* KM-15	Liquid culture	121	(Zhou et al., 2013)
	K. marxianus CBS 6556	Liquid culture	1400 U/OD_{600}	(Martins et al., 2002)
	K. marxianus CBS 712	Liquid culture	333.8 U_{ONPG}/g lactose	(Rech et al., 1999)
	K. marxianus CBS 6556	Liquid culture	129.7 U_{ONPG}/g lactose	(Rech et al., 1999)
	K. marxianus NRRL Y-1109	Liquid culture	2800 ± 250 U/g, 32,700 ± 2000 U/L	(Cortés et al., 2005)
	K. marxianus MTCC 1388	Liquid culture	1.14 U/mg	(Bansal et al., 2008)
	K. marxianus CCT 7082	Liquid culture	463 U/g	(Manera et al., 2011)
	K. marxianus ATCC 16045	Submerged cultivation	10.4	(Braga et al., 2012)

β- Xylosidase	*K. marxianus*	Liquid culture	333 IU/g cells on xylose	(Rajoka, 2007)
Pectinase	*K. marxianus* CCT 3172	Liquid culture	21.69 µmol galacturonic acid/µg protein/ min	(Schwan et al., 1997)
	K. marxianus P 5656	Liquid culture	0.78	(Harsa et al., 1993)
Lipolytic enzyme	*K. marxianus* CECT 1018	Liquid culture	80	(Deive et al., 2003)
Endo-poly-galacturonase	*K. marxianus* CCT 3172	Continuous production packed bed reactor (PBR)	7.82	(Almeida et al., 2003).
	K. marxianus CCT 3172	Continuous stirred tank reactor (CSTR)	1.01	(Almeida et al., 2003).
	K. marxianus ATCC 36907	Liquid culture	1 UE/mg	(Dinnella et al., 1996)

Polygalacturonase	*K. marxianus* 166	Liquid culture	14.2 µmol of galacturonic acid/µg protein/ min	(da Silva et al., 2005)
Protein phosphatases	*K. marxianus* (strain not indicated)	Liquid culture	437.62 nmol/min/mg	(Jolivet et al., 2001)
Carboxy peptidases	*K. marxianus* (own isolate)	Liquid culture	5.43 U/mg	(Ramírez-Zavala et al., 2004)
Amino peptidases	*K. marxianus* (own isolate)	Liquid culture	22.53 U/mg	(Ramírez-Zavala et al., 2004)
Endo-*β*-1,4-glucanase	*K. marxianus* NBRC 1777	Liquid culture	1.6	(Hong et al., 2007)
***β*-glucosidase**	*K. marxianus* NBRC 1777	Liquid culture	83 m U/mL	(Hong et al., 2007)
Cellobiohydrolase	*K. marxianus* NBRC 1777	Liquid culture	60 µU/mL	(Hong et al., 2007)

Lactate dehydrogenase	*K. marxianus* KM-1	Liquid culture	8.4 U/OD_{600}	(Pecota et al., 2007)
α-Galactosidase	*K. marxianus* CBS 6556	Liquid culture	153	(Bergkamp et al., 1993)
Cu/Zu superoxide dismutase	*K. marxianus* NBIMCC 1984	Liquid culture (batch)	996 U/mg of protein	(Nedeva et al., 2009)
Serine protease	*K. marxianus* IFO 0288	Liquid culture	4.47 I U/mg	(Foukis et al., 2012)

[a] U mL^{-1} unless otherwise mentioned; Initial dry substrate (ids);

Apart from that the inoculum size is also important in fermentation process to produce inulin because a lower inoculum size may result in an insufficient biomass production and permit the growth of unexpected organisms while a higher inoculum density can produce too much biomass and reduce the nutrients required for production formation (Selvakumar and Pandey, 1999). Selvakumar and Pandey (1999) observed that the inulinase production by *K. marxianus* reached a maximum value of 116.43 U/gds with a 4% inoculum size at 72 h. Cruz-Guerrero et al. (1995) reported that *K. marxianus* CDBB-L-278 was a hyperproducing strain for inulinase. This strain demonstrated a high resistance to 2-deoxyglucose, however, inulinase production was under catabolic repression. The catabolic repression was created when glucose or fructose were enhanced from 0.25 to 1% in the medium. Furthermore, a batch fermentation with a 4% inulin and 4% glucose confirmed that the stain CDBB-L-278 was under catabolic repression because inulinase was not produced until the level of glucose were low enough (Cruz-Guerrero et al., 1995). Kushi et al. (2000) achieved the highest yield of inulinase with sucrose as substrate in carbon and energy-limited continuous cultures. Nevertheless, the lower enzyme activities were observed with higher the higher concentrations of residual substrate, indicating that the enzyme synthesis is regulated by catabolic repression/residual sugar concentration in the medium (Rouwenhorst et al., 1988, Kushi et al., 2000). It is possible, however, to produce pyruvate and acetate while *K. marxianus* subjected to excess sugar. This tendency may be a problem during large-scale fermentation because of the presence of sugar gradients in the reactor (Rouwenhorst et al., 1988). The distribution of enzyme is also influenced by the growth parameters. Indeed, it has been reported that important content of enzyme in the growth medium after centrifugation with a concomitant decrease in the amount of cell wall enzyme was obtained when growth (production) temperatures below the optimal temperature interval were used. The inverse situation was observed when growth temperatures higher than the optimal values were used. Also, it has been anticipated that growing of *K. marxianus* at higher pH values can contribute to enhance the relative amount of enzyme excreted into the culture medium. This was explained by the fact that the increased pH stimulated the process of inulinase release from the yeast cell wall (Rouwenhorst et al., 1988). Moreover, carbon-limited growth on glucose or lactose or nitrogen-limited growth on sucrose resulted in the higher percentages of

inulinase production in the culture medium and low amounts of enzyme in the cell wall (Rouwenhorst et al., 1988).

The inulinase production from *Staphylococcus* sp. RRL-1 and *K. marxianus* ATCC 52466 was investigate by Selvakumar and Pandey (1999) using agro-industrial wastes such as rice bran, wheat bran, corn flour, and coconut oil cake via solid-state fermentation. It was found that bacterial culture took a relatively shorter time (48 h) than the yeast culture (72 h) to attain the maximal yield (90.53 U/gds), however, the yeast culture produced comparatively higher yield (106.37 U/gds) of enzyme at 37 ℃ (Selvakumar and Pandey, 1999). Furthermore, *K. marxianus* var. *bulgaricus* ATCC16045 produced higher amounts of inulinase while grown on glucose, sucrose, fructose, inulin, and raffinose as carbon sources (Kushi et al., 2000). Nevertheless, they observed that the inulinase activity was significantly higher for the inulin from Dahlia tubers than that of other carbon sources. Similarly, Gao et al. (2009) reported that the inulin was the best carbon source (inulin>lactose>fructose>mannitol>glucose>sucrose) to produce inulinase using *Bacillus smithii T7*, *K. marxianus*, *Cryptococcus aureus G7a*; which indicated that the inulinase is an inducible enzyme. They also demonstrated that the enzyme activity was increased (69.5 IU/mL) with increase of inulin concentration up to 2% and then decreased in activity was noticed. Furthermore, the inulinase production was enhanced to 104.3 IU/mL by using $(NH_4)H_2PO_4$ up to 0.5% (w/v) as nitrogen source, however, the greater concentration of $(NH_4)H_2PO_4$ had an inhibitory effect on inulinase synthesis (Gao et al., 2009).

Although it has been reported by several authors (Grootwassink and Hewitt, 1983, Parekh and Margaritis, 1986, Gao et al., 2009) that the production of inulinase was inducible, but the inulinase production was partially constitutive for the strains *K. marxianus* CDBB-L-278 and *K. marxianus* NCYC-1429, since the inulinase (up to 20% of the total activity attained with inulin) was produced using glycerol as carbon source without inulin, which does not present neither induction nor catabolite repression (Cruz-Guerrero et al., 1995). Glucose and fructose, on the other hand, acted as both inducers and repressors (Grootwassink and Hewitt, 1983, Parekh and Margaritis, 1986, Gao et al., 2009). It has been generally known that the expression of most of the inulinase genes in a native producer was repressed by glucose and fructose and induced by inulin and sucrose (Liu et al., 2013). The inulinase activity by *K. marxianus* KM-0 was increased when glucose concentration increased to 20

g/L from 10 g/L, however, the activity was decreased for further increment of glucose to 80 m/L (Zhou et al., 2014b). This attributed to the fact that the biosynthesis and secretion of inulinase in this strain was, indeed, repressed by glucose. The transcriptional repressor *Mig1* encoded by *MIG1* played as a central component for glucose repression. However, *K. marxianus* KM-69 with the disrupted *MIG1* gene, produced higher inulinase (101.7 U/mL) compared to the wild type strain *K. marxianus* KM-0 (84.3 U/mL) (Zhou et al., 2014b). They also showed that the overexpression of the native inulinase gene from the wild type strain KM-0 into the disruptant KM-69 could further enhance the inulinase activity to 119.3 U/mL using a recombinant strain, namely *K. marxianus* KM-526 (Zhou et al., 2014b). The secretory expression of inulinase enzyme in *K. marxianus* could be further improved by increasing the efficiency of the inulinase-encoding gene (*INU1*) promoter and signal sequence engineering (Zhou et al., 2018). The lower Michaelis–Menten constant (Km) value (3.04 mM) of the purified inulinase from *K. marxianus* CDBB-L-278 (Cruz-Guerrero et al., 1995) compared to inulinases from other microorganisms, including *C. aureus* G7a (20.06 mg/mL) (Sheng et al., 2008), *B. subtilis* 430A (8 mM) (Uzunova et al., 2001), and *B. smithii* T7 (4.17 mM) (Gao et al., 2009), could make *K. marxianus* a better candidate for inulin hydrolysis. Moreover, the enzymes from *K. marxianus* have a good thermal stability up to 50-55 °C (Cruz-Guerrero et al., 1995, Kushi et al., 2000). Therefore, the inulinases produced by *K. marxianus* could be broadly used in biotechnology, pharmaceutical, food, feed, chemical and biofuel industries (Chi et al., 2011).

1.4.3.1.2 β-galactosidases production

β-galactosidases are the mostly used enzymes in the food processing industries. *β*-galactosidases (EC 3.2.1.23), generally well-known as lactase, that catalyzes the hydrolysis of lactose, and producing a mixture of galactose and glucose. In particular, *β*-galactosidases have significant applications in the food and pharmaceutical industries as it is used for saccharification of whey and, in the treatment of milk for reducing lactose content (Singh et al., 2016). The latter use is mainly related to the populations with the genetic deficiency for lactose metabolism, such as the black populations in Brazil, United States, and Central Africa (Belem and Lee, 1998, Bayless et al., 2017). Various strains of *Kluyveromyces* were reported as efficient for the industrial production of *β*-galactosidases (Hensing et al., 1994, Oliveira et al., 2011). Various approaches with different cultivation strategies were used to produce *β*-

galactosidases from industrial media like molasses (Morrissey et al., 2015) and cheese whey (Rech et al., 1999, Padilla et al., 2015). The hydrolytic and transgalactosylic activities, which are indispensable in food processing, usually carried out using commercial *β*-galactosidases (Pivarnik et al., 1995). The hydrolytic activity is used to reduce lactose content in milk in the food industry, while the transgalactosylation activity is performed to synthesize the galactose and galacto-oligosaccharides containing chemicals (Oliveira et al., 2011). Recently, Padilla et al. (2015) described that the *β*-galactosidases from *K. lactis* and *K. marxianus* could be successfully used for the production of lactulose oligosaccharides through isomerization of transgalactosylated whey permeate.

The regulation of *β*-galactosidases expression in *K. marxianus* occurred by its natural inducers, galactose and lactose. However, the production of *β*-galactosidases was dependent on substrate concentration. A repressive mechanism was superimposed to the inducing effect of the substrate while *K. marxianus* were exposed to a higher concentration of lactose or D-glucose, consequently, the *β*-galactosidases activity was decreased (Martins et al., 2002). This might be due to the accretion of intermediate glycolysis metabolites when microbial cells consumed D-glucose or lactose at a high rates (Martins et al., 2002). In a previous study, Zhou et al. (2013) reported that the biosynthesis of *β*-galactosidase suppressed by glucose, however, its production was derepressed after removal of the *MIG1* gene. They also found that, after disrupting the *MIG1* gene of *K. marxianus* KM, the disruptant (*mig1* mutant) *K. marxianus* KM-15 strain achieved more *β*-galactosidase than *K. marxianus* KM. A galactosidase activity of 121 U/mL was obtained by the disruptant KM-15 in 60 h under the optimal conditions. Likewise, Bergkamp et al. (1993) achieved a high *β*-galactosidase activity of 153 mg/L and secretion efficiency of 99% by addressing the *INU1* promoter and signal sequence in *K. marxianus*.

Besides the substrate concentration, other operational parameters including pH, incubation time, temperature, inoculum size, and age significantly influenced the *β*-galactosidases activity. The optimum growth and higher *β*-galactosidases activity were noticed at a temperature of 37 ℃ and a pH of 5.5. using *K. marxianus* CBS6556 and CBS712 in cheese whey (Rech et al., 1999). Panesar (2008) obtained the maximum activity (1580 IU/g dry weight) with an optimal conditions of temperature 30 ℃, pH 5.0, inoculum size 6% (v/v) having 20 h age, shaking 100 rpm after 28h of incubation time using *K. marxianus*

NCIM 3465 in whey. Gupte and Nair (2010) observed maximum *β*-galactosidase activity of *K. marxianus* NCIM 3551 at 25 ℃ temperature, pH 5.0, and inoculum size of 10% after a 20 h of incubation time. An incubation period extending up to 30 h was also reported for *K. marxianus* MTCC 1388 (Bansal et al., 2008). However, a decrease in the enzyme activity was noticed with further increase in the incubation time (24 h to 36 h), attributed to the fact that the growth of culture reached the stationary phase (Panesar, 2008, Gupte and Nair, 2010). The earlier workers reported that the enzyme activity probably started after the lag phase and highest yield obtained at the beginning of the stationary phase of growth (Pinheiro et al., 2003). The enzyme activity remained quasi-constant during the stationary phase, thereafter the yield of the enzyme decreases (Rech et al., 1999, Panesar, 2008, Gupte and Nair, 2010). Temperature also strongly influenced the enzyme activity and an optimum temperature of 28-31 ℃ has been mostly used in the earlier studies (Panesar, 2008, Gupte and Nair, 2010).

The enzyme activity was increased with the agitation mode attributed to the uniform distribution of yeast culture in the medium resulting in oxygen transfer rate (OTR) and nutrient availability (Panesar, 2008). According to some authors (Barberis and Gentina, 1998, Pinheiro et al., 2003), the expression of *β*-galactosidases enzyme was correlated to the OTR because the growth of aerobic cultures could be enhanced by air pressure raise in limited OTR (Belo and Mota 1998). However, in many cases, improved oxygen partial pressure can be toxic to the aerobic cultures and inhibits growth and product formation because of the formation of reactive oxygen species (ROS) (Onken and Liefke, 1989). *K. marxianus* possess several defensive mechanisms such as induction of antioxidant enzyme superoxide dismutase (SOD) in air pressure raise (Pinheiro et al., 2003, Dellomonaco et al., 2007). Pinheiro et al. (2003) investigated the impact of total air pressure increase on cell growth and *β*-galactosidases activity of *K. marxianus* CBS 7894 in batch cultures. They demonstrated that the specific *β*-galactosidase productivity was increased from 5.8 to 16.9 U/g/h using a 6-bar air pressure instead of air at atmospheric pressure Therefore, the rise in air pressure up to 6-bar could be an alternative for preventing the oxygen limitation in *β*-galactosidase production. The influence of the oscillating dissolved oxygen tension (DOT) on the metabolism of *K. marxianus*, in particular, on the *β*-galactosidases production was also investigated by Cortés et al. (2005). They observed that the faster oscillations of DOT can increase the final volumetric and specific enzyme activity. The findings of their study imply

that the *β*-galactosidase production by *K. marxianus* in industrial scales would be more robust in respect to the oxygen variations (Cortés et al., 2005). Furthermore, the extraction methods also have an influence on the activity of *β*-galactosidase since it is an intracellular enzyme. SDS-Chloroform technique was observed to be the best method followed by Toluene-Acetone, sonication, and homogenization with glass beads out of the four methods for extraction (Bansal et al., 2008). In addition, the thermostable characteristics of β-galactosidases produced from *K. marxianus* was studied by Tomáška et al. (1995). The results of this study suggest that *Kluyveromyces* β-galactosidase was thermostable at a temperature of more than 45 ℃ and could be further improved by confinement and stabilization inside the cells or by combining with immobilization technique.

1.4.3.1.3 Pectinase production

Endo-polygalacturonases (EC 3.2.1.15), commonly known as pectinases, hydrolyze pectins. Pectins are heteropolysaccharides comprising the main structural elements of the plant cell walls. Pectinases have been used in the juice and wine manufacturing because of their ability to degrade the cell wall (Alimardani-Theuil et al., 2011). These enzymes are mostly produced by plants and different microorganisms including bacteria, yeasts, and filamentous fungi. Among them, yeast pectinases are of great interest for last one decade (Alimardani-Theuil et al., 2011). Based on the environmental ang genetical background, the pectolytic yeasts can produce different kinds of enzymes. Four species of yeast, namely *Torulopsis kefir*, *S. fragilis*, *S. cerevisiae*, *K. marxianus* have been widely exploited for pectolytic activity. They produce polygalacturonases (PG), pectinlyases (PL), pectinesterase (PE) or pectate lyase (PaL), depending on the temperature, pH, and substrate availability. For instance, *Candida, Sacharomyces,* and *Kluyveromyces* produce PG (mainly endopolygalacturonase), whereas *Rhodotorula* produces both PG and PE (Blanco et al., 1999, Alimardani-Theuil et al., 2011)

K. marxianus was reported as the prominent pectinolytic yeast with 85% of total secreted protein, containing of a constitutive endopolygalacturonase (endo-PG), while compared with 12 cocoa pulp-degrading yeasts (Schwan et al., 1997). Moreover, a simple one-stage purification scheme attained more than 90% recovery of a highly purified PG enzyme from *K. marxianus* fermentation process (Harsa et al., 1993).In a study, Garcia-Garibay et al. (1987) observed that *K. marxianus* can produce endo-PG using whey as carbon

source. They observed that the dissolved oxygen significantly influenced the biomass production and endo-PG synthesis by *K. marxianus*. The production of biomass was very low under anaerobic condition whereas the enzyme activity was maximum at an intermediate level of aeration as enzyme activity associated with growth. However, the enzyme was fully repressed when *K. marxianus* was cultivated under the highest aeration conditions. Although the enzyme was repressed at the high aeration levels, significant amounts of PG produced under such conditions when pectin was complemented as inducer. Under these conditions, the productivity was 4 times higher than the anaerobic fermentation in absence of the inducer. Some other studies exhibited endo-PG activity in glucose as the carbon source, however, PG activity was increased by replacing glucose with galactose in the culture medium (Radoi et al., 2005). Moreover, the transcription of *PGU1* was also improved in the yeast strains when PG activity intensified on galactose compared to glucose (Louw et al., 2009).

The synthesis of microbial enzymes at the industrial scale typically requires highly productive strains to reduce the cost and to enhance the efficacy, however, the regulatory mutants have rarely been exploited. Most of the pectinases were induced by pectins and exposed to catabolic repression, however, the PG of *K. marxianus* is interesting because the production was constitutive and not repressed by carbohydrates. A maximum yield was obtained in a 10% (w/v) glucose under self-induced anaerobic conditions (Schwan and Rose, 1994). Furthermore, PG activity could be increased by adding pectins or their degradation products on fermentation medium (Radoi et al., 2005). Oliveira et al. (2012) demonstrated that *K. marxianus* CCMB 322 can use pectin as a carbon source. Although glucose can considerably affect the regulation of the PG synthesis, and the product of the citrus pectin hydrolysis (galacturonic acid) was the most effective product for induction. Furthermore, the extracellular PG activity was increased (from 0.2126 to 0.7457 μmol/mL/min) while the strain was cultivated in the media combined with glucose (1%) and pectin substances (1%) compared to the individual substrates. This fact suggests that the production of PG using *K. marxianus* CCMB322 was partially constitutive. Likewise, Wimborne and Rickard (1978) demonstrated that the enzyme secretion efficiency *K. marxianus* could be increased to 100% by adding pectin in the culture medium. Low PG activity (except for galacturonic acid) were produced in the absence of glucose suggesting that the product of citrus pectin hydrolysis (galacturonic acid) was the most efficient for PG production by *K. marxianus* CCMB322,

although glucose had an impact on the regulation PG synthesis (Oliveira et al., 2012). However, different results were previously reported by McKay (1988), who demonstrated that the PG activity was constitutive during the growth of *K. marxianus* NCYC 587 on glucose, which unable to grow on polygalacturonic acid as sole carbon source. Likewise, Schwan and Rose (1994) did not observe any significant change in PG activity by addition of pectin, pectic acid or polygalacturonic acid to a medium containing 1% (w/v) glucose using *K. marxianus* CCT 3172. To sum up, the potential effect of carbon sources on PG activity seems to be strain dependent and is associated with other complex means of regulation.

Beside medium composition, the excretion and synthesis of PG enzymes depend on the variables of fermentation and culture medium such as temperature, pH, inoculum size, incubation time, or type of microbial strain. For example, the pectinolytic enzyme secretion capacity was highly efficient for *K. marxianus* isolated from tropical fruits (da Silva et al., 2005). Also, Schwan et al. (1997) observed that the optimum PG activity can be obtained at a pH of 5.0 and a temperature of 40 ℃ using *K. marxianus* CCT 3172. However, the optimum pectinolytic activity was achieved at a pH of 7.36 and a temperature of 70 ℃ using *K. marxianus* CCMB 322 after 48 h of incubation, and 93% of its original activity can be retained for 50 min at 50 ℃ (Oliveira et al., 2012). Moyo et al. (2003) revealed that the PG from *K. wickerhamii* had an optimum pH and temperature of 5.0 and 32 °C, respectively. The results imply that the optimum temperature and pH of *K. marxianus* CCMB 322 were higher than those of *K. wickerhamii* for PG activity. Interestingly, most of the microbial PG enzymes showed optimum activity in the acidic region (pH 4-5). The most interesting factor for biotechnological applications, is the optimum temperature for the activity of these enzymes because some yeast PG can act over a wide range of temperature (0-60 ℃) (Barnby et al., 1990). However, Cordeiro and Martins (2009) revealed that the PG of *Bacillus* sp. SMIA-2 maintained only 70% of activity after heating for 120 min at 50 ℃. Whereas, the PG produced by *K. marxianus* CMB 322 displayed more thermal stability (~93% at 50 ℃), thus it can hydrolyze polygalacturonic acid at the usual commercial temperature (50 ℃) that render this enzyme from *K. marxianus* particularly interesting for use in the fruit juice industry (Oliveira et al., 2012). Furthermore, Sieiro et al. (2014) observed that the endo-PG from *K. marxianus* KMPG enhanced the total compounds responsible for the aroma in white

wines compared to the commercial pectic enzyme. In addition, endo-PG enzyme from *K. marxianus* CCT3172 causes a drastic reduction in the pectin viscosity, is responsible for the depolymerization of pectin, and therefore, has industrial importance in the wine, juice, vegetable and animal feed industry (de Mansoldo et al., 2019). Indeed, the yeast pectinases could potentially be used in various kinds of industries such as, in vegetables and fruits processing, citrus processing, wine making, tea and coffee fermentation, textile processing, and paper making industries (Alimardani-Theuil et al., 2011).

1.4.3.1.4 Lipase production

The term lipolytic enzymes refers to the lipases and carboxylic ester hydrolases. The lipases, also known as triacylglycerol-acyl-hydrolases (EC 3.1.1.3) are hydrolytic enzymes that catalyze both the hydrolysis and the synthesis of esters. They are generally accountable for the hydrolysis of acyl-glycerides, which are indispensable for the bioconversion of lipids (triacylglycerol) in nature (Vakhlu, 2006). Lipases possess several unique features, which includes the stereospecificity, substrate specificity, regiospecificity, and the ability to catalyze a heterogenous reaction at the interface of water insoluble and soluble systems (Sharma et al., 2002). Like the carbohydrases and proteases, the lipases of microbial origin enjoy greater industrial importance as they are more stable than the animal or plant lipases. Moreover, they can be produced in bulk at a low cost (Vakhlu, 2006). In addition, the lipases from microbial origin are more beneficial because of a wide ranging catalytic activities available, rapid growth of microbes on low cost media, absence of seasonal fluctuations, and ease of genetic manipulations (Šiekštelė et al., 2015). Microbial lipases are mostly extracellular and obtained from bacterial and fungal species. Among them, the fungal lipases are extensively exploited due to their exclusive characteristics such as pH and thermal stability, economical extraction process, substrate specificity, and efficient activity in organic solvents (Sarmah et al., 2018).

Lipase production is prevalent among yeasts, however, only few species can produce lipases with suitable traits and in sufficient amounts to be industrially useful. *C. rugosa*, *Y. lipolytica*, *C. antarctica*, *C. utilis*, and *Saccharomyces* were reported as the most promising lipase producing yeasts (Sarmah et al., 2018). Deive et al. (2003) investigated the ability of *K. marxianus* to produce extracellular lipolytic enzymes and to observe the effect of several lipidic compounds and surfactants on enzyme secretion. They observed that *K. marxianus*

showed lipolytic activity in a complex liquid medium with several potential inducers such as triacylglycerols, fatty acids. However, the tributyrin and oleic acid were identified as the best inducers in their study. The highest extracellular lipolytic enzyme production (about 80 U/mL in 3 d) was obtained while the medium was supplemented with a 2 g urea/L plus 5 g tributyrin/L. However, addition of surfactants did not improve production. Furthermore, the enzymes demonstrated high thermal stability in aqueous solution (73% residual activity after 9 d at 50 ℃; 16 min half-life time at 100 ℃), good tolerance to organic solvents (70% residual activity, after 2 d in n-hexane of cyclohexane) and stability at acidic pH (>85% residual activity after 24 h of incubation time at 25 ℃ for pH 2.0-8.0, 100% activity at pH 4.0). However, the lipase produced by *K. marxianus* was sensitive to alkaline pH and nearly to be inactivated after 24 h at pH above 8 (Deive et al., 2003). The stability at acidic pH could be of some commercial interest, since most lipases from some other microbial strains were rapidly inactivated in this condition (Corzo and Revah, 1999, Sharma et al., 2002).

Apart from the physicochemical factors such as temperature, pH, and dissolved oxygen, the production of lipases from *K. marxianus* was significantly affected by medium composition. In respect to this, Stergiou et al. (2012) optimized several process parameters influencing the extracellular lipase production using *K. marxianus* IFO 0288. They observed the productivity was increased by18-fold with an optimized nutritional (0.5 % olive oil) and cultivation (pH 6.5, 35 °C, 150 rpm) conditions during the 65 h of fermentation of olive oil as substrate. Recently, a bioinformatics analysis of the genes of lipases from *K. marxianus* L-2029 was also performed to analyze biochemical characteristics, properties, and phylogeny of the extracellular lipases from *K. marxianus* L-2029 (Martínez-Corona et al., 2019). The phylogenetic analysis of *K. marxianus* lipases showed evolutionary affinities with the lipases from abH15.03, abH23.01, and abH23.02 families. Furthermore, Cardoso et al. (2015) observed that *K. marxianus* 83F showed higher lipolytic activity in traditional Serro Minas cheese; hence, it could be used as a good starter culture in cheese production due to their effects on the sensory properties. Indeed, microbial lipases are very versatile enzymes having many promising applications in different industries, such as detergent industry, baking and food industry, organic synthetic industry, dairy and flavor industry, paper manufacturing industry, medical and pharmaceutical industry, biosurfactant synthesis cosmetics and

perfumery, leather industry; oleochemical and agrochemical industry, biosensors, and bioremediation (Sarmah et al., 2018).

1.4.3.1.2 Cell factory applications of *K. marxianus*

Many recent studies highlighted the potential of *K. marxianus* to be used as effective cell factory to produce valuable metabolites by means of engineered manipulations. For example, Kim et al. (2014) reported a study in which 2-Phenylethanol, which is an aromatic alcohol with a rose scent, was successfully produced by using genetically modified yeast strains by the Ehrlich pathway. In the study of Kim et al. (2014), K. marxianus was genetically engineered in order to be able to overproduce 2-Phenylethanol by using glucose as carbon source. To achieve this objective, the authors induced an overexpression of phenylpyruvate decarboxylase and alcohol dehydrogenase genes of S. cerevisiae in the used K. marxianus strain. Thus, approximately 1.0 g/L of 2-Phenylethanol was produced. A similar level of 2-Phenylethanol was also produced from evolved K. marxianus, which was genetically engineered by using specific genes from Klebsiella pneumoniae. This modification allowed an overexpression of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthase in the used K. marxianus. Thus, by using this cell engineering technique, the authors reported a yield of 1.3 g/L of 2-Phenylethanol when a 20 g/L glucose solution without addition of phenylalanine to the medium was used (Kim et al., 2014). In another study, Zhang et al. (2020) used different sugars as carbon source such as glucose, xylose, and fructose for glycerol production. They reported that a TPI1 gene encoding triose phosphate isomerase was deleted from K. marxianus NBRC1777 and that this procedure allowed the newly engineered K. marxianus to be able to grow with glucose, fructose, and xylose as sole carbon sources. Thus, under aerobic conditions at a temperature of 42 °C, the engineered *K. marxianus* YZB115 strain fermented 80 g/L glucose, fructose, and xylose solutions, yielding 40.32, 41.84, and 18.64 g/L glycerol without by-product, respectively (Zhang et al., 2020). In a study reported by Zhang et al. (2017), *K. marxianus* was used as cell factory to produce fructose from inulin. They hypothesized their research on the fact that in yeast, the hexose assimilation is started at hexose phosphorylation. However, in K. marxianus, the hexokinase and glucokinase genes were not identified. Thus, in the study they reported, the KmHXK1 and KmGLK1 genes were over-expressed in different *K. marxianus* strains. They showed that glucose and fructose assimilation ability decreased significantly in

the KmHXK1 genre disrupted *K. marxianus* YLM001 strain. However, this ability was not changed obviously in KmGLK1 disrupted *K. marxianus* strain YLM002. In the case of over-expressing KmGLK1 in YLM001, only the glucose assimilation ability was recovered in the engineered *K. marxianus* YLM005 strain. They also showed the engineered strains by the KmHXK1 gene could phosphorylate glucose and fructose, and that the KmGLK1 gene induced only a glucose phosphorylation. The authors were also able to obtain a thermo-tolerant *K. marxianus* YGR003 strain which produced glucose-free fructose solution from inulin in one step (Zhang et al., 2017).

1.4.3.2 Cell proteins production

Single cell proteins (SCPs) are known as dietary single-cell microorganisms whose biomass or protein extracts are derived from pure or mixed microalgae, yeasts, mushrooms or bacterial cultures. These microorganisms can be used as protein-rich foods or food ingredients or dietary supplements for human and animal consumption (Ritala et al., 2017). Therefore, large-scale production of microbial biomass could be advantageous for replacing proteins of agricultural origin (plant and animal proteins) for food or feed due to the high multiplication rate and a high protein content (30-80% protein in terms of dry weight) of microorganisms, the ability to utilize the large number of different low-cost carbon sources including waste materials (Karimi et al., 2018). Furthermore, SCPs could be obtained in a shorter time compared the proteins from agricultural origin due to short life cycle of the microorganisms and relatively smaller amount of space and labor needed. More importantly, it does not need huge arable land, thus the conflicts with world food production could be avoided (Karim et al., 2018c).

Among the SCP producing microorganisms, yeasts have been considered as more suitable candidate due to the high protein content, small particle size, ease handling and relatively low production costs. *K. marxianus* is a candidate of great interest for SCP production and being used as feed organism in various countries. *K. marxianus* (*K. fragilis*) was found to have a higher specific growth rate than that of *S. cerevisiae* in continuous production of a novel yeast diet. *K. fragilis* showed a maximum biomass yield of 4.81 g/L/h in an aerobic continuous fermentation of 2.5% fructose medium for producing a high nutritious protein diet with a protein content of 50-55% (Kim et al., 1998). Yadav et al. (2014) used *K. marxianus* GQ 506972 for SCP production and concurrent COD removal of cheese

whey and obtained a biomass production of 6 g/L with a 55% COD removal efficiency after 36 h of fermentation in batch system at 40 ℃ and pH 3.5. Moreover, an increase in the inoculum concentration resulted in the increase of biomass production of 15 g/L with a COD removal of 80%. In another study, whey permeate was fermented by *K. marxianus* to produce SCP under batch and aerobic condition at a low pH of 4.5 and a temperature of 35 ºC where ammonium sulphate was added as nitrogen source to increase biomass yield (Yadav et al., 2014a). Also, Yadav et al. (2014b) evaluated the potentiality of co-culture to obtain improved quality SCP and to enhance the COD removal during the batch and continuous fermentation of whey at extreme culture conditions (low pH, 3.5 and high temperature, 40 ℃). The batch system of mixed culture (*K. marxianus* and *C. krusei*) resulted in a 19% higher biomass yield with 33% increased productivity and simultaneously 8.8% higher removal of COD than the monoculture. In addition, the SCP obtained from mixed culture was enriched with required protein content and all necessary amino acids including lysine. The results revealed that the mixed culture of thermo-tolerant and acid resistance yeasts can be a potential approach to produce SCP and concurrent removal of COD from wastewater under extreme conditions (Yadav et al., 2014a,b). In another study, the co-culture of *K. marxianus* and *Trichoderma reesei* was more efficient for production of SCP (51%) from beet pulp compared to a monoculture of *T. reesei* (49%) and the protein contained all essential amino acids (Ghanem, 1992). In another study, a mixed culture of *S. cerevisiae* and *K. marxianus* was used for producing food-grade SCPs at at 30 ℃ and pH 6.5. The result showed that 92% of total whey proteins was recovered by the co-culture while 84% by monoculture under these optimized conditions (Yadav et al., 2016).

Furthermore, several microorganisms (*S. cerevisiae, K. marxianus* and *C. kefir*) with industrial interest were grown in the food wastes through solid state fermentation, and *K. marxianus* was found to contain the highest protein and fat concentration (59.2% w/w dm), thus it could be utilized for its high fat content and livestock feed enrichment (Aggelopoulos et al., 2014). The essential amino acid composition of SCP from *K. marxianus* using sugar cane molasses was found to be similar with other yeast species (Anderson et al., 1988). Øverland et al. (2013) evaluated the performance of *C. utilis, K. marxianus* and *S. cerevisiae* yeasts as protein sources in diets for Atlantic salmon (Salmo salar).

1.4.3.3 Single cell oil and fatty acids production

Single cell oil (SCO) or microbial lipids are considered as potential substitutes of vegetable oils and animal fats to produce non-fossil biofuels (i.e., biodiesel) and oleochemicals as the oil properties are similar in types, structure, and composition of fatty acids. Some microorganisms attain a higher oil content compared to vegetable oils, but they do not need fertile land, thus avoiding conflicts associated with the food vs. fuel issue. Furthermore, they are not disturbed by the climate and the seasons (Karim et al., 2019a).

K. marxianus CBS 6556 was capable of producing SCOs using deproteinized whey (sweet and sour) concentrates as carbon source and showed an increase in quantity of essential amino acids (Schultz et al., 2006). *K. marxianus* NCYC 1424 was most efficient for conversion of whey to SCO while an investigation with several organisms was conducted by Willetts and Ugalde (1987). The oxygen availability in the medium was found to have a great influence in the conversion efficiency of whey to biomass (Willetts and Ugalde, 1987). Arous et al. (2017) observed that *K. marxianus* CC1 was the most efficient to assimilate and to ferment a wide variety of carbon sources and, showed a strong lipolytic activity to grow on fats. It obtained 6 g/L of biomass comprising 12.9% w/w lipids after 72 h fermentation at an initial medium pH of 6 ± 0.1 and at 28 °C temperature. This quantity was considerably higher than that of reported by Fonseca et al. (2007) in *K. marxianus* ATCC 26548 (=CBS 6556) (5.2% w/w lipids). However, the lipid accumulation was higher for *S. cerevisiae* (7% wt/wt lipids) compared to *K. marxianus* in batch culture (Fonseca et al., 2007). These variations in lipid production capabilities might be ascribed to the specific physiological behavior of each microorganism or to the differences in the culture conditions.

Saccharomyces cerevisiae is a yeast that played a central role in human society due to its use in food production such as bread, beer, and wine. In modern scientific research, this yeast is also widely used as a model microorganism to perform various genetic manipulations and achieve biotechnological goals that require microbial engineering (Cernak et al., 2018). However, various studies have shown that *S. cerevisiae* is genetically difficult to manipulate to develop new strains capable of metabolizing unconventional carbon sources to obtain specific metabolites. This yeast also requires fairly specific conditions for optimum growth and tolerates little specific industrial applications where it is difficult to work under the optimum conditions for its growth. Also, it has been reported that yeasts capable of solving

many of these problems specific to *S. cerevisiae* remain difficult to manipulate genetically, hence the interest of *K. marxianus* as a potential substitute of *S. cerevisiae* as a biotechnological tool (Cernak et al., 2018).

In this context, Cernak et al. (2018) successfully designed the thermotolerant yeast *Kluyveromyces marxianus* as a novel platform for microbial engineering and synthetic biology. They used the CRISPR-Cas9 technique and showed that wild strains of *K. marxianus* can be made heterothallic for sexual crossing. By selecting two mating-type *K. marxianus* strains, these authors were able to combine three complex traits: thermotolerance, lipid production, and easy transformation with exogenous DNA into a single host. This has made it possible through microbial engineering to use *K. marxianus* as a cell plant for the production of lipids at temperatures exceeding those of other fungi, opening up highly potential prospects for large-scale industrial applications. These results showed that *K. marxianus* can be used as a substitute for S. cerevisiae, as it exhibits more robust metabolic characteristics with potential for the industrial production of ingredients of high nutritional and functional value such as fatty acids and antimicrobial peptides. In addition, unlike *S. cerevisiae*, these authors concluded that the yeast *K. marxianus* can easily grow at high temperatures while being able to utilize a wide range of carbon sources, making it a promising microorganism for industrial biotechnology and the production of specific metabolites from renewable raw materials such as plant biomass (Cernak et al., 2018).

1.4.3.4 Bioethanol production

Bioethanol production through fermentation at a high temperature has received attention nowadays since rapid fermentation at elevated temperature can reduce cooling cost and continuous change from fermentation to distillation, decrease the risk of contamination, carry out simultaneous saccharification and fermentation, and be used in tropical countries (Fonseca et al., 2008). The high temperature optimum for growth (P_{max} of 0.86/h at 40 °C) of *K. marxianus*, is particularly interesting because this facilitate cooling during large-scale fermentations for which heat transfer is proven to be limiting factor. Currently, industrial ethanol production mostly depends on conventional strains of *S. cerevisiae* because of its high production rate and better tolerance to high ethanol titers (upwards of 120 g/L), however, the suitable temperature of this strain is comparatively low (only 25 to 30 °C) (Qiu and Jiang, 2017). In this regard, there has been a significant interest in the yeast species which

are able to produce ethanol at high temperature, and the isolates of *K. marxianus* appear to be particularly promising (Madeira-Jr and Gombert, 2018). *K. marxianus* species can grow at 47 ºC (Nonklang et al., 2008, da Silva et al., 2018), 49 ºC (Hughes et al., 1984), and even 52 ºC (Banat et al., 1992) and can produce ethanol at temperature more than 40 ºC (Banat et al., 1992, Madeira-Jr and Gombert, 2018). In addition, *K. marxianus* can utilize a broad range of low-cost substrate such as corn silage juice (Hang et al., 2003), molasses (Martínez et al., 2017) , whey permeate (Zafar and Owais, 2006, Ozmihci and Kargi, 2007) to produce ethanol. Considering these advantages, *K. marxianus* is currently being promoted as a feasible alternative to *S. cerevisiae* as an ethanol producer.

Recently, it was revealed that bioethanol production at high temperatures (~48 ºC) using *K. marxianus* NCYC 3396 from sugarcane can decrease contamination levels, cooling costs, use of antibiotic, use of H_2SO_4 in cell recycling, water usages, and energy use in distillation ; which reduced ultimate cost of bioethanol production in Brazilian biorefineries (Madeira-Jr and Gombert, 2018). They obtained a similar yield (0.40 g ethanol/g glucose) using *K. marxianus* at 48 °C to those displayed by *S. cerevisiae* CEN.PK113-7D at 37 ºC temperature. Although the ethanol production was similar (0.43 g/g glucose) by *K. marxianus* K213 and *S. cerevisiae* using glucose at 30 ºC, however, *S. cerevisiae* almost lost its ability to produce ethanol at 45 ºC whereas *K. marxianus* K213 still maintained same conversion efficiency (0.43 g/g glucose) (Yan et al., 2015). An optimization study showed that the temperature (32.5-35 ºC) was the most significant factor for ethanol production from cheese whey using *K. marxianus* URM7404, followed by pH (4.8-5.3) and lactose concentration (61-65 g/L) (Murari et al., 2019). A recent study by Suzuki et al. (2019) revealed that the recombinant *K. marxianus* DMB13 strain converted xylose to ethanol rapidly, particularly after depletion of glucose, and achieved the maximum ethanol yield (0.402 g/g) in a xylose/glucose co-fermentation at 40 ºC.

Furthermore, *K. marxianus* is of particular interest because of its ability to utilize xylose as a carbon source at temperatures as high as 45-52 ºC, at which the fermentation efficiency was similar to that of at *S. cerevisiae* 30 ºC (Suzuki et al., 2019). Therefore, *K. marxianus* could be advantageous for second generation bioethanol production, which uses lignocellulosic biomass (LCB) as substrates, because the temperature is typically higher for the saccharification process of LCB than usual fermentation temperatures and the hydrolytic

enzymes have their optimum activity at these high values (Madeira-Jr and Gombert, 2018). In another study, *K. marxianus* DSMZ-7239 was the most suitable strain for ethanol production (3.3%, v/v) using whey permeate (50 g/L) as substrate (Ozmihci and Kargi, 2007). In addition, *K. marxianus* CCT7735 showed a higher efficiency compared to *K. lactis*, due to its high ability to express LAC4 gene (*β*-galactosidase) and *RAG6* gene (pyruvate decarboxylase) and, genes of Leloir pathway under hypoxia and high lactose concentrations (Diniz et al., 2012). Nevertheless, it is still not evident whether the oxygen concentration regulates the expression of the lactose transport in the *Kluyveromyces* genera. However, *K. marxianus* is preferred to produce ethanol from both LCB (mainly, xylose) and whey (mainly, lactose) at high temperature (>40 ℃), however, in contrast to *S. cerevisiae*, *K. marxianus* cannot endure high ethanol concentrations which is the major drawback of this strain to be used at industrial level as its growth is strongly inhibited by an ethanol concentration higher than 6%. This is because the low membrane stability and down regulation of some gene-encoding enzymes of the ergosterol biosynthesis pathway under high ethanol stress (Diniz et al., 2017).

Nowadays, different techniques including the pre-treatment techniques for substrates, simultaneous saccharification and fermentation (SSF), co-production of bioethanol and other bioproducts, coculture inoculum, immobilization technique, and evolutionary engineering, have introduced to improve the productivity of bioethanol production using *K. marxianus*. For example, SSF of taro waste exhibited higher bioethanol productivity (2.23 g/L/h) and maximum ethanol concentration (48.98 g/L) after 22 h when 5% of *K. marxianus* K21 inoculum was employed at 40 °C (Wu et al., 2016). The SSF of *Agave teuilana* fructans (ATF) using *K. marxianus* strains has been proved as a potential approach for industrial application due to the production of specific exo-fructanhydrolase activity for ATF hydrolysis, and simultaneous production of bioethanol as well (Flores et al., 2013). Furthermore, the ethanol production (7.34 g/L) was 1.78-fold higher for *K. marxianus* K213 at 42 °C than *S. cerevisiae* at 30 °C using $NaOH/H_2O_2$ -pretreated water hyacinth (Yan et al., 2015). Moreover, *K. marxianus* ATCC36907 produced 7.53 g/L ethanol using alkaline pretreated Carnauba straw residue through SSF, which was 3-fold higher than *S. cerevisiae* CAT-1 (2.3 g/L) at 45 °C (da Silva et al., 2018). In addition, the coproduction of bio-oil and bioethanol by *K. marxianus* through valorizing the steam-exploded wheat straw displayed

it's potentiality for a biorefinery approach (Tomas-Pejo et al., 2017). Nevertheless, the coculture of *K. marxianus* and *S. cerevisiae* resulted in higher ethanol production (21.12 g/L) and better sugar consumption (88%) than monoculture from enzymatic rice waste hydrolysates (Saratale et al., 2017). Recently, a coculture of immobilized *K. marxianus* and *S. cerevisiae* resulted in a higher hydrolysis of whey and produced higher ethanol than the single cultures during the batch fermentation (Beniwal et al., 2018). Moreover, the development of high lactose utilizing osmotolerant yeast strain and its further use to ferment lactose rich whey has gained interest for higher ethanol titer and to lower energy consumption because expression of GPD1, TPS1and TPS2 upregulated in lactose adapted *K. marxianus* MTCC 1389 strain and it accumulated the trehalose and glycerol in response to lactose stress. Consequently, the osmotolerant *K. marxianus* cells leads to efficient conversion of whey lactose into bioethanol. More recently, the strain *K. marxianus* MTCC 1389 (ATCC64884) was reported as more resistant to osmotic and oxidative stresses than *S. cerevisiae* MTCC170 because the genes related to glutathione biosynthesis and glycerol synthesis were upregulated and, resulted in high glutathione level (6.8 µg/mg protein) and high intracellular glycerol (2.2 g/g cell dry weight) in the presence of osmotic and oxidative stress (in 150 g/L lactose), that is indicating the outcome of stress protectants at the transcriptional level (Saini et al., 2017a).

1.4.4 Applications in food and feed industry

1.4.4.1 Bioemulsifier-mannoprotein production

Bioemulsifiers (biosurfactants) are surface-active molecules or proteins, lipoproteins, lipopolysaccharides, amphipathic polysaccharides, or complex mixtures of these biopolymers synthesized by different microorganisms. Increasing environmental concern about chemical emulsifiers prompts attention to the bioemulsifiers mainly due to their environmental-friendly nature, biodegradability, and low toxicity (Nitschke and Costa, 2007). Presently, the bioemulsifiers are mostly used in the remediation of pollutants, however, the interest in these compounds have considerably been increasing as substitute to the chemical surfactants such as sulphonates, carboxylates, and sulphate acid esters in the food, pharmaceuticals, and oil industries, specially to be used as emulsifiers, solubilizers, wetting, foaming, antiadhesive and antimicrobial agents (Gudiña and Rodrigues, 2019). Although the bacteria have been reported as a major source of various microbial emulsifiers in several literatures (Shekhar et al., 2015), many emulsifiers from bacterial sources are not

recommended to use in the food industry because of pathogenic nature of the producers (Shepherd et al., 1995). In contrast, yeasts have widely been used to produce the emulsifiers. Generally, bioemulsifier producing strains include *C. petrophilum* (Iguchi et al., 1969), *C. tropicalis* (Käppeli and Fiechter, 1977), *Torulopsis petrophilum* (Cooper and Paddock, 1984), *C. lipolytica* (Cirigliano and Carman, 1985) and *C. bombicola* (Brakemeier et al., 1998). However, commercial application of bioemulsifiers from these species is unappealing due to the requirements of water-immiscible substrates (i.e., oils and alkanes) for facilitating metabolism, difficulties in isolation, foam fractionation, requirement of enzyme digestion, and repetitive extraction process with solutions of methanol-chloroform (Cameron et al., 1988); moreover, the yields of these complex procedures were very low (Cameron et al., 1988). Although the cell wall proteins of *S. cerevisiae* were reported as a bioemulsifier in foods (Shekhar et al., 2015), more understanding is required to evaluate their potentials as food ingredients.

Recently, the interest on other nonconventional yeast species including *K. marxianus, K. lactis, K. fragilis* etc. have been increasing due to their high ability to utilize low-cost substrates and high biomass production, which could ultimately lead to high bioemulsifier yields (Lukondeh et al., 2003). However, only a few studies have been conducted on protein production from *Kluyveromyces* sp. so far (Akanni et al., 2015, Galinari et al., 2018, Hajhosseini et al., 2020). Lukondeh et al. (2003) revealed that the emulsification properties of mannoprotein extracted from *K. marxianus* FII 510700 cell wall were like to those mannoprotein obtained from the cell walls of traditional source, *S. cerevisiae*. The optimization study of Hajhosseini et al. (2020) demonstrated that the carbon and nitrogen concentrations, fermentation time, and medium pH significantly influenced the mannan production as bioemulsifier by *K. marxianus* IBRC-M 30114. They observed that a mannan yield of 245.98 mg/100 mL could be obtained at the optimized conditions (pH: 4.99, glucose: 55.15 g/L, yeast extract: 9.35 g/L, and fermentation time: 168 h). Galinari et al. (2018) described that the cell wall polysaccharides such as α-D-mannan fractions from yeast *K. marxianus* CCT7735 showed hydroxyl-radical scavenging, superoxide radicals scavenging, copper- and iron-chelating activities, and reducing power as well as total antioxidant capacity. Thus, *K. marxianus* can be considered as an ideal source renewable and natural

polysaccharides with pharmacological properties (antioxidant, antiproliferative, and immunostimulatory properties).

1.4.4.2 Baker's yeast and biomass for bread production

Yeast are considered as one of the most important single cell nutrition sources because they contain low amounts of nucleic acids but all essential amino acids especially lysine in higher amount compared to algae and bacteria. *S. cerevisiae* is the most common food grade yeast, also called baker's yeast, which has been used worldwide to produce bread and baking products. It contains 35-45% of carbohydrates (high β-glucan), 40-58% of proteins, 6.5-9.3% of nitrogen, 5-7.5% of minerals, 4-6% of lipids, various kinds of vitamins, and high volumes of glutathione depending on its types and growth conditions (Öztürk et al., 2017). Several efforts have been made to utilize the low-cost substrate whey to produce baker's yeast, however, *S. cerevisiae* is not able to use lactose as carbon source (Caballero et al., 1995). Therefore, several methods have been proposed to overcome this limitation, such as hydrolysis of lactose (Reed, 2012), or its conversion to lactic acid through an addition fermentation step (Champagne et al., 1990). In the contrary, using native lactose fermenting yeast could be an attractive approach for bread production. Caballero et al. (1995) carried out an experiment with *K. marxianus* stains since this yeast possess high growth in whey without any previous treatment. The efficiency of two *K. marxianus* strains (NRRL-Y-1109 and NRRL-Y-2415) as baker's yeast were assessed and compared with the strains of *S. cerevisiae,* which were isolated from compressed yeast and active dry yeast, respectively. The dough proofing activity (both in rich and lean doughs) and sensory evaluation of breads were tested and observed that both *K. marxianus* stains displayed a higher proofing activity in the rich doughs (prepared with whey or lactose) than the commercial baker's yeast strains (Caballero et al., 1995). The improved aroma of breads was attained by applying *K. marxianus* (IFO 288) as starter culture for making sourdough bread. Furthermore, the use of mixed cultures (*K. marxianus* and *Lactobacillus delbrueckii* ssp. *bulgaricus*, or *L. helveticus*) as starter cultures to make sour dough bread could lead to have longer shelf-life and better sensorial qualities of sourdough breads (Plessas et al., 2008).

On the other hand, the reduction of oligo-, di-, and monosaccharides and polyols (FODMAPs) levels in whole wheat bread is imperative because a diet low in FODMAPs could reduce the abdominals symptoms to about 70% of the patients suffering from irritable

bowel syndrome (Struyf et al., 2018). The main source of FODMAPs in our diet is usually from wheat bread since wheat contains relatively high levels of fructans. Recently, Struyf et al. (2017) proposed a yeast-based approach to reduce the FODMAPs in wheat bread, and the strains of *K. marxianus* showed a superior efficiency compared to the control *S. cerevisiae.* They observed that the fructan levels in the final products could be reduced to 90% using an inulinase secreting *K. marxianus* in dough fermentation whereas only 56% reduction was achieved by the control *S. cerevisiae*. This is might be due to the higher inulinase enzyme secretion capacity of *K. marxianus* having both secreted form and cell wall associated form of inulinase production capacity, while the invertase produced by *S. cerevisiae* is preserved in the cell wall instead of secreting into the dough (Caballero et al., 1995). As a result, the wheat grain fructans (that present in the dough) were more accessible to *K. marxianus* inulinase compared to *S. cerevisiae* invertase; and subsequently, a higher degradation of fructans might be obtained by *K. marxianus* inulinase (Struyf et al., 2017). Moreover, *K. marxianus* CBS6014 produced different levels of five volatile flavor compounds than the conventional bakery strain *S. cerevisiae* during the fermentation of whole meal breads low in FODMAPs (Struyf et al., 2018). Furthermore, a coculture of *S. cerevisiae* and *K. marxianus* could efficiently be used in bread preparation since the breads prepared by the coculture had a very low fructan level (≤0.2% dm) and the volume of loaf was almost same as control. Thus, the reduction of FODMAPS level in bread using K. marxianus could be an attractive approach.

1.4.4.3 *K. marxianus* as a probiotic and its stimulation by prebiotics

The live microbes, which when administrated in sufficient amounts to confer a health benefit on the host is called probiotics (Hill et al., 2014) and mostly includes bifidobacterial, clostridia, lactobacilli, *Faecalibacterium*, enterococci, and recently, propinonibacteria (Altieri, 2016). Although *S. cerevisiae* var. *boulardii* has been described as a probiotic for a long time (Moré and Swidsinski, 2015), there is an increasing interest toward the probiotic potential of the other nonconventional yeasts in the recent years. It is verified that some strains of yeasts can produce the bile salt hydrolase (BHS) enzyme like the probiotic bacteria, and subsequently, deconjugating the bile salts (Liu et al., 2012). This capacity can increase the secretion of endogenous cholesterol, which may stimulate the hepatic bile salts synthesis. Consequently, the amount of absorbed cholesterol would be reduced and the development of

micelles will be compromised (Kumar et al., 2012). *K. marxianus* is very promising to be used as a probiotic due to the capacity of modifications in the cell immunity, adhesion, and human gut microbiota; and having the antioxidative, anti-inflammatory, and hypocholesterolemic properties (Xie et al., 2015, Cho et al., 2018).

K. marxianus may have the ability of surviving in the digestive tract to safely reach the intestines and to function as prebiotics because of its resistance to acid and bile that could be encounter in the gastrointestinal environment. The capacity of surviving in the acid and bile, and a higher ability to adhere to the Caco-2 cells suggested that it might have higher antioxidant activities (Cho et al., 2018). *K. marxianus* isolated from kefir showed cholesterol lowering ability Cho et al. (2018) observed that *K. marxianus* isolated from kefir showed cholesterol lowering ability and the strain KU140723-04 reduced 30% of cholesterol, which was even higher than *S. cerevisiae* ATCC 6037 and *K. lactis* ATCC 34440. Liu et al. (2012) revealed that the amount of eliminated cholesterol from culture media was proportional to the BSH enzyme activity of *K. marxianus* strains. In another study, the impacts of *K. marxianus* on diet-induced hypercholesterolemia in rats were studied, which revealed that *K. marxianus* M3 isolated from Tibetan mushrooms had a protective effect in hyperlipidemic rats (Xie et al., 2015). Furthermore, the synergistic effects of *K. marxianus* KU140723-02 isolated from kefir and polyphenol rich grape seed extracts (GSE)/grape seed flour (GSF) on radical scavenging ability suggested that *K. marxianus* performed as a probiotic while GSE/GSF as probiotics. *K. marxianus* performed as a probiotic and GSF/GSE as prebiotics. Thus, *K. marxianus* together with the GSE/GSF would be used as an efficient functional food ingredients to enhance the anti-oxidant activity in the gut (Cho et al., 2018). Furthermore, *K. marxianus* AS4, isolated from traditional dairy products like yogurts and cheese, displayed an outstanding tolerance to high bile salts (with a survival rate of 83%) and low pH (with a survival rate of 71%), a high antipathogenic activity, a satisfactory antifungal susceptibility. Besides, it exhibited a higher anticancer activity in gastric cancer cells (~54% mortality) due to the secreted metabolites, downregulated Bcl-2 gene, and upregulated BAD and CASP9 gene expression system (Saber et al., 2017). Nevertheless, *K. marxianus* displayed an increase in HDL-cholesterol and a reduction in serum TC, LDC-cholesterol and TAG concentration (Xie et al., 2015).

Furthermore, the strains of *K. marxianus* (VM003, VM004, VM005), isolated from whey, were able to survive under gastrointestinal conditions and they showed weak auto-aggregation and co-aggregation with pathogenic bacteria (*Escherichia coli, Serratia* sp., *Salmonella* sp., and *Salmonella typhimurium*) (Díaz-Vergara et al., 2017). Fadda et al. (2017) found that artisanal cheese-derived *K. marxianus* stains have significant functional characteristics and lack of undesirable properties, consequently, it could be used as a suitable probiotic. Recently, the strain *K. marxianus* B0399, isolated from milk, was reported as a potential probiotic strain as it was also able to survive in gastrointestinal track, retaining its vitality and fermentation capability (Tabanelli et al., 2016). This strain has also exhibited its capability to increase the bifidobacterial concentration in the colonic model system, to affect the colonic microbiota, and to induce the formation of higher quantities of short chain carboxylic acids, acetate and propionate. In addition, it is highly adhesive to human enterocyte such as Caco-2 cells and can modulate immune response; and hence, fermented milk containing *Bifidobacterium animalis* sp., *K. lactis* BB12, *K. marxianus* B0399 was suggested for the patients with irritable bowel syndrome in some studies. Thus, *K. marxianus* can be introduced as a potential probiotic yeast (Maccaferri et al., 2012a, Maccaferri et al., 2012b).

A substrate that is selectively utilized by host microorganism conferring a health benefit is called a prebiotic (Gibson et al., 2017). To be considered as a prebiotic, a substance must have the ability to manipulate the host microbiota for some beneficial health effects. Currently, the fructans (fructo-oligosaccharides and inulin) and the glactans (galacto-oligosaccharides or GOS) are being considered as dominating prebiotics as evidenced by several studies on their prebiotic effects (Collins and Reid, 2016). *K. marxianus* can produce high value-added bioingredients such as oligosaccharides (OS), that is used as prebiotics to increase the growth of *Bifidobacterium* sp. in the human and animal intestines; oligonucleotides (ON), usually used as enhancer of flavors in food products; and oligopeptides (OP), added to dairy products as immuno-stimulators (Belem and Lee, 1998). When added to foods, these compounds (i) act as immunopotentiators; (ii) lower the low density lipoprotein-cholesterol (a risk for cardiovascular diseases); (iii) promote protection against bacterial infections; (iv) enhance food flavors; and (v) stabilize food emulsions (Collins and Reid, 2016).

It is proven that the mortality from acute myocardial infarction (heart attack) could be reduced by lowering the plasma cholesterol levels since hypercholesterolemia is a risk factor for the coronary heart disease (Inzucchi et al., 2015); hence, the demand for biological ingredients that is able to lower plasma cholesterol is rising in the field of medicine, food, and nutrition. Nowadays, the heat inactivated dried yeasts are used as nutrition supplement because whole yeast cells are rich in protein, vitamin B, and dietary fiber (Inzucchi et al., 2015). It was seen from some previous reports that the yeasts or constituents of yeasts possess hypocholesterolemic activity (Nicolosi et al., 1999), anti-tumor activity (Mifuchi et al., 1969) or immuno-stimulation activity (Williams et al., 1992), or can prevent constipation (Nakamura et al., 2001) in animals or humans (Inzucchi et al., 2015). However, not all but only a few species are widely used as dried yeasts including *S. cerevisiae*, *C. utilis*, and *K. marxianus* (Yoshida et al., 2004, Inzucchi et al., 2015). Recently, Yoshida et al. (2004) investigated the hypocholesterolemic activities of some yeasts (81) strains from different species in rats fed, a high cholesterol diet. In this study, some yeasts species such as brewer's and baker's yeasts, that are predominantly used for food, did not show the hypocholesterolemic activity even when administered at a high concentration of 10%. In contrast, the highest potentiality in hypocholesterolemic activity of *K. marxianus* YIT 8292 was observed. Moreover, plasma total cholesterol as well as liver total cholesterol were significantly reduced by this strain when introduced as a dietary admixture at a concentration of 3%. Therefore, *K. marxianus* could be used as a novel food supplement with the ability to prevent hypercholesterolemia.

1.4.4.4 Fructose and fructo-oligosaccharides production

Fructose could be an alternative sweetener to sucrose because of its higher sweeting capacity (1.5-2 times than sucrose) and can increase iron absorption in children, whereas fructo-oligosaccharide (FOS) could be a promising source of dietary fiber with a bifidogenic effect. Interestingly, both compounds can be obtained through the enzymatic hydrolysis of inulin. However, the production of FOS and fructose typically performed at a high temperature (around 60 °C) because inulin shows a limited solubility at room temperature. Therefore, the isolation and characterization of thermostable inulinases are of great interest to hydrolyze inulin at high temperatures (Flores-Gallegos et al., 2015).

FOS are used as food ingredients because of its health benefits like inducing proliferation of intestinal bifidobacterial community (probiotics), promoting a good balance in the intestinal flora. Moreover, oligosaccharides have recently achieved 'GRAS' status by Food and Drug Administration (Flores-Gallegos et al., 2015). Inulin could be an efficient feedstock to produce the inulo-oligosaccharides using the endo-inulinases; but insolubility of inulin in cold water or slightly soluble (only 5%) in water at 55 ℃ remained the main challenge to hydrolyze it. Only a few inulinases can be found that own an optimal temperatures of 50 ℃ or higher (Gao et al., 2009), thus exploring of thermostable inulinase procedures are of interest for industrial applications. In this regard, *K. marxianus* can be a promising candidate to produce thermostable inulinases. Furthermore, the development of new techniques to produce fructose syrups has received more attention because it is less viscous, highly soluble and less cariogenic than sucrose, and can be utilized by diabetics (Vandamme and Derycke, 1983, Flores-Gallegos et al., 2015). However, the production of fructose by conventional methods using starch required several enzymatic stages, such as α-amylase, amylo-glucosidase, and glucose isomerase activity; but yielding only a maximum of 45% fructose solution. In contrast, fructose production by acid hydrolysis is not suggested because of the undesirable coloring of the inulin hydrolysate and the formation of di-fructose anhydride without any sweetening property. Therefore, the enzymatic hydrolysis of inulin using microbial enzymes could be alternative method to produce fructose syrups containing 95% fructose (Vandamme and Derycke, 1983).

Microbial inulinase enzymes from various organisms (yeasts, fungi) are known to split up the β-(2,1)-fructofuranosidic bonds of inulin (Chi et al., 2009). Yeasts are preferred for fructose production because the inulinases produced by yeasts were capable of exohydrolysis of inulin (Liu et al., 2013). These inulinases commonly obtained from several nonconventional yeast species including *K. marxianus* or *K. fragilis* (Holyavka et al., 2016), *C. kefyr* (Negoro and Kito, 1973), *Debaryomyces antarelli* (Beluche et al., 1980). Among them, *K. marxianus* can grow on fructans such as inulin, thus inulinases might have the ability to saccharify the fructans of plant origin (Hensing et al., 1994, Chi et al., 2011). A large volume of inulin is generally found in the tubers of many plants like chicory, dahlia, yacon, and Jerusalem artichoke. The inulinases can hydrolyze the fructo-oligosaccharides and inulin into fructose by breaking down the glycosidic linkages of their molecules (Holyavka et al.,

2016). The pure inulin and raw inulin (from roots of *Asparagus racemosus*) were hydrolyzed by using an extracellular exoinulinase produced by *K. marxianus* YS-1 to produce a high-fructose syrup. The exoinulinase successfully hydrolyzed the pure inulin (84.8%) and raw inulin (86.7%) for production of 43.6 and 41.3 mg/mL of fructose in a batch system, respectively (Singh et al., 2007b). The fructose production through enzymatic hydrolysis of poly- and oligo-saccharides of plant extracts by immobilized inulinases of *K. marxianus* would be an efficient and advantageous approach for commercial sugar production (Holyavka et al., 2016). The extracellular exoinulinase (from *K. marxianus* YS-1) was immobilized on Duolite A568 after partial purification and the immobilized biocatalyst was used to produce a high-fructose syrup, which yielded 40.2 and 39.2 g/L of fructose in 4 h using pure and raw inulin (from roots of *A. racemosus*), respectively (Singh et al., 2007c). Furthermore, the inulinases produced by *K. marxianus* using xylose medium as carbon source could be another promising options to produce high concentration fructose syrup at industrial level (Hoshida et al., 2018).

1.4.4.5 Aroma compounds production

Production of flavor and fragrance compounds through biotechnological process plays a significant role in several industries including food, pharmaceuticals, cosmetics and chemical industries because of the growing demand for natural food additives and other products of biological origin (SÁ et al., 2017). Two aromatic alcohols, namely 2-phenyethanol (2-PE) and 2-phenyethyl acetate (2-PEA) having a rose like flavor, are widely used in the food and cosmetics industries. Therefore, the attention is renewed to the microbial production of 2-PE and 2-PEA (SÁ et al., 2017, Hoşoğlu, 2018a). The mostly used biotechnological route of producing 2-PE and 2-PEA is the bioconversion of L-phenylalanine (L-phe) using food-grade yeasts via Ehrlich pathway (Etschmann et al., 2004). In this process, L-phe is transformed into phenylpyruvate (an intermediate metabolite), that is later decarboxylated to phenylacetaldehyde and then reduced to 2-PE though dehydrogenation. Thereafter, 2-PE can be transesterified to 2-PEA (Martínez et al., 2018a). Therefore, different yeast strains are receiving increased interest in catalyzing the bioconversion of L-phe to L-PE for developing an efficient biotechnological production process (Güneşer et al., 2016a). Among various yeasts, several *Kluyveromyces* strains are promising candidate to synthesis significant amounts of aromatic compounds, particularly *K. marxianus* possess high potential

to produce different aroma compounds such as 2-PE, alcohols, furanones, fruit esters, ketones, carboxylic acids, and aromatic hydrocarbons (Morrissey et al., 2015, Güneşer et al., 2016a).

Martínez et al. (2018a) achieved a maximum 10.21 mg/g of 2-PE and 8.20 mg/g of 2-PEA through the solid-state fermentation process of sugarcane bagasse supplemented with L-phenylalanine using *K. marxianus* as inoculum. In another study, *K. marxianus* CCT7735 was the most outstanding strain among 267 strains to produce the maximum 2-PE (3.44 g/L) titer under optimized conditions of 30 ºC temperature, 3.0 g/L of glucose, and L-phe concentrations of 4.0 g/L (De Lima et al., 2018). The growth of *K. marxianus* CCT7735 was impaired by the concentration of 2-PE in the medium, however, this effect was less pronounced compared to that of reported for certain *S cerevisiae* strains (De Lima et al., 2018). The growth inhibition of *K. marxianus* was observed while the concentration of 2-PE reached a critical value of ~1.4 g/L (Fabre et al., 1998). **Table 1.10** shows several flavor compounds produced by *K. marxianus* during fermentation.

Garavaglia et al. (2007) observed that the yield of 2-PE was significantly affected by medium pH, temperature, L-phe concentration, and oxygen concentration. An optimum yield of 2-PE (0.59 g/L) was achieved using *K. marxianus* CBS6556 on grape must at a pH of 7.0, L-phe concentration of 3.0 g/L, temperature of 37 ℃, and oxygen mass transfer of 2.0/h. In a recent study, Gethins et al. (2015) noticed that the carbon and nitrogen source had a pronounced effect on the yield of volatile and flavor metabolites production using *K. marxianus*. The highest levels of the 2-PE and isoamyl alcohol obtained while yeast extract used as supplementary nitrogen source, however, ammonium showed a repressing effect on the 2-PEA production (Gethins et al., 2015).

Table 1.10: Flavor compounds produced by *K. marxianus* in different medium during fermentation.

Aroma compounds	Flavors	Substrate used	Applications	References
2-Phenylethanol (2-PE)	Rose-like smell	Whey	Food industry: fruit formulas, ice cream, candy, soft drinks, gelatins, puddings, rubber gum; Pharmaceutical industry: antiseptic and local anesthetic; Perfumes and cosmetics	(Conde-Báez et al., 2017)
2-Phenethyl acetate (2-PEA)	Floral and rose-like odor	Sugarcane bagasse	Food, fragrance, cosmetic industries	(Martínez et al., 2018a)
Phenylethyl propanate	Caramel aromas	YPD medium	Flavors, Fragrances	(Hoşoğlu, 2018a)
Ethyl acetate	Fruity and sweet	Whey permeate	Manufacturing inks, adhesives, photoresists, coating formulations, and utilized as an extracting agent.	(Löser et al., 2013a)
Isoamyl alcohol	Banana like smell	YPD medium	Flavors, Fragrances	(Hoşoğlu, 2018a)
Isoamyl acetate	Sweet aromatic, fruity smell like banana or pear	YPD medium	Flavors, Fragrances	(Hoşoğlu, 2018a)
2-phenylethyl-isobutyrate	Floral smell	Whey and pomaces	Flavoring and fragrance agent used in decorative cosmetics, fine fragrances, shampoos, toilet soaps, other toiletries, and non-cosmetic products	(Güneşer et al., 2016a)

In contrast, nitrogen source did not show any influence on isoamyl acetate or ethyl acetate production, attributed to the fact that more than one alcohol acetyl transferase activity was present in *K. marxianus*. Moreover, the lower production of all acetate esters in a growth medium containing lactose as a unique carbon source compared to glucose or fructose, indicates a lower pool of the acetyl-CoA (Gethins et al., 2015). The ethyl acetate is derived from the esterification of acetyl-CoA and ethanol by the action of alcohol acyltransferase enzyme which catalyzes the esterification reaction of aliphatic and aromatic alcohols and acyl-CoA into esters. Thus, probably *K. marxianus* DSM5422 synthesized the ethyl acetate from acetyl-CoA. However, it was observed that impairing the activity of the TCA cycle by limiting the availability of iron (Fe) or copper (Cu), the production of ethyl acetate can be triggered (Löser et al., 2012). This might be attributed to the diversion of acetyl-CoA to ester synthesis from the tricarboxylic acid cycle in a Fe-limiting condition because of the reduced activity of aconitase which catalyses the stereo-specific isomerization of citrate to isocitrate, and succinate dehydrogenase (both enzymes depend on Fe) as well as for a limited oxidation of NADH in the respiratory chain because the electron transferring proteins depend on Fe and Cu (Löser et al., 2015). Recently, Löbs et al. (2018) demonstrated that the alcohol acetyl transferase *Eat1* is the critical enzyme for ethyl, isoamyl, and phenylethyl acetate production by using *K. marxianus* and that high ester biosynthesis is contingent on *Eat1* mitochondrial localization.

Lately, the efficiency of fermentation was enhanced using cell immobilization technique while *K. marxianus* was cultivated on apple/chokeberry, which resulted in higher yield of aroma compounds by immobilized *K. marxianus* stain (Wilkowska et al., 2015). Furthermore, delignified cellulosic supported *K. marxianus* strain demonstrated to be suitable for whey fermentation at high temperature. The whey after fermentation by *K. marxianus* observed to have a low level of amyl alcohols and an improved aroma with an alluring flavor compared to unfermented one (Kourkoutas et al., 2002). In addition, the higher concentration of volatile metabolites such as 2-phenylethyl isobutyrate, phenylethyl acetate, phenyl ethyl alcohol , ethyl acetate, isoamyl acetate, isovaleric acid, and isoamyl alcohol could be synthesized by a strain of Na-alginate entrapped *K .marxianus* LOCK0024 using agro-industrial wastes such as pepper and tomato pomaces, grape, and acid whey as substrate (Güneşer et al., 2016a).

1.4.5 Bio-environmental applications

1.4.5.1 Organic load reduction from agri-food industry wastewater

Environmental pollution caused by extensive industrial wastewater generation is a serious issue and it cannot be avoided due to population growth, industrialization, and food production practices (Karim et al., 2019a). As such, the agro-industries including dairy industry, brewery industry, apple industry, etc. generate a huge amount of waste in North America, especially, in Canada. Although there are different kinds of agro-industries in Canada, the large amounts of wastewater generation are related to the dairy industries. Generally, these dairy industries are associated with the transformation of the raw milk to the milk, yoghurt, cheese, ice cream, butter, milk powders, and other milk products by distinct kinds of production processes (Davarnejad et al., 2018). The dairy effluents are considered as high strength wastewater as they contain a higher degree of chemical oxygen demand (COD), biochemical oxygen demand (BOD), and various kinds of nutrient compounds, mainly phosphorus and nitrogen. According to Ghaly and Singh (1989), the higher level of lactose concentration in dairy wastewater might be responsible for the high COD, for example, a lactose content of 50 g/L corresponds to a COD of 40,000-60,000 ppm and, which can interfere with the biological process of sewage disposal plants. For years, the disposal of wastewater has been problematic and often discharged into local water or fields (Yousuf et al., 2017, Price, 2019). Therefore, the effective treatment of effluents must be required, not only to minimize the environmental burden but also for the purpose of water recovery from industrial processes (Davarnejad et al., 2018). Apart from lactose, the whey also contains lipids, soluble proteins, some nitrogenous compounds, vitamins and detergents, and various kinds of mineral salts. Consequently, these abundant, inexpensive, micronutrient rich substrates could potentially be used as alternate carbon sources for bioprocessing.

The bioconversion of wastewater by using yeast could be a promising technique due to the higher sugar contents of whey (Kourkoutas et al., 2002, Zafar and Owais, 2006). Whey, a wastewater from dairy industry, contains a number of minerals and vitamins in addition to the basic sugar lactose, which may efficiently enhance the physiological activity of the cells. The bioconversion of whey to ethanol using the yeasts, particularly, *Kluyveromyces* species (Zafar and Owais, 2006) appears to be potential through the efficient bioremediation of plant effluents (Kourkoutas et al., 2002). *K. marxianus* URM 7404 showed a reduction of 78.94%

COD from cheese whey during the bioethanol production (0.50 g/g, 2.57 g/L/h) (Murari et al., 2019). Moreover, *K. marxianus* is a suitable species which was able to produce alcohol and fodder yeast by fermentation of deproteinized and concentrated whey due to its special physiological characteristics. It can uptake and hydrolyze lactose to the monomers, glucose and galactose by a lactose permease and a β-galactosidase enzyme, respectively. A combined approach of biomass harvesting (such as SCO or SCP or others) and pollution potential reduction (e.g., COD reduction) could be employed using deproteinized whey as a substrate for *K. marxianus*. Schultz et al. (2006) reported that 80% COD was reduced by *K. marxianus* using deproteinized sour and sweet cheese whey as substrates to produce SCP. In another study, 42% total COD, 65% soluble COD, 53% total solids, and 90% ammonium nitrogen were reduced by successful fermentation of the whey permeates. Nevertheless, the significant reduction of suspended solids and organic nitrogen were also achieved to 60% and 17%, respectively (Ghaly and Singh, 1989).

Furthermore, the maximum COD reduction of 80.20% was achieved at a retention time of 24 h through a high biomass productivity (0.17 g/L/h) by a coculture of *K. marxianus* and *C. krusei* using whey as carbon source (Yadav et al. 2014a; Yadav et al., 2015). The removal efficiency of COD for several *K. marxianus* strains via biomass production from different substrates is presented in **Table 1.11**. Nevertheless, *K. marxianus* is also able to treat the highly acidic food processing waste effluents by removing the organic pollutants from wastewater. The lactic acid could be rapidly removed by a flocculent strain of *K. marxianus* from sauerkraut processing effluents (Hang et al., 2003, Nowak and Hang, 2003). The biodegradation of lactic acid by *K. marxianus* from sauerkraut brine was studied by Nowak and Hang (2003) and they found that the lactic acid concentration reduced by 81.2 to 90.17% after 48 h of fermentation. They observed that *K. marxianus* was the best candidate to produce a higher amount of biomass over other microbial cultures used (over 13 g of CDW per liter substrate) and, significantly consumed the organic pollutants (acetic acid, lactic acid, and ethanol) from the corn silage juice (Hang et al., 2003).

Table 1.11: COD removal efficacy and simultaneous biomass (SCP) production performances of several *Kluyveromyces* species from waste effluents.

Strains	Substrates	Objectives	Initial COD (gL^{-1})	Removal efficiency (%)	Conversion rate (%)	Process conditions	References
K. marxianus GQ 506972	Diluted cheese whey	COD removal and biomass (SCP) production	50	55-78.50	12-19	Batch fermentation with high cell densities and continuous process	(Yadav et al., 2014b)
K. marxianus CBS 6556	Deproteinized sweet whey	COD removal and SCP production	193	90	52	Batch fermentation	(Schultz et al., 2006)
K. marxianus CBS 6556	Deproteinized sour whey	COD removal and biomass (SCP) production	150	83	48	Batch fermentation	(Schultz et al., 2006)
K. marxianus	Deproteinized cheese whey	COD removal and SCP production	-	96.26	30	Batch, $(NH_4)_2$. SO_4 as nitrogen supplementation	(Anvari and Khayati, 2011)
K. fragilis	Deproteinized sweet cheese whey	SCP production	-	-	55	Batch, Enriched matrix	(Kebbouche-Gana and Touzi, 2001)

K. marxianus	Whey permeate, 4.5% (w/v) solution	SCP production	~48	75.60	84	Batch fermentation, 0.22% (w/v) urea as nitrogen source	(Yadav et al., 2016)
K. fragilis	Acid cheese whey	COD removal and biomass (SCP) production	74.22	42.98	40.98	Batch, without supplementation	(Ghaly and Kamal, 2004)
K. marxianus CHY 1612	Diluted cheese whey	COD removal and SCP production	30	78	26	Batch fermentation, 0.15% (w/v) urea as nitrogen source	(Yadav et al., 2014a)
K. marxianus ATCC 36907	Cassava wastewater	COD removal and and 2-phenylethanol	16.09	80.42	13.08	Batch, glucose and phenylalanine as supplement	(de Oliveira et al., 2013)
K. marxianus + *S. cerevisiae*	Whey permeate, 4.5% (w/v) solution	SCP production	~52	73.30	92	Batch fermentation, 0.22% (w/v) urea as nitrogen source	(Yadav et al., 2016)
Serratia marcescens + *K. fragilis*	Fresh sweet whey	Protease production and COD removal	88	76	46	Batch fermentation	(Ustáriz et al., 2007)

K. marxianus + *C. krusei*	Diluted cheese whey	COD removal and biomass (SCP) production	30	86.80	31	Batch fermentation, 0.15% (w/v) urea as nitrogen source and TSB media	(Yadav et al., 2014a)
K. marxianus + *C. krusei*	Diluted cheese whey	COD removal and SCP production	30	26-80.2	17-27	Continuous process, 6 h to 24 h HRT	(Yadav et al., 2014a)
K. marxianus + *C. krusei*	Diluted cheese whey	COD removal and SCP production	30	80.20	37.20	Batch fermentation, without any supplement	(Yadav et al., 2015c)

1.4.5.2 Heavy metals recovery from agri-food industry wastewater

The wastewater with the presence of heavy metals or/and radionuclides from different industrial effluents poses serious environmental threat due to the metal toxicity effects, non-degradability; and moreover, their accretion through the food chain may lead to terrible health and ecological crises. There are various kinds of physical and chemical methods to treat the wastewater for heavy metals; however, remediation through physiochemical methods is not cost effective, high energy consuming (Pal et al., 2009), and less feasible for the effluent containing complex organic matters (Aksu and Dönmez, 2000, Fonseca et al., 2008). Therefore, the bioremediation (biotechnological approaches) of heavy metals and other pollutants has been getting renewed attention in the recent time because of its potential application in many industries (Fonseca et al., 2008, Islam et al., 2018). Several microorganisms including *S. cerevisiae*, *C. utilis*, *K. marxianus* have much potential for removing metals from wastewater either by active or passive uptake mechanisms. There are two ways of bioremediation by microorganisms, bioaccumulation (a metabolism-dependent slow uptake) and biosorption (a metabolism-independent rapid surface reaction) (Aksu and Dönmez, 2000). Yeast cells show a better performance over other microorganisms due to their special characteristics of metal uptake (Pal et al., 2009), faster growth rate on cheap media, ease of cultivation at large scale, and their capability of accumulating a wide range of heavy metals under different external conditions (Aksu and Dönmez, 2000, Karim et al., 2018c).

Aksu and Dönmez (2000) proposed a microbiological approach to remove copper (II) or Cu^{2+} ions using *K. marxianus* in molasses. They observed that, at a constant initial Cu^{2+} concentration (100 mg/L), *K. marxianus* efficiently removed copper ions with a maximum uptake of 8.0 and 9.8 mg/g of dry biomass at an initial sucrose concentration of 5 and 20 g/L, respectively. Increase in the biomass biomass production, Cu^{2+} accumulation, and specific Cu^{2+} uptake was observed with increasing initial sucrose concentration attributed to the defense mechanism of *K. marxianus* cells, namely acclimation to toxicity. Furthermore, the rate of Cu^{2+} bioaccumulation was also observed to increase (4.3 to 55.0 mg/g) with increasing initial Cu^{2+} concentration (from 50 to 500 mg/L); however, the growth rate was decreased from 0.082 to 0.038 h^{-1} at same initial sucrose concentration (5 g/L). This might be due to the inhibitory effects of excess Cu^{2+} concentration in the medium. The maximum growth and

bioaccumulation by *K. marxianus* were determined at an optimum pH of 4.0. It achieved a maximal Cu^{2+} uptake capacity of 63.6 mg/g at the end of the exponential phase, while the initial Cu^{2+} and initial sucrose concentrations were 512.2 mg/L and 20.4 g/L, respectively. However, the Cu^{2+} concentration was only decreased by 48.4% after 8 days of biomass growth. The overall results of that study imply that *K. marxianus* possess a high level of copper (II) resistance, which probably facilitated by constitutive production of metallothionein as well as synthesis of other copper-binding proteins. Moreover, they could have enough energy reserves for active transport of Cu^{2+} metal that eventually deposited into the vacuoles, which become expanded with the increasing time of exposure to the Cu^{2+} solution.

Similarly, Dönmez and Aksu (1999) reported that the performance of accumulating copper (II) and the ability of microbial growth were mostly dependent on the initial concentration of Cu^{2+} and the pH of medium. The maximum Cu^{2+} accumulation was obtained at the optimal pH vales of 5.0, 4.0, 4.0, and 4.0 for *K. marxianus*, *S. cerevisiae*, *Chizosaccharomyces pombe*, and *Candida* sp., respectively. *K. marxianus* and *Candida* sp. were more efficient than *S. cerevisiae* and *C. pombe* in respect to heavy metal resistance and bioaccumulation of Cu^{2+} at higher Cu^{2+} concentrations without losing their biological activities. The maximum removal efficiency of Cu^{2+} was achieved to 25, 72.6, 74.2, and 90.3% for *S. pombe*, *Candida* sp., *S. cerevisiae*, and *K. marxianus*, respectively. *Candida* sp. was highly resistant to Cu^{2+} concentration of 708 mg/L compared to *S. cerevisiae* (291 mg/L), *S. pombe* (101 mg/L), and *K. marxianus* (488 mg/L). On the contrary, an inhibitory effect on the growth of cell was observed for lead (II) uptake by *K. marxianus* from the contaminated molasses. However, the decline in biomass production did not lead to decrease lead (II) uptake; and the biosorption ability was greater at higher initial lead (II) concentrations (Fonseca et al., 2008). The capability of *K. marxianus* in biosorption of uranium was also reported by Bustard et al. (1997) where 120 mg U/g (dry weight basis) of biomass was achieved in the same time.

Furthermore, the bioaccumulation of metal cations such as Cu (II), Zn (II), Co (II) using both free and immobilized *K. marxianus* cells was studied by Yusef (1997). However, it was established that the main mechanism of metal accumulation in *K. marxianus* might be the absorption of metals by insoluble cellular materials (Yazgan and Özcengiz, 1994). The

role of functional groups (i.e., carboxylic acids, amines, phosphates, sulfhydryl etc.) for biosorption of several heavy metals including lead, arsenic, cobalt, mercury, and cadmium using *K. marxianus* in different aqueous solutions was demonstrated and optimized by Pal et al. (2009) to ease the biosorption process in metal recovery process. Perhaps the most significant practical limitation to biological uptake is inhibition of cell growth in high metal ion concentration. Another important constraint can be the toxicity of wastewater to living cells such as extremes pH and high salt concentration. However, such limitations would not preclude the applications of living cells in the wastewater treatment processes through the bioremoval of heavy metals. The problem of metal toxicity may be overcome using a metal-resistant microbe like *K. marxianus*. Indeed, the tolerance and uptake capacities of a living organisms are the most preferential characteristics to be effectively utilized in a metal-ion removal process.

1.4.5.3 Paper waste and sludge treatment

Paper and pulp mill industries are responsible for the production of huge amount of wastes in many countries. The paper sludge is the solid waste stream, which generally contains short cellulose fibers, contaminants, and other paper making components such as clays and fillers (Chen et al., 2014). The disposing of this waste stream (highly contaminated sludge or biosolids) makes the paper production costly; and hence, it provoked the entrepreneurs and the government to find out new options to use these biosolids (Kádár et al., 2004, Chen et al., 2014). However, paper sludge could be an attractive biomass source for the production of various value added products such as fuel ethanol, due to its high and easily accessible cellulose content of 50-60% (Kádár et al., 2004), low cost, and lack of special pretreatment requirement (Chen et al., 2014).

Kádár et al. (2004) investigated the efficiency of *K. marxianus* to utilize various paper mill substrates, such as paper sludge, old corrugated cardboard (OCC) waste, and Solka Floc in the SSF to produce ethanol. The result showed that *K. marxianus* was as good as *S. cerevisiae* in SSF at 40 °C using paper sludge and OCC, and the cellulose conversions of 55-60% were achieved for all substrates. No significant differences in the yield of ethanol production were observed between *K. marxianus* and *S. cerevisiae*, and the yield was 0.31 to 0.34 g/g cellulose added for both strains. These results showed that the conversion of lignocellulosic industrial wastes like paper sludge and OCC would efficiently be utilized to

produce bioethanol in SSF. In another study, SSF experiments were performed at a higher temperature (32-45 ℃) by utilizing Solka Floc (10%) as cellulose substrate, and the highest ethanol production (38 g/L) was achieved in 78 h using both *K. marxianus* and *K. fragilis* at a temperature of 42 ℃ (Ballesteros et al., 1991). As it was mentioned elsewhere, *K. marxianus* was not able to efficiently convert cellobiose to ethanol; however, this result demonstrated that *K. marxianus* can convert cellulose to ethanol in SSF batch system, even at 45°C (Barron et al., 1995). Barron et al. (1995) showed that a maximum ethanol production (10 g/L; 39% of theoretical yield) can be achieved using milled paper as nutrient source (5% (w/v) cellulose with 0.75% (v/v) cellulase) at a high temperature (45 ℃). The yield of ethanol production could be further increased by pretreating the milled paper with phosphoric acid (Nilsson et al., 1995), as the accessibility of substrate was improved after the pretreatment which resulting in a considerably higher ethanol yield. It is worth noting that *K. marxianus* was reported to utilize not only the paper wastes, but also a wide range of low-cost substrates from diversified sources as demonstrated in **Table 1.12**.

1.4.5.4 Biosorption of dyes

The textile industries have been discharging large volumes of wastewater into natural water bodies after dyeing process, which leads to serious environmental pollution. Generally, the effluents from textile industries contain huge amounts of toxic chemicals such as azo dyes, and reactive dyes. The excessive discharge of these effluents may adversely affect the natural resources, aquatic species, water quality, and soil fertility; and strongly disturb the integrity of ecosystems (Droste and Gehr, 2018). Discharging of effluents without adequate removal of these dyes might be responsible for severe environmental issues (Holkar et al., 2016). Therefore, the degradation of the dyes in textile effluents is indispensable to avoid toxicity. So far, various methods including physiochemical (such as coagulation, flocculation, flotation, ion exchange, irradiation, electrochemical destruction, adsorption, ozonation, precipitation, and chemical oxidation etc.) and biological methods were adopted for the reduction of azo dyes to achieve decolorization (Holkar et al., 2016, Droste and Gehr, 2018). Some of those methods have been proven to be efficient; however, the excess usage of chemicals, long time requirement, large amounts of sludge generation, high plant installment cost, and high operating and maintenance costs limited their application. The

activated carbon has been reported to be a suitable dye absorbent but the manufacturing and regeneration of this absorbent is expensive (Priya and Selvan, 2017).

Recently, the microbial degradation and decolorization of textile effluents to detoxify the azo dyes has gained great attention from both industries and the scientists' community due to eco-friendly nature, general simplicity, and inexpensive technologies (Sudha et al., 2014). The toxicity and carcinogenicity of textile dyes is mostly due to the several toxic compounds including benzidine and other aromatic components, which could be successfully converted into simple compounds through microbial metabolism (Sudha et al., 2014). Therefore, biosorption technique could be a promising low-cost alternative to efficiently remove color from textile effluents. Certain types of microbial biomass displayed a strong biosorbent behavior towards some metallic ions and other contaminants, like the dyes from textile, as a function of the chemical make-up of the microbial cells of which the biomass consists (Mrudula et al., 2016). Furthermore, several charged pollutants such as acetamide groups of chitin; amido, amino, sulfhydryl and carboxyl groups in proteins; hydroxyl groups in polysaccharides; phosphate groups in nucleic acids could be attracted and sequestered by several chemical groups in biomass (Roane et al., 2015). According to Kakuta et al. (1998), several yeasts were found to be suitable as metal absorbent because of the ability to degrade the synthetic dyes. Meehan et al. (2000) observed that *K. marxianus* was capable of decoloring the solution of Remazol Black-B dye. The solution with Remazol Black-B was completely decolorized within 24 h by actively growing *K. marxianus* IMB3 cells under an aerobic condition and a maximum of 98% color removal was achieved at a temperature of 37 ºC. They also demonstrated that the decolorization was mainly due to the physical adsorption of the dyes to cellular biomass, not due any chemical enzymatic activity (Meehan et al., 2000). However, the mechanism by which the cellular biomass takes-up the dye components was unexplained. In addition, the main challenge in biosorption based processes could be that, the use of biomass may lead to a large amount of sludge generation, which may require a further treatment like the use of solid-state fermentation. Therefore, the problem would be overcome using the microbes that capable of carrying out solid state fermentation including white rot fungi, *K. marxianus*, etc. which have been shown to be effective for textile dye decolorization (Senthilkumar et al., 2014).

Table 1.12: Valorization of different low-cost waste by-products of the food and agricultural industries by *K. marxianus*.

Strains	Substrates	Process	Purpose	References
K. marxianus K21	Agro-industrial waste -Taro waste	Simultaneous saccharification and fermentation	Bioethanol production	(Wu et al., 2016)
K. marxianus IFO 0288	Dairy waste -Cheese whey	Hybrid fermentation–enzymatic bioprocess	Ethanol and lactic acid produced in fermentations were esterified to ethyl lactate	(Koutinas et al., 2014)
K. marxianus IMB3	Agro-industrial wastes -Dairy industry (Cheese whey) -Sugar industry (Molasses) -Solid wastes (brewer's spent grains and malt spent rootlets) -Vegetable and fruit processing wastes (potato and citrus)	Solid-state fermentation	Single cell protein, aroma volatiles and fat production	(Aggelopoulos et al., 2014)
K. marxianus LOCK0024	Agro-industrial wastes (tomato, pepper, grape, and acid whey)	Liquid fermentation	Ethanol and volatile metabolites (flavor compounds) such as ethyl acetate, isoamyl alcohol, isoamyl acetate, 2-	(Güneşer et al., 2016a)

			phenylethyl isobutyrate, phenylethyl acetate, and phenylethyl alcohol production	
K. marxianus NRRL Y-8281	Agro-industrial wastes -Olive pomace	Solid-state fermentation	Tannin acyl hydrolase (tannase, E.C.3.1.1.20) and gallic acid production	(Mahmoud et al., 2018)
K. marxianus var. marxianus CBS 712.	Dairy industry wastes -Whey effluent -Scotta effluent	Semi-continuous fermentations	Bioethanol production	(Zoppellari and Bardi, 2013)
K. marxianus ATCC 10022	Agro-industrial wastes -Sugarcane bagasse -Sugar beet molasses	Solid-state fermentation (Batch, intermittent mixing and fed batch)	Aroma compounds production	(Martínez et al., 2018b)
K. marxianus KCTC 7118	Vegetable waste -Chinese cabbage	Liquid fermentation	Microbial biomass production	(Choi and Park, 2003)
K. marxianus BY25569	Agricultural wastes -Rice waste biomass	Solid-state fermentation	Bioethanol production	(Saratale et al., 2017)
K. marxianus Y01070	Industrial wastes -Old corrugated cardboard -Paper sludge	Simultaneous saccharification and fermentation	Bioethanol production	(Kádár et al., 2004)
K. marxianus	Dairy industry -Whey	Liquid fermentation	Biomass production	(Koutinas et al., 2009)

K. marxianus (own isolates)	Agro-industrial waste -Overripe mango pulp	Liquid fermentation	Bioethanol production	(Buenrostro-Figueroa et al., 2018)
K. marxianus (own isolates)	Agro-industrial waste -Cheese whey (raw whey, wastewater and swab samples)	Liquid fermentation	Bioethanol production	(Hesham et al., 2014)
K. marxianus CBS1555 (KCTC7001)	Lignocellulosic material -Empty palm fruit bunches (EFBs)	Simultaneous saccharification and fermentation	Bioethanol production	(Jung et al., 2015)
K. marxianus var. marxianus CBS 397	Dairy industry waste -ricotta cheese whey (“Scotta”) -Raw cheese whey -Deproteinized whey	Liquid fermentation (batch bioreactor)	Bioethanol production	(Sansonetti et al., 2009)

1.4.6 Concluding remarks and future perspective

S. cerevisiae is a model yeast species which has been exploited for the applications in the biotechnology in addition to basic research in the fields of cell biology, biochemistry, and genetics. However, due to the advancement of biotechnological tools in addition to its intrinsically exceptional characteristics, *K. marxianus* has become one of the top interesting nonconventional yeasts, comparable to *S. cerevisiae*, at least for industrial applications (Nurcholis et al., 2020). Thermotolerance, high ethyl acetate production, utilization of a wide-ranging inexpensive carbon sources (e.g., inulin, lactose, and xylose), a shorter doubling time and faster growth rate make *K. marxianus* as a versatile tool in biotechnological, food and environmental applications. Moreover, the survivability of *K. marxianus* in the presence of relatively higher ethanol concentration and/or elevated temperature indicating its improved adaptation capacity. It was displayed in several literatures that *K. marxianus* possess tolerance to the different stressing conditions such as lower pH, elevated temperature, severe oxygen restraint condition, and higher concentration of ethanol which could be very beneficial for the biorefinery approach-based future industrial applications. In addition, the bioingredients such as flavor and fragrance molecules, SCPs, SCOs production capability and protein secretion performance of *K. marxianus* could be beneficial to the food-based industries by diversifying the product portfolio in future (Güneşer et al., 2016a, Saini et al., 2017a, Madeira-Jr and Gombert, 2018).

On the other hand, the metabolically engineered *K. marxianus* strains with inulin-assimilating capacity, can produce lactic acid by digestion of Jerusalem artichoke tuber powder (Bae et al., 2018). This approach to harvest optically pure L-lactic acid and/or D-lactic acid using recombinant stains could lead to the efficient production of bioplastics in a cost-effective way. However, most of the studies with *K. marxianus* dedicated on the liquid fermentation (LF) to date. The major setback of this technique was the inhibition of microbial cells due to a high volume of final product (such as 2-PE, bioethanol) concentration in the medium. Several strategies like stain development, cell immobilization, in situ product removal, and culture optimization were commonly employed using synthetic media as substrate to overcome this problem (Martínez et al., 2018a). Solid-state fermentation or dry fermentation (DF) using waste biomass as substrate can be of particular interest in this regard, over LF due to the smaller reactor capacity requirements, lower water content, no additional

stirring and so on. However, the larger amounts of inoculum requirements and much longer retention time might be challenging in DF. Therefore, further study is required to engineer the proper techniques and appropriate reactor design for a large-scale application of DF. Nowadays, bioethanol production from LCB hydrolysates using *K. marxianus* has also been getting more importance due to the availability of LCB in nature. But then again, the fermentation of LCB might be difficult to digest due to the unique crystalline structure of LCB. The study with different kinds of pre-treatment methods, in this regard, could be attractive in future research to make the biomass more accessible for the microorganisms. Furthermore, the evolutionary adaptation and immobilization techniques of *K. marxianus* would be evaluated to increase the efficiency of various value-added product through economically viable fermentation processes (**Table 1.13**).

Table 1.13: List of the equivalent strain repository numbers of *K. marxianus* strains (www.atcc.org).

ATCC strain of *K. marxianus*	**Equivalent strain repository**
ATCC 200963	HA 63 [NRRL Y-8281, CBS 712]
ATCC 22296, ATCC 56501	CBS 5671 [K.210, NRRL Y-8287, UCD 71-15]
ATCC 8554, ATCC 34439	CBS 5795, CCRC 21480
ATCC 2340	NRRL Y-6
ATCC 26548	NRRL Y-7571 [CBS 6556, KCTC 17555]
ATCC 26548	KCTC 17555, CBS 6556
ATCC 64885	NRRL Y-1175
ATCC 74080	pKD1
ATCC 200964	HA 732 [CBS 2231]
ATCC 28912	CCY 21-40-1
ATCC 46537, ATCC 56497, ATCC 56752	CBS 397 [CCRC 2147]
ATCC 36907	NCYC 587 [351]

ATCC 60480	SG 120 [4-67-2/4]
ATCC 200965	HA 729 [CBS 600]
ATCC 10022, ATCC 28126	NRRL Y-665 [CBS 6432, CCRC 21628]
ATCC 28244	5850 [VTT C-81111]

Considering all these aspects, it can be argued that the practical research on *K. marxianus* towards industrial application has come into a new era. On the contrary, the diversity of *K. marxianus* species at the genetic, metabolic, and physiological levels is relatively little explored, which would be designed via new engineering tools for producing valuable materials or productivity based on genomic and transcriptomic information and data from modeling of the metabolism (Nurcholis et al., 2020). For a fruitful commercial production platform, it is emergent to instigate the different aspects of strains and process development. In particular, the knowledge gaps around genes, pathways, enzymes and their regulation are sorely needed to fill for a comprehensive understanding of how *K. marxianus* produces relevant metabolites (Morrissey et al., 2015). To set the genetic and phenotypic diversity of *K. marxianus* into context, the metabolic pathway and its mechanism showed variation and functional diversities, hence there are still many opportunities for future study. Exploring of the relationship between genetic variation and functional diversities at species level could be the potential research opportunity for innovative and advanced knowledge. The pilot scale study as well as extensive lab-scale evolution of metabolic engineering would be employed to understand the mechanism of metabolism to justify the applicability in industrial level. Finally, it seems very likely that a wide range of applications of *K. marxianus* is exists but a greater depth of knowledge in the genetic diversity or population genetics is still required. This review on the diversified applications of *K. marxianus*, perhaps will pave the way for more in-depth studies in the biotechnological and environmental arena.

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Chapitre 2: Problem statement, research hypothesis and objectives

2.1 Problem statement

The dairy industries, e.g., cheese manufacturing, is one of the largest industries which produces huge volume of effluents in Canada and worldwide. This is mainly concerning whey which has high pollution load and requiring recovery or treatment before discharging into environment. The production of whey continuously increasing due to global demand of cheese and whey proteins for their nutritional value and wide range of functionality. Another co-product is whey permeate (WP) that is remained after recovering whey proteins from liquid whey by ultrafiltration-diafiltration, which has so far been limited applications and major parts of this is simply discarded as dairy effluents with serious environmental issues. The high organic load of whey/WP is mainly due to the high concentration of lactose, and their recovery remain as a real challenge, especially in its whole and without any pre-treatment and/or fractionation, which is the existing worldwide trend. Recovery of lactose from the whey/WP is one of the simple options offered, but the world market is saturated which makes this route less appealing with an end-product of low commercial value. Therefore, the isomerization of lactose for a large-scale production of prebiotic lactulose has attracted extensive research interests in recent times.

At present, the commercial lactulose is produced through the chemical synthesis from lactose by following an isomerization reaction in an alkaline medium using high quantity of base catalysts and several complexing reagents. Most of these processes are generally characterized by huge challenges for low yield of lactulose (less than 25%), and subsequent by-products formation such as epilactose, galactose, glucose, isosaccharinic acid due to the high level of lactose degradation. However, presence of any side products is undesirable, especially for food, pharmaceutical, and medical applications. Moreover, using catalysts lead to the extensive separation and purification steps, and subsequent increment in the production cost. In this context, this research project is intended to develop a sustainable process for complete valorization of whey/WP through *in situ* isomerization of lactose into lactulose by electro-activation (EA) technology, without having to resort to upstream treatment and/or fractionation and/or downstream during the process. The end-product targeted by this study

could have prebiotic and antioxidant properties for a range of uses in the food, pharmaceutical, cosmetic, and even livestock feed industries, which could alleviate pressure on the environment. Although the EA is a promising green technology and chemical method is mostly used for lactose isomerization into lactulose, no comparative study is existing in the literature that showed which method is more efficient at equivalent reaction conditions. Therefore, a comparative study is required to determine the most effective method for lactulose production *in situ* of whey/WP.

Furthermore, the microbial conversion of whey/WP into valuable fermented metabolites such as organic acids, enzymes, volatile flavour substances, and single-cell proteins could be a sustainable approach for whey management and valorization. However, raw whey/WP is a tricky type of waste by-product to handle, which can be quickly acidified because of its high organic content with inadequate bicarbonate alkalinity. Another main problem is low total solid content and high lactose to glucose ratio, that makes fermented beverage watery and unpleasant taste. Furthermore, many microorganisms including probiotic bacteria showed poor growth in the milk-based substrates because of their limited lactose assimilation ability, weak proteolytic activity, and inadequate oxidation-reduction potential of the substrates. Hence, there is still an immense need to explore the efficient substrates or suitable supplements to incorporate with substrates for promoting the growth of microorganisms. Several recent studies demonstrated that the EA of whey/WP could convert lactose into lactulose and other simple sugars such as galactose, glucose, and fructose at a desired alkaline condition, which in turn may promote microbial growth by ease assimilation of readily available monosaccharides and prebiotic lactulose. However, no report is found to explain the proficiency of electro-activated whey (EA-whey) and electro-activated whey permeate (EA-WP) compared to typical whey/WP. Further research is needed to evaluate the efficiency of electro-activated substates to produce specific metabolites through fermentation.

2.2 Research hypothesis

Based on the literature, it can be hypothesized that electro-isomerization of lactose into lactulose directly *in situ* of whey and WP using the EA technology is a suitable way for integral valorization of whey as well as the EA-whey and EA-WP produced in the EA process

can be used as suitable growth media to produce highly valuable metabolites using a whole Kefir culture and *Kluyveromyces marxianus* as fermenting agents.

2.3 General Objective

The main objective of this study is aimed to compare the electro-activation technology with a chemical process at equivalent solution alkalinity for lactulose production using lactose, whey, and WP as feedstocks and to valorize the electro-activated materials into valuable metabolites using a whole Kefir culture and a pure culture of *K. marxianus*.

2.4 Specific objectives

To verify the research hypothesis and to achieve the main objective, the following specific objectives are formulated:

Specific objective 1: To study the efficiency of lactose isomerization into lactulose by comparing the electro-activation and the chemical isomerization processes at equivalent solution alkalinity by using pure lactose solution.

Specific objective 2: To study the efficiency of lactose isomerization into lactulose *in situ* of whey by comparing the electro-activation technology and the chemical isomerization processes at equivalent solution alkalinity.

Specific objective 3: To study the efficiency of lactose isomerization into lactulose *in situ* of WP by comparing the electro-activation technology and the chemical isomerization processes at equivalent solution alkalinity.

Specific objective 4: To study a bioprocess of using EA-whey/WP/lactose as substrates to produce valuable metabolites by using a starter culture originated from a whole kefir grain.

Specific objective 5: To study a bioprocess of using electro-activated Whey/WP/Lactose as substrates to produce valuable metabolites by using *K. marxianus* as a starter culture.

Chapitre 3: Sustainable electroisomerization of lactose into lactulose and comparison with the chemical isomerization at equivalent solution alkalinity

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RÉSUMÉ

La demande de production de lactulose augmente continuellement en raison de son utilisation pharmaceutique et de son pouvoir bifidogène en tant que prébiotique reconnu et éprouvé. Par conséquent, l'isomérisation du lactose pour produire de la lactulose par électro-activation (EA) en solution, une technologie efficace et potentiellement respectueuse de l'environnement, est d'actualité. Dans cette étude, il a été démontré que la production de lactulose par électro-isomérisation du lactose est affectée par plusieurs conditions opératoires et que le processus peut être optimisé. Dans ce contexte, la technologie d'électro-activation (EA) a été appliquée pour isomériser le lactose en lactulose dans un réacteur modulé par des membranes échangeuses d'anions et de cations. L'effet de la concentration du lactose (5, 10, 15 et 20 %), de l'intensité du champ électrique appliqué (300, 600 et 900 mA) et du temps de traitement (0-60 min) sur la cinétique d'électro-isomérisation du lactose et la formation de lactulose, ainsi que des co-produits de la réaction (formation de glucose, de galactose et de fructose) a été étudiée. Différents paramètres physico-chimiques tels que le pH, l'alcalinité titrable, la température, la migration des ions et le potentiel d'oxydoréduction (ORP) lors de la conversion du lactose en lactulose ont été mesurés et corrélés avec la formation de lactulose pour comprendre le mécanisme d'action du processus impliqué. La conversion du lactose en lactulose était dépendante de la concentration en lactose, du courant électrique appliqué et du temps d'EA. Il a été possible d'atteindre un rendement de lactulose de 38 % après 40 min d'EA en utilisant une intensité de courant électrique de 900 mA dans une solution de10% de lactose. Les résultats ont ensuite été comparés avec ceux obtenus avec une isomérisation chimique classique utilisant du KOH comme agent alcalinisant en maintenant des conditions alcalines similaires à ceux de l'électro-activation (EA) à température ambiante (22 ± 2 °C). Les résultats obtenus ont montré que le rendement en lactulose a été plus élevé avec le procédé d'électro-activation (EA) en un temps de réaction plus courte par rapport à celui de l'isomérisation chimique. Les résultats de cette étude suggèrent que l'EA est une approche prometteuse pour l'amélioration de la production de lactulose à partir du lactose.

Mots-clés: Electroactivation; Lactose; Lactulose; Alcalinité; Migration d'ions; Potentiel d'oxydoréduction; Température; Isomérisation chimique.

ABSTRACT

The demand of lactulose production is increasing tremendously because of its bifidogenic (prebiotic) functionality. Therefore, the isomerization of lactose to synthesize lactulose through electroactivation (EA) technology is of great interest nowadays. However, lactulose production through electroisomerization is affected by several operational and experimental conditions, and the process needs to be optimized. In this context, the EA technique was applied to isomerize lactose into lactulose in an EA reactor modulated by anion and cation exchange membranes. The effect of lactose concentrations (5, 10, 15, and 20%), applied electric fields (300, 600, and 900 mA), and processing time (0-60 min) on lactose electroisomerization rate (lactulose formation) and coproduct (glucose, galactose, and fructose) formation has been investigated. The effect of different physicochemical parameters such as pH, alkalinity, temperature, ion migration, and oxidation-reduction potential (ORP) on the conversion of lactose into lactulose was correlated with the lactulose formation to understand the involved process mechanism of action. The conversion of lactose into lactulose was lactose-concentration-, electric-current-, and EA-time-dependent and reached the highest lactulose yield of 38% at 40 min using a 900 mA current intensity in a 10% lactose solution. The results were then compared to conventional chemical isomerization maintaining similar alkaline conditions at ambient temperature (22 ± 2 °C). A higher yield of lactulose was achieved in the EA process within a short reaction time compared to that of the chemical isomerization. The outcome of this study suggests that EA is a promising technique for the enhanced production of lactulose from lactose.

Keywords: Electroactivation; Lactose; Lactulose; Alkalinity; Ion migration; Oxidation-reduction potential; Temperature; Chemical isomerization.

3.1 Introduction

Nowadays, the demand of lactulose production is increasing tremendously because of its bifidogenic (prebiotic) functionality with many applications in food, nutraceuticals, and pharmaceutical industries. Lactulose (4-O-β-d-galactopyranosyl-d-fructose) is a synthetic disaccharide composed of a galactose moiety linked to a fructose moiety by a 1-4 β-glyosidic linkage (Kareb and Aïder, 2018, Djouab and Aïder, 2019a). In pharmaceutical industries, lactulose is widely used as an effective drug against different diseases like acute and chronic constipation, hepatic encephalopathy, inflammatory bowel disease, and liver disease (Aider and de Halleux, 2007, Kareb and Aïder, 2018). Furthermore, it lowers blood glucose and insulin levels (antidiabetic), increases mineral absorption, and has been reported as antiendotoxin, effective in tumor prevention, as well as in hypocholesterolemia (Nooshkam and Madadlou, 2016). In food industries, lactulose is used as a bifidus factor and has purported high stability under thermal-acidic conditions and thus can be used as an excellent ingredient for acidic foods, such as fruit juices (Nooshkam and Madadlou, 2016). Therefore, the isomerization of lactose for large-scale production of lactulose has attracted extensive research interests in recent times.

Currently, the commercial lactulose is produced through the chemical synthesis from lactose by following an isomerization reaction in an alkaline medium according to the Lobry de Bruyn-Alberda van Ekenstein (LA) transformation (Aider and de Halleux, 2007, Kareb and Aïder, 2018). Most of these processes are generally characterized by a huge challenge for the low yield of lactulose and subsequent byproduct formation such as epilactose, galactose, glucose, and isosaccharinic acid due to the high level of lactose degradation. However, the presence of side products is undesirable, especially for food, pharmaceutical, and medical applications (Kareb and Aïder, 2018). Furthermore, a substantial amount of catalysts, such as calcium hydroxide, sodium and potassium hydroxides, sodium carbonate, magnesium oxide, tertiary amines, borates, sodium aluminates, zeolites, and eggshell powders have been used (both in homogeneous and heterogeneous catalysis) to improve the reaction yield, which led to the extensive separation and purification steps, and subsequent increment in the production cost (Aider and Gimenez-Vidal, 2012). Although the uses of several complexing reagents such as aluminates and borates could accelerate the reaction with a minimum of secondary reactions and result in a high yield of lactulose by eliminating

lactulose from the reaction mixture in the form of a complex, however, they are considered to be unsatisfactory from the industrial viewpoint because of the toxicity and complexity of eliminating the aluminate and borate (Zokaee et al., 2002). In addition, the lack of reaction selectivity of chemical isomerization has limited its application on a large scale (Aissa and Aïder, 2013a). On the other hand, the lactulose synthesis by the enzymatic process could be a suitable alternative to overcome the limitations associated with the chemical synthesis since it is usually carried out under mild conditions, which could limit the formation of side products. Thus, it would provide a high-purity final product and, consequently, could simplify the purification steps (Cardelle-Cobas et al., 2016). However, the major problems are the low yield, extended reaction time, and high production cost depending on the microbial source of enzyme catalysts (Aissa and Aïder, 2013a).

Recently, an emerging technology called electroactivation (EA) has been introduced and attracted particular attention for the isomerization of lactose into lactulose without adding any alkalinizing chemicals (Aider and Gimenez-Vidal, 2012, Kareb and Aïder, 2018). EA is a science devoted to studying the physicochemical and reactive properties of aqueous solutions excited by an external electric field in a reactor that is modulated by the appropriate disposition of electrodes and ion-exchange membranes to modify the activation energy required for the targeted chemical reactions (Aider et al., 2012b). Fundamentally, the charged species migrate toward the electrode of opposite charge, when an aqueous solution is subjected to an external electric field (Aider and Gimenez-Vidal, 2012). In fact, water splitting occurs at the interfaces of electrodes with a simultaneous generation of protons (H^+) and hydroxyl (OH^-) ions (Hung et al., 2012). Thus, the EA process is able to self-generate acid and alkaline conditions following the electrolysis of water molecules at the solution/anode and solution/cathode interfaces, respectively (Aissa and Aïder, 2013a). In the case of the lactose isomerization reaction, the alkaline solution (catholyte) can be employed either in pure lactose or directly in situ of whey to produce lactulose by following lactose isomerization (Djouab and Aïder, 2019b). Indeed, a high alkaline condition is a prerequisite to creating enough proton acceptors, required for the isomerization reaction to occur and to neutralize the acids contained in the medium that can inhibit the isomerization reaction (Paseephol et al., 2008b, Djouab and Aïder, 2019a). The formed OH^- ions at the cathode interface of the EA reactor were capable of creating required alkaline conditions and act as

proton acceptors in the isomerization of lactose into lactulose (Bologa et al., 2008, Sprinchan et al., 2011, Djouab and Aïder, 2019b, a).

Generally, the conventional isomerization of lactose into lactulose was operated at a higher temperature and required longer reaction time. In contrast, the EA process can be performed under relatively low temperature (0 to 30 °C) and approximately 35-45% lactulose yield with a purity of 95% can be achieved in short reaction time (Aissa and Aïder, 2013c). Kareb et al. (2016b) achieved a maximum yield of 35% lactulose after 40 min of reaction at a temperature of 10 °C under a 400 mA electric current and using 100 mL of 7% sweet whey as feed solution. In a recent study, Djouab and Aïder (2019b) obtained a yield of 38.66% lactulose at a current intensity of 330 mA for 14 min in a 5% lactose solution. The optimization of several parameters such as current intensity, reaction time, electrolyte concentration, and reactor configuration resulted in an increased lactulose yield of up to 45% (Aissa and Aïder, 2014b). These results were quite higher than those obtained by chemical synthesis (~16-25% lactulose yield), which were operated at a higher temperature (~70 to 130 °C) and prolonged reaction time (~60-150 min) in the presence of strong bases (Hashemi and Ashtiani, 2010a, Song et al., 2013b, Sakkas et al., 2014b). Lately, the effect of several process parameters of EA on the reaction yield, product purity, and process efficiency such as current intensity, electric tension, concentration, and the volume of feed solution, temperature, and reactor configuration were studied by several researchers (Aider and Gimenez-Vidal, 2012, Aissa and Aïder, 2014b, Kareb et al., 2016b, Djouab and Aïder, 2019b); however, the chemical mechanisms behind this process are still not understood completely. Moreover, the effect of several physicochemical parameters such as solution alkalinity, ion migration, oxidation-reduction potential (ORP), etc. on the process performance is not studied to date. Furthermore, no study was devoted to evaluating the EA process efficiency in comparison with the conventional chemical isomerization at equivalent solution alkalinity. In this context, a detailed study of the physico-chemical principles involved in the EA process to produce lactulose using the lactose aqueous solution is imperative to understand the process mechanism.

In this study, several EA process parameters such as lactose concentration, electric current intensity, and EA duration were studied to understand their effect on solution pH, alkalinity, temperature, ion migration, ORP, and sugar profile. Furthermore, the conversion

rate of lactose into lactulose was compared with that of the conventional chemical isomerization under equivalent solution alkalinity.

3.2 Materials and methods

3.2.1 Chemicals and reagents

The high-purity chemicals and reagents (purity≥95%) of analytical or high-performance liquid chromatography (HPLC) grade were obtained from different suppliers. Lactose, lactulose, fructose, glucose, and galactose (HPLC grade) were purchased from Sigma-Aldrich (Ottawa, Ontario, Canada). Phenolphthalein ($C_{20}H_{14}O_4$) was procured from MAT Laboratory Inc. (Laboratoire Mat Inc., Quebec, Canada). Potassium chloride (KCl) and hydrochloric acid (HCl) were procured from Fisher Chemical (Geel, Belgium). Potassium sulfate (K_2SO_4) and lactose ($C_{12}H_{22}O_{11}.H_2O$) powder used in this study were obtained from Sigma-Aldrich Co. (St. Louis, MO). All solutions were prepared in deionized (DI) water. The cation exchange membrane (CEM) and anion exchange membrane (AEM) were purchased from Membrane International Inc. (Ringwood, NJ), and were used directly in the reactor without any pretreatment.

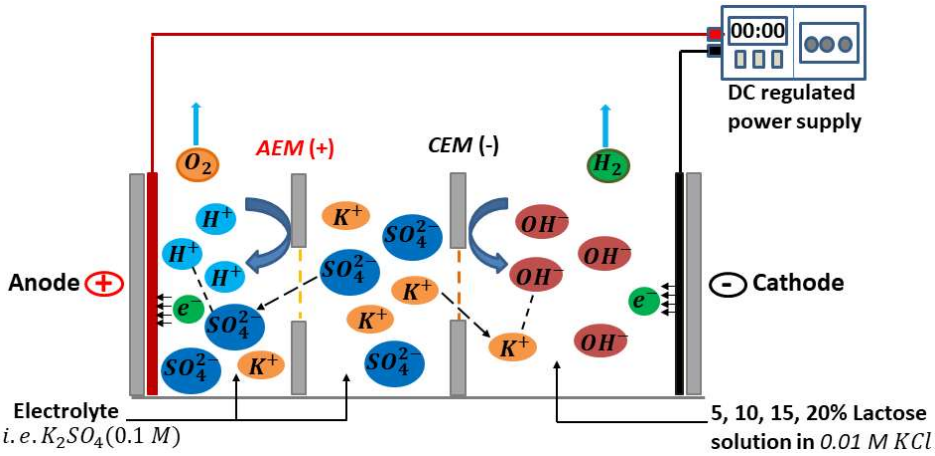

Figure 3.1: Schematic diagram of the electro-activation reactor used for the isomerization of lactose to lactulose.

3.2.2 Electro-activation protocol

An EA reactor made of Plexiglas, comprising of three compartments (anodic, central, and cathodic compartments), was used in this study (**Figure 3.1**). In brief, the anodic compartment was linked to the positive side of a DC-regulated power generator (model: CSI12001X, CircuitSpecialists.com) through a titanium electrode coated with ruthenium-

iridium (RuO_2-IrO_2-TiO_2), and the cathodic compartment was connected to the negative side using a food-grade stainless steel electrode. The anodic and cathodic compartments were separated by the central compartment, and it was communicating with the anodic and cathodic compartments through an anion (AMI 7001S) and a cation (CMI 7000S) exchange membrane, respectively. The freshly prepared lactose solution (350 mL) of different concentrations (5, 10, 15, and 20%; w/v) in 0.01 M KCl was placed in the cathodic compartment, whereas the central and anodic compartments were filled with the 0.1 M K_2SO_4 solution. The experiments were carried out under different current intensities set at 300, 600, and 900 mA for 60 min. The samples were collected from the cathodic and central compartments in a regular interval of 5 min and were kept at 4 ℃ until further analysis. All experiments were conducted at ambient temperature (22 ± 2 ℃). Prior to each batch, the EA reactor was properly cleaned with DI water and filled with DI water after each use to maintain high membrane hydration.

3.2.3 Evaluation of pH, alkalinity, oxidation-reduction potential (ORP), and temperature

The temperature, ORP, pH, and alkalinity of the lactose solution in the cathodic compartment were measured in 5 min intervals during the 60 min of the EA process. The pH was determined using an Oakton pH 700 digital pH meter equipped with a pH probe (Oakton, Vernon Hills, IL). The temperature and ORP were measured using an ORP meter (Ultrapen, Myron L Company, Carlsbad, CA). Titratable alkalinity (catholyte) of the electroactivated lactose solutions was determined using a titration method. In brief, 5 mL of the corresponding solution was collected from the cathodic compartment in a beaker. Thereafter, two drops of phenolphthalein were added to form a pink color, and the sample was titrated from the burette filled with 0.1 M HCl. The catholyte was titrated until the pink color disappeared. The final volume of 0.1 M HCl in the burette was recorded when the end point reached. Finally, the total alkalinity was calculated using **Eq. 3.1** and expressed in mmol/L

$$Total\ Alkalinity_{eq} = \frac{C_{titrant} \times V_{titrant} \times 1000}{V_{sample}} \quad \text{(Eq. 3.1)}$$

$V_{titrant}$: the total volume of the titrant *(*0.1 M HCl*)* used for titration in mL; $C_{titrant}$: the titrant concentration in mol/L; V_{sample}: the volume the of sample that was taken for titration in

mL; $\text{Total Alkalinity}_{eq}$: Equivalent NaOH/KOH concentration in the electroactivated solutions in mmol/L(equiv).

3.2.4 Determination of potassium concentration

The concentration of potassium (K^+) ions in the central compartment was determined using atomic absorption spectrometry. The samples (that were collected from the central compartment in a regular interval of 5 min during the 60 min of EA process) were analyzed according to a standard protocol for the atomic absorption spectrometer (PerkinElmer Instruments, model: AAnalyst 200).

3.2.5 Conventional chemical isomerization of lactose

Conventional chemical isomerization was carried out using similar lactose concentrations (5, 10, 15, and 20%) in the feed solutions and adding the equivalent (to the total alkalinity in EA) amounts of potassium hydroxide (KOH) to the feed. In brief, the total alkalinity (mmol/L) of the EA lactose solutions was expressed to the equivalent amounts of KOH (mg/L). The equivalent amount of KOH was added to the feed in a regular interval of 10 min, and the mixture was stirred at ambient temperature (22 ± 2 °C). The pH and ORP were monitored in 5 min intervals during the 60 min of reaction. The samples were collected from the feed solution at a regular interval of 5 min and were kept at 4 °C until further analysis.

3.2.6 Determination of sugars composition

The sugar contents of all samples (i.e., EA lactose solutions and chemically isomerized lactose solutions) were determined using a high-performance liquid chromatography (HPLC) system (Waters, Millipore Corp., Milford, MA). The system was equipped with a refractive index detector (Hitachi, model: L-7490) and a carbohydrate analysis column (Waters Sugar Pak-I, 300 × 6.5 mm^2, Waters Co.). The column temperature was maintained at 90 °C. The isocratic mobile phase consisting of a solution of 50 mg/L ethylenediaminetetraacetic acid (EDTA) was used as the mobile phase at a flow rate of 0.5 mL/min. The injection volume was 50 μL, and the running time was set at 30 min per sample. The identification and quantification of sugars were accomplished by comparing their retention times with the standard solutions of lactose, lactulose, glucose, galactose, and fructose.

3.2.7 Statistical analysis

Statistical analysis was performed using a complete randomized factorial design with repeated measurements. The factors were current intensity, lactose concentration, and reaction time. The dependent variables were the pH of the catholyte, alkalinity, K^+ ion migration, temperature, ORP, lactulose yield, as well as the yield of byproducts (galactose, glucose, and fructose). Each experiment was carried out in triplicate, and mean values ± standard deviation was used. Differences at $p < 0.05$ were considered to be significant. Analysis of variance (ANOVA) of the data was performed using SAS software (V9.3, SAS Institute Inc., Cary, NC).

3.3 Results and discussion

3.3.1 Evolution of pH

The evolution of pH in the cathodic compartment during 60 min of EA for different lactose solutions (5, 10, 15, and 20%) at three different current intensities (300, 600, and 900 mA) is presented in **Figure 3.2**. It can be seen that the current intensity, EA time, and lactose concentration have a significant effect on the pH of the medium in the cathodic compartment. The obtained data showed that the pH increased drastically within the first 5 min for all current intensities and concentrations. Thereafter, the pH was differently increasing during the remaining 55 min EA time and was dependent on the current intensity and lactose concentration. During this period, the pH was increasing slowly until 60 min of EA and reached a plateau for 300 and 600 mA current intensities. However, the pH evolution reached a maximum value after 30 min of treatment under a 900 mA current intensity, followed by a slight decreasing pattern after 30 min of treatment whatever the lactose concentration used. The rate of increment was observed to be higher for greater current intensities while it was slightly lower for the higher lactose concentrations.

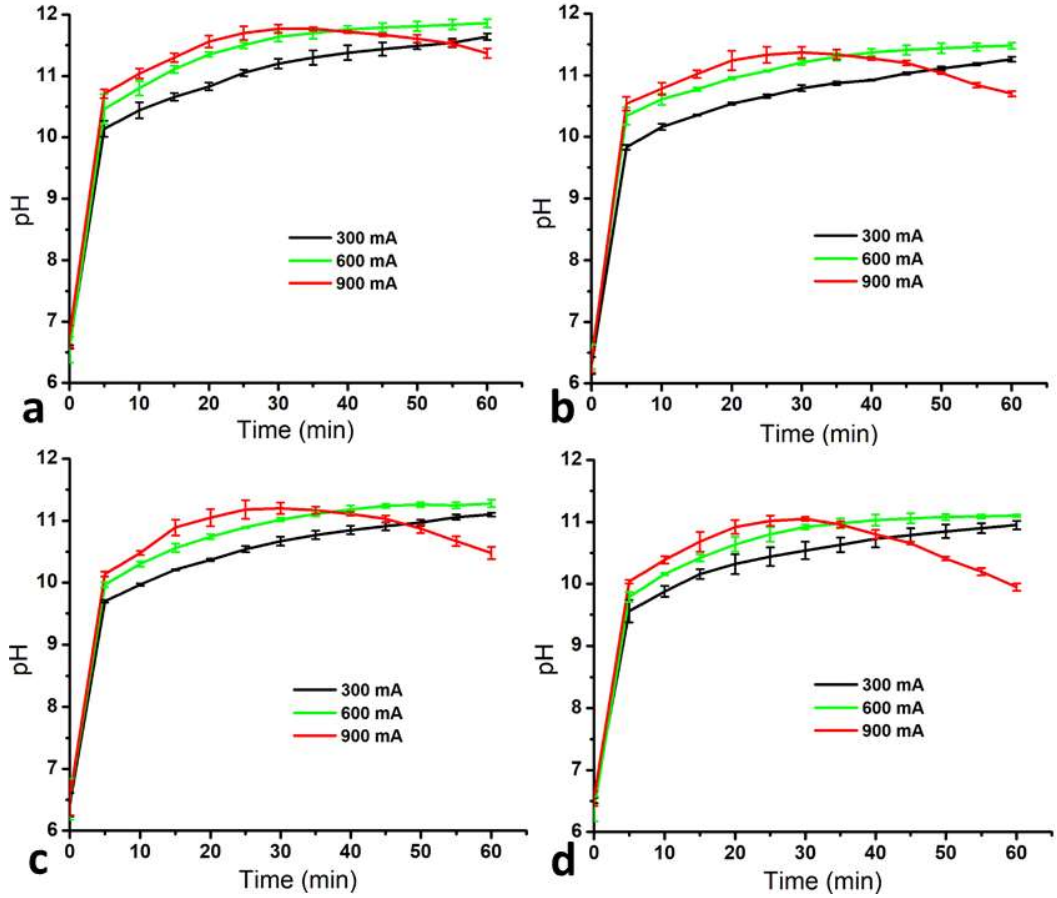

Figure 3.2: Evolution of pH as a function of the EA time for (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions at different current intensities (300, 600, and 900 mA).

Two phenomena of reduction and oxidation have occurred during the electrolysis of water or any aqueous solution. A reduction reaction occurs at the negatively charged electrode (cathode) (Aider and Gimenez-Vidal, 2012, Aider et al., 2012b), and electrons (e^-) from the cathode are donated to the positively charged ions, like hydrogen cations to form hydrogen gas (H_2) and OH^- (**Eqs. 3.2 and 3.3**). The formation of OH^- ions was responsible for the pH increase in the cathodic compartment (Lu et al., 2008, Aider and Gimenez-Vidal, 2012). Indeed, pH is the most important parameter in lactose isomerization for lactulose synthesis, and it should be as high as possible (>pH 9) for isomerization to occur (Aider and Gimenez-Vidal, 2012).

Cathode (reduction):

$$2H^+ + 2e^- \rightarrow H_2(g) \quad \text{(Eq. 3.2)}$$

$$2H_2O(l) + 2e^- \rightarrow H_2(g) + 2OH^-(aq) \quad \text{(Eq. 3.3)}$$

The rate of pH increment that was higher for greater current intensities might be due to the formation of OH^- ions from the water electrolysis (**Eq. 3.3**). Consequently, an increase in the concentration of the OH^- ions was obtained in the cathodic compartment, which leads to an increase of the solution pH (Djouab and Aïder, 2019b). This phenomenon was expected because the rate of water electrolysis is directly proportional to the electric current applied (Aissa and Aïder, 2013c, Kareb et al., 2016b), because the flow of electrons migrating through the electrochemical reactor might be increased by amplifying the electric current intensity, and subsequently, a higher dissociation of water molecules has occurred at the electrode/solution interface (Fiegenbaum et al., 2013b, Djouab and Aïder, 2019b). On the other hand, an oxidation reaction has occurred at the positively charged electrode (anode), and free electrons (e^-) migrated to the anode (Aider and Gimenez-Vidal, 2012, Aider et al., 2012b). This migration produces oxygen gas (O_2) by transferring electrons to the anode (**Eq. 3.4**) and, consequently, lowering the pH of the anodic compartment by increasing the H^+ ions (**Eq. 3.5**). However, the inference of this acidic pH in the anodic compartment and alkaline pH in the cathodic compartment has been avoided using an anion exchange membrane (AEM) between the anodic and the central compartments, and a cation exchange membrane (CEM) between the cathodic and the central compartments (**Figure 3.1**).

Anode (oxidation):

$$2H_2O \rightarrow O_2(g) + 4H^+ + 4e^- \quad \text{(Eq. 3.4)}$$

$$4OH^-(aq) \rightarrow O_2(g) + 2H_2O(l) + 4e^- \quad \text{(Eq. 3.5)}$$

A drastic increase of the pH within the first 5 min of EA can be explained by the generation of a higher amount of OH^- ions due to the intensive water electrolysis at the beginning of the reaction to allow the electric current transfer in the cathode-solution interface. Thereafter, a lower rate of pH increase was observed because the solution became saturated with OH^- ions (Aissa and Aïder, 2013c, Djouab and Aïder, 2019b). Similar observations to this study were reported by Djouab and Aïder (2019b), and they found that the evolution of pH followed a drastic increase (0-21 min) and a slower increase (21-63 min) during the EA of whey permeate (WP) with different current intensities (110, 220, and 330 mA). They obtained a pH of 11.59 after 63 min EA at a 330 mA current intensity for a 5% lactose solution. However, the geometrical parameters in their study were different from those used in the present one, mainly the cathodic compartment volume and the distance between the cathode

and the cation exchange membrane. These parameters seem to be highly significant in terms of the overall process performance. In the present study, the highest pH values of 11.64 ± 0.05 (60 min), 11.86 ± 0.07 (60 min), and 11.77 ± 0.07 (30 min) were achieved for a 5% lactose solution at 300, 600, and 900 mA current intensities, respectively. The highest pH values of 11.26 ± 0.04 (60 min), 11.48 ± 0.05 (60 min), and 11.37 ± 0.09 (30 min) were achieved for a 10% lactose solution, whereas they were 10.95 ± 0.07 (60 min), 11.10 ± 0.02 (60 min), and 11.05 ± 0.03 (30 min) for a 20% lactose solution at 300, 600, and 900 mA current intensities, respectively. These results were in good agreement with those previously reported (Aider and Gimenez-Vidal, 2012, Djouab and Aïder, 2019b). It was observed that the rate of pH increment was slightly lower for the higher lactose concentrations. Similar to this observation, Aider and de Halleux (2007) reported that the pH of the concentrated lactose solutions was more difficult to increase. Likewise, Aider and Gimenez-Vidal (2012) reported that the pH was instantly raised to the mean values of 11.46 ± 0.09 and 11.16 ± 0.11 during the first 10 min of EA at a 100 mA current intensity for 5 and 10% lactose solutions, respectively. The rate of pH increment was slightly lower for the solutions with a higher lactose concentration because the rate of water electrolysis was lower for higher concentration of lactose.

In the present study, the phenomenon consisting of a decline of pH due to the rapid degradation of lactulose into galactose and acidic compounds, which was previously reported for chemical lactulose synthesis (Paseephol et al., 2008b, Hashemi and Ashtiani, 2010a), was not observed to have occurred for 300 and 600 mA current intensities. However, the pH was observed to be decreased after 30 min of treatment for 900 mA. The pH decrease could be attributed to the formation of reaction byproducts with an acid character during this stage (Aider and de Halleux, 2007) or to water splitting at the cation exchange membrane interface facing the cathodic compartment. The latter hypothesis is more realistic because it has already been reported in a recent study by Djouab and Aïder (2019b) that only a low level of galactose can be formed as a reaction byproduct during the lactose electroisomerization in situ of whey permeate (WP).

3.3.2 Migration pattern of potassium ions

The variation of potassium concentration in the central compartment was studied during the EA process of different lactose solutions (5, 10, 15, and 20%) at different current intensities (300, 600, and 900 mA), and the obtained results are presented in **Figure 3.3**. It appears that the concentrations of K^+ were decreasing in the central compartment over the running time for all current intensities and solution concentrations. The decreasing rate was appeared to be higher at the beginning of the reaction whatever the current intensities. However, the decreasing rate was relatively higher for greater current intensities and higher solution concentrations. It was observed that the K^+ concentrations were decreasing gradually following a quasi-linear behavior for 300 and 600 mA during the 60 min of EA process, whereas they were decreasing more drastically during the first 30 min for a 900 mA current intensity. Thereafter, it has steadily declined until the end of the EA process. This observation regarding the migration of potassium ions from the central compartment toward the cathodic one was correlated with the evolution of pH when 900 mA was applied to the EA reactor. Indeed, under this electric current intensity, the pH and solution alkalinity decreased approximately after 30 min of EA. This may be caused by water splitting at the CEM-solution interface to compensate for the lack of the current carriers toward the cathode. Thus, this water splitting created enough H^+ ions, which competed for electromigration with the K^+ ions.

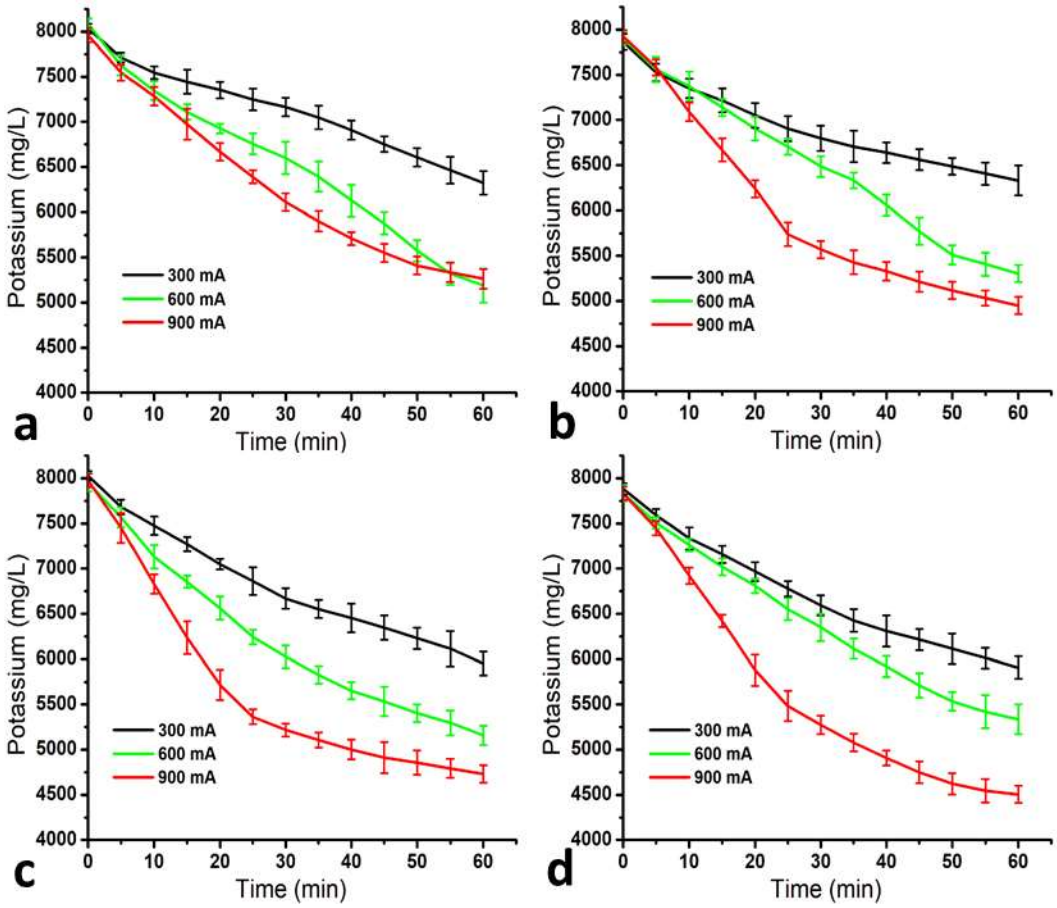

Figure 3.3: Variation of potassium concentration in the central compartment for (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions during the EA process at different current intensities (300, 600, and 900 mA).

The concentration of K^+ was decreasing in the central compartment because the cations (K^+) of the used electrolyte (K_2SO_4) migrated toward the cathode through the CEM by the attraction of the negatively charged cathode (Djouab and Aïder, 2019b), where there were OH^- ions and some other ions such as K^+ and Cl^- ions, which came from 0.01 M KCl added at the beginning of the process to ensure the conductivity of the lactose solution. Similarly, the anions (SO_4^{2-}) might be migrated toward the anodic compartment (where there were H^+ ions) through the AEM by the attraction of the positively charged anode (**Figure 3.1**). The rate of K^+ ion migration was slowed down at the end of the EA process. This could be explained by the concentration polarization phenomenon, which was created by a difference between the ion transfer numbers in the solution and in the membranes, leading to a variation of the electrolyte concentration near the membrane surface and a considerable

potential drop in the polarized region known as the Nernst layer, thus decreasing ion migration and solution demineralization (Cifuentes-Araya et al., 2011b). However, it is obvious that the migration of K^+ ions was more intensive during the first 30 min for a 900 mA current intensity because of higher current intensity, and thereafter, it would have been reached to a limiting current density (Cifuentes-Araya et al., 2011b). As a result, the resistance of the system was significantly increased (**Figure A1**). At this stage, water dissociation might be occurred at the interface between the CEM and solution in the central compartment due to a continuous current regime (Cifuentes-Araya et al., 2011b). Consequently, more H^+ and OH^- ions would have been produced by water splitting at the interface of the CEM to avoid the ion depletion in the central compartment (**Eq. 3.6**). The newly generated H^+ ions might be migrated to the cathodic compartment through the CEM by the attraction of the negatively charged cathode (Djouab and Aïder, 2019b). The H^+ ions might be competing with K^+ ions to travel toward the cathodic compartment due to the higher electrical mobility of the H^+ ions than K^+ ions. Thus, the rate of K^+ ion migration has been slowed down more apparently after 30 min of EA at a current intensity of 900 mA. This fact of H^+ ion migration from the central compartment could also be correlated to the decrease in pH after 30 min of EA for that causing acidification of the solution of the cathodic compartment (Djouab and Aïder, 2019b).

$$2H_2O \rightarrow 2H^+ + 2OH^- \quad \text{(Eq. 3.6)}$$

3.3.3 Evolution of alkalinity

The variation of alkalinity in the cathodic compartment was determined for different lactose solutions (5, 10, 15, and 20%) during the EA process at different current intensities of 300, 600, and 900 mA, as shown in **Figure 3.4**. It appeared that the current intensity and running time have a highly significant effect ($p < 0.001$) on solution alkalinity. It was linearly increasing during the 60 min of EA for a 300 mA current intensity and reached 28.53, 31.73, 32.27, and 34.13 mmol/L of alkalinity for 5, 10, 15, and 20% lactose solutions, respectively. For a 600 mA current intensity, it has been increased during the 60 min of EA but showed some incurving behavior that could be interpreted as a tendency to reach a plateau. A maximum alkalinity of 43.47, 43.20, 43.20, and 40.80 mmol/L was achieved at the end of the reaction for 5, 10, 15, and 20% lactose solutions, respectively. Contrary to 300 and 600 mA, the alkalinity of the catholyte (electroactivated lactose solution) reached a maximum

value of 35.73, 30.13, 34.13, and 27.20 mmol/L after 30 min of treatment, and thereafter, it was decreasing gradually down to 16.27, 7.73, 10.93, and 4.53 mmol/L at the end of the EA treatment for 5, 10, 15, and 20% lactose solutions, respectively.

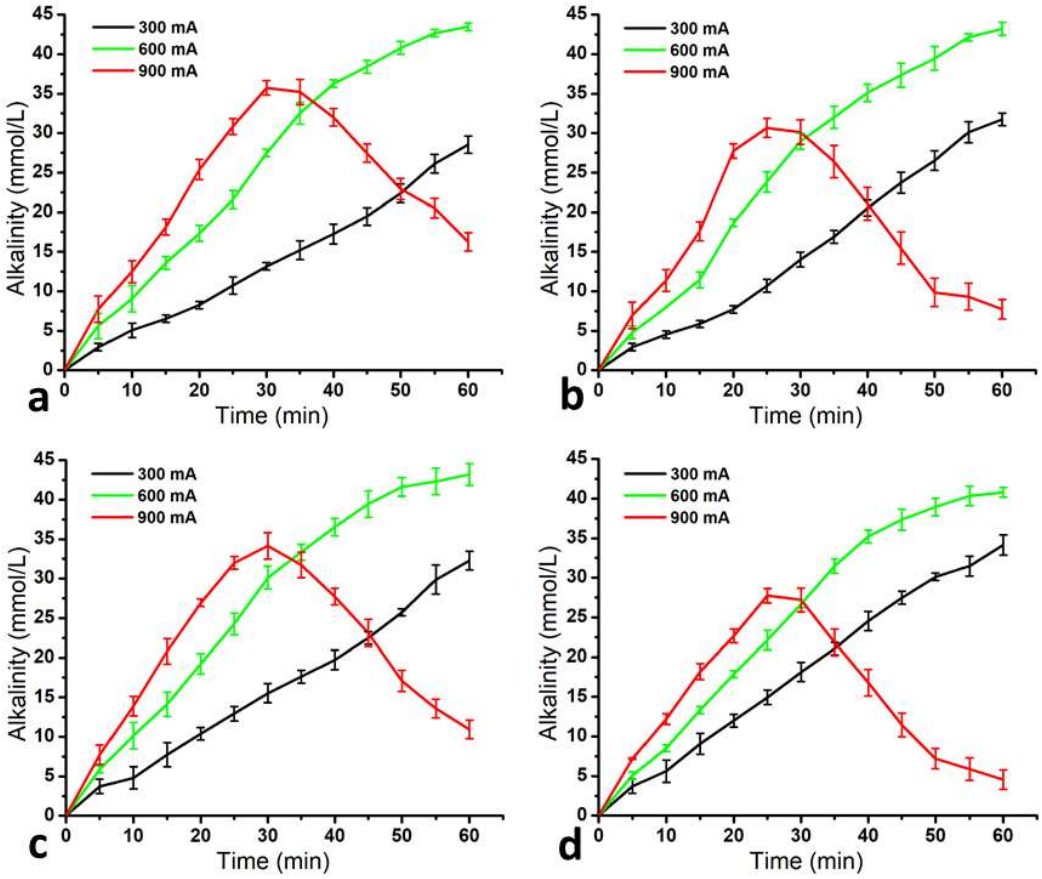

Figure 3.4: Variation of alkalinity in (a) 5%, (b) 10%, (c) 15%, and d) 20% lactose solutions during the EA process at different current intensities (300, 600, and 900 mA).

Generally, the isomerization reaction requires proton acceptors (OH^- ions), and this can be achieved in a high alkaline medium through water electrolysis at the cathode/solution interface under the influence of an external electrical field (Aissa and Aïder, 2013c). As seen from **Figure 3.4**, the evolution of alkalinity was significantly higher for greater current intensities, except when the conditions leading to water splitting were reached such as the case, after 30 min of EA when 900 mA was used. This is attributed to the fact that the higher current intensity resulted in faster decomposition of water (Kareb et al., 2016b). The alkalinity was gradually increased during the 60 min of EA for 300 mA and 600 mA because

the dissociation of water at both electrode interfaces produces H^+ and OH^- ions, as discussed in Section 2.1. The OH^- ions in the cathodic side might be attracted by the positively charged anode, but the ions transported by the electric current repulsed by the negatively charged CEM. Thus, the high concentration of OH^- ions at the cathode interface was able to create an alkaline condition and could act as proton acceptors, which is a key condition for the occurrence of the isomerization reaction of lactose into lactulose (Aider and Gimenez-Vidal, 2012, Djouab and Aïder, 2019b). Moreover, the K^+ ions were continuously migrating to the cathodic compartment, which reacted with the OH^- ions to make high alkalinity of the catholyte (**Eq. 3.7**). However, they reached a plateau after 30 min of EA for a 900 mA current intensity, which might be attributed to the saturation of the catholyte with the OH^- ions. Thereafter, the alkalinity was gradually decreasing because the migrated H^+ ions from the central compartment caused acidification of the solution, as discussed in the previous Section 2.2. This result is in concordance with the pH decline after 30 min of EA for a 900 mA current intensity.

$$2K + 2HOH \rightarrow 2KOH + H_2(g) \qquad \text{(Eq. 3.7)}$$

3.3.4 Evolution of oxidation-reduction potential (ORP)

The evolution of ORP in the cathodic compartment during the EA of different lactose solutions (5, 10, 15, and 20%) at different current intensities (300, 600, and 900 mA) is shown in **Figure 3.5**. The ORP values in the cathodic compartment were decreased drastically to a value of around -800 mV within the first 5 min of EA for all current intensities and concentrations used and then reached a quasi-steady state. Thereafter, it remained almost constant during the 60 min of treatment for 300 and 600 mA. However, a minor increasing tendency was observed after 30 min for 900 mA. No significant changes were observed for the solution concentrations.

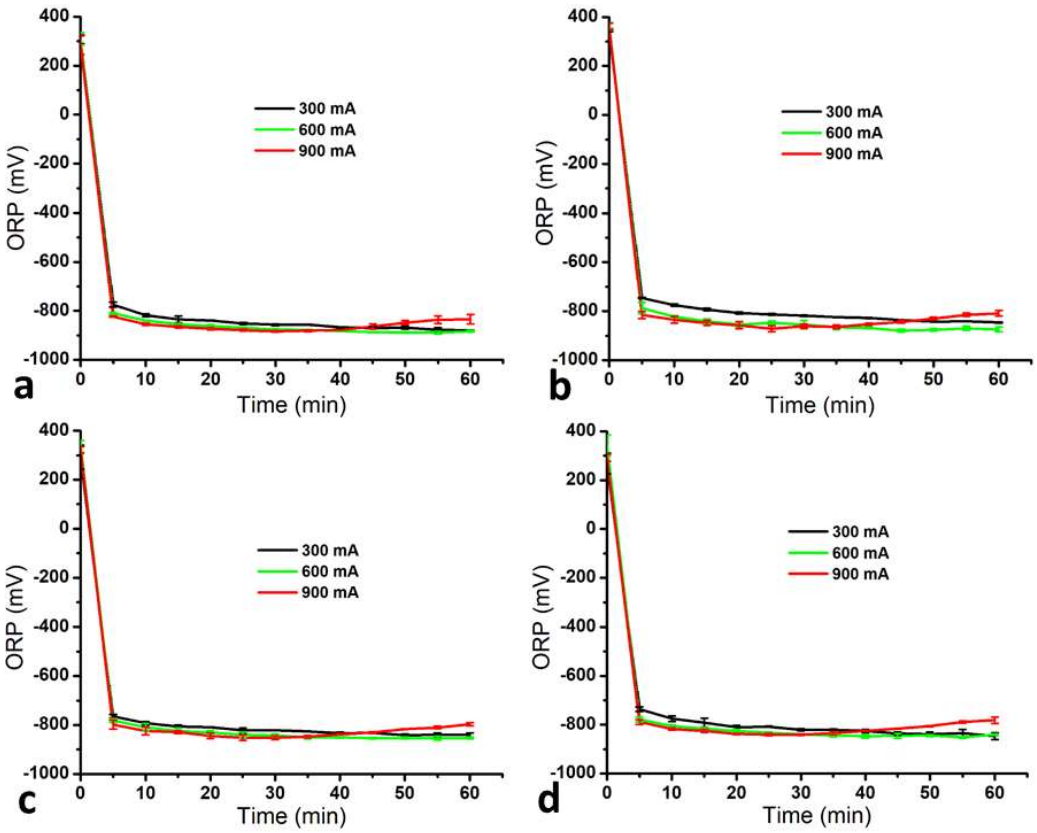

Figure 3.5: Evolution of ORP in (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions during the EA process at different current intensities (300, 600, and 900 mA).

The ORP is one of the most important parameters of water or any aqueous solution that can be modified by means of EA. In fact, EA is the process of transferring any solution into a nonequilibrium thermodynamic state, which is accompanied by a change in the internal energy of the system (Dykstra, 1999). The observed reducing ORP of the EA solution in the cathodic compartment is also due, to ahigh extent, to its saturation by hydrogen gas that is formed following water electrolysis at the cathode-solution interface. Indeed, it is well known that hydrogen gas is a reducing agent when it reacts with non-metals. The results obtained in the present study were in good agreement with those previously reported by Nabok and Plutahin (2005). They obtained the ORP values of -767 and +905 mV by means of electroactivation of aqueous solutions in the cathodic and anodic chambers, respectively, while the control aqueous solution exhibited an ORP of +220 mV. This phenomenon can be explained by the formation of unstable complexes, such as (OO), $(OO)^{+}$, and $(HH)^{+}$ due to the dissociation of water molecules and their vibrational modes (Antonchenko et al., 1991,

Aider et al., 2012b). Shironosov and Shironosov (1999) also explained the anomalies in the pH and ORP of EA water by the stable, high-energy resonant water microclusters due to covibrating dipoles of water molecules and charged species near-electrode interfaces. In another study by Hricova et al. (2008), an acidic electrolyzed water (pH: 2-3, ORP>1100 mV) and a basic electrolyzed water (pH: 10-13, ORP: -800 to -900 mV) were obtained by the electrolysis of dilute NaCl solution. The electroactivated solutions in the cathodic chamber were characterized by a negative ORP, which probably related to a training effect of excess electrons (e^-) formed after electrochemical activation (Podkolzin et al., 2001a). Moreover, EA is an electrochemical process that can cause the formation of different ionic species and radicals, e.g., the formation of highly active reducers such as OH^-, H^-, $\cdot H$, $\cdot HO$, $\cdot O^-$, $\cdot O_2^-$, $\cdot HO_2^-$, $H_2O_2^-$, H_3O^{2-} which may lead to a high reduction potential (Aider and Gimenez-Vidal, 2012).

In the present study, the ORP values in the cathodic compartment were decreased sharply to a value of around -800 mV within the first 5 min of the EA process. The drastic change in ORP within the first 5 min was attributed to the generation of excessive electrons and the formation of other highly active reducers because of the rigorous electrolysis of the solution. Besides the formation of diverse compounds, the EA induced dynamic water electrolysis in the cathodic compartment, resulting in an enhancement of the negative charge concentration through the accumulation of the hydroxyl groups (Kareb et al., 2017c). Thereafter, it reached at a quasi-steady state when the system became saturated with the charged species. The slight increase in ORP (i.e., reactivity decreased) after 30 min of EA for 900 mA might be due to the migration of H^+ H_3O^+ $H_3SO_4^+$ toward the cathodic compartment from the central compartment because the newly migrated ions might reduce the number of electrons (e.g., $2H^+ + 2e^- \rightarrow H_2(g)$) in the cathodic compartment.

3.3.5 Evolution of temperature

The changes in temperature in the cathodic compartment were observed during the EA process of different lactose solutions (5, 10, 15, and 20%) at different current intensities (300, 600, and 900 mA), as presented in **Figure 3.6**. The temperature was gradually increased during the 60 min of the EA process for all current intensities and solution concentrations. The rate of increment was significantly higher for the greater current intensities. As can be seen for a 5% lactose solution (Figure 5a), the temperature was significantly increased to

29.30, 37.97, and 48.70 °C for 300, 600, and 900 mA current intensities, respectively. Some increase in temperature was also observed for the higher concentrations of lactose solutions; however, the difference was not too significant even if some tendency was noticed. For instance, the temperature was slightly increased to 37.97, 38.80, 38.99, and 40.87 °C when a 600 mA current intensity was used for 5, 10, 15, and 20% lactose solution concentrations, respectively.

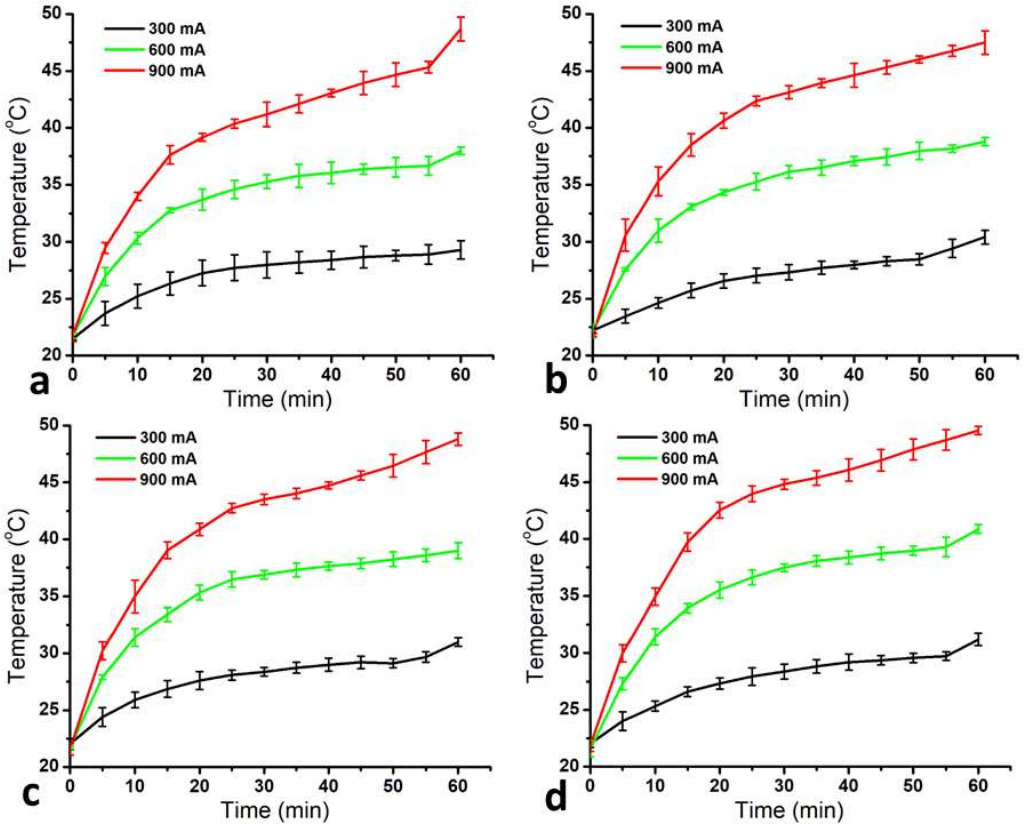

Figure 3.6: Variation of temperature in (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions during the EA process at different current intensities (300, 600, and 900 mA).

The observed temperature rise is mainly the result of the Joule effect in the electrodes, and the generated heat was dissipated in the solution. It was not due to the system electrical resistance because the solutions used in the EA reactor were conductive enough and allowed easy current transfer through the system, which led to a greater water dissociation to create high alkalinity in the cathodic side of the reactor. Some initial resistance of the ion-exchange membranes used could also at some extent contribute to the temperature rise. Indeed, Cifuentes-Araya et al. (2011b) reported that the initial system global resistance in a

membrane process can be due to the intrinsic resistance of the membranes and the resistance of the feed solutions, whereas the system global resistance during and at the end of the process was correlated with an evolutionary demineralization and the presence of fouling. Nevertheless, Djouab and Aïder (2019b) did not reveal any fouling of membrane in the EA of pure lactose and WP. Therefore, the global electrical resistance (**Figure A1**) occurred in the present study, which might be due to the intrinsic resistance of the membranes and the resistance of the feed solutions. The demineralization due to the ion migration could be another possible reason for decreasing conductivity in the central compartment, as discussed in Section 2.2. As a result, the resistance of the system was increased significantly. Thus, the variation of temperature during the isomerization time might be a consequence of the decreased conductivity of the lactose solution during the passage of the electric current (Aissa and Aïder, 2013c). The rate of temperature increment was significantly higher for the greater current intensities because the heated energy dissipation (i.e., the increase in temperature) is proportional to the increase in the electric current and electric tension, as described by Joule's law (Aissa and Aïder, 2013c). An increase in temperature was also observed for the higher lactose concentrations, which might be due to the higher resistance for greater concentrations of the feed solution.

3.3.6 Evolution of lactulose formation

The isomerization yield of lactose into lactulose during the EA process at different current intensities (300, 600, and 900 mA) was studied over time (60 min) for different lactose concentrations (5, 10, 15, and 20%), as presented in **Figure 3.7**. As can be observed, the current intensity, running time, and solution concentration had a significant effect ($p < 0.001$) on the conversion rate of lactose into lactulose.

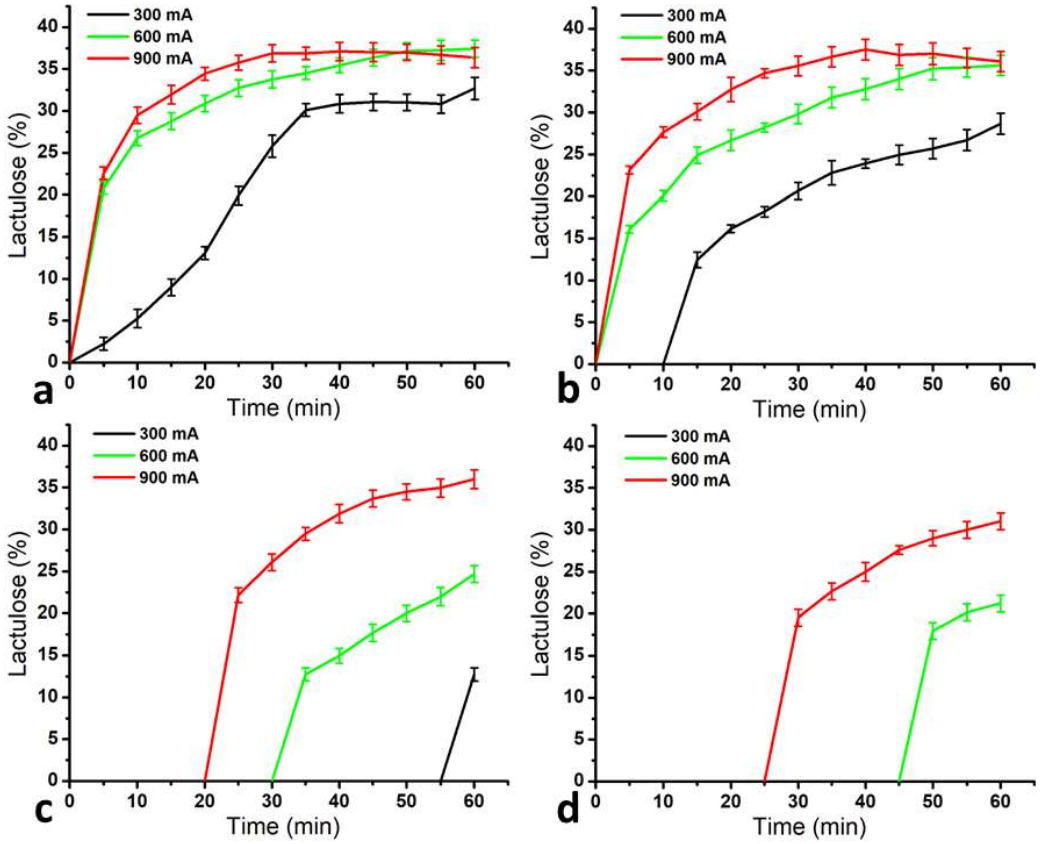

Figure 3.7: Evolution of lactulose yield as a function of the EA time for (a) 5%, (b) 10%, (c) 15%, and d) 20% lactose solutions at different current intensities (300, 600, and 900 mA).

From **Figure 3.7a** for a 5% feed solution, it can be seen that the lactulose was gradually increased until 50 min for 600 mA and thereafter reached a plateau (~37% lactulose); however, it reached the plateau at 30 min for 900 mA (~37% lactulose) and then slightly decreased at the end of the EA process (~36% lactulose). Whereas, the formation of lactulose was sharply increased to ~30% until 35 min for 300 mA, and no significant enhancement was observed afterward (~33% at 60 min). For 10% lactose (**Figures 3.7b** and **3.8**), the lactulose yield was increased until the end for 600 mA and thereafter reached a plateau (~36% lactulose), but it reached the plateau at 40 min for 900 mA (~38% lactulose) and later slightly decreased (~36% lactulose). However, the formation of lactulose was started at 15 min for 300 mA (~12% lactulose) and then gradually increased until the end (~29% lactulose). As can be seen from **Figure 3.7c** (for a 15% lactose solution), the lactulose began to form at different times for different current intensities such as 25, 35, and 60 min for 900, 600 and 300 mA, respectively. It can be observed that ~36% lactulose was produced for 900 mA at

60 min, while it was only ~13% for 300 mA and ~25% for 600 mA. On the other hand, no lactulose was formed for 300 mA during the 60 min EA process when a 20% lactose solution was used (**Figure 3.7d**). However, ~31% lactulose was produced at 60 min for 900 mA, while it was only ~21% for 600 mA.

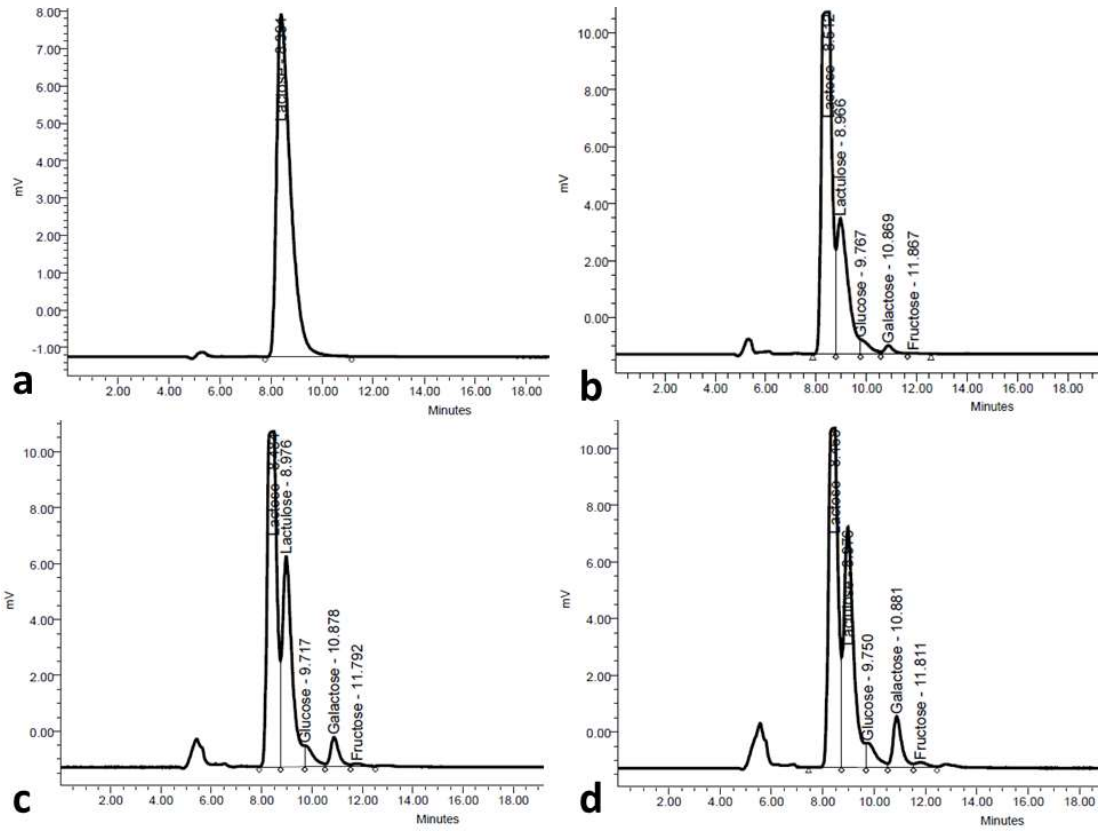

Figure 3.8: High-performance liquid chromatography (HPLC) chromatograms of lactose electroisomerization into lactulose in EA for a 10% lactose solution; (a) initial feed solution, (b) at 300 mA after 60 min (29%), (c) at 600 mA after 60 min (36%), and (d) at 900 mA after 40 min (38%).

3.3.6.1 Effect of solution pH

The pH of the lactose solution had a significant ($p < 0.001$) influence on lactulose formation. However, different phenomena were observed depending on the lactose solution concentration. For a 5% lactose solution, lactulose was started to form while the pH was above 10 (pH>10.00). It can be seen from **Figure 3.2a** that the pH reached 10 within 5 min of EA for all three current intensities, and lactulose was started to form at the same time (**Figure 3.7a**). Whereas, for 10% lactose, the lactulose was found to be created while the pH achieved a value higher than 10.30 (pH>10.30). Although the pH reached 10.30 within 5 min

of EA for 600 mA and 900 mA current intensities (**Figure 3.2b**), however, it reached beyond 10.30 at 15 min for 300 mA and the lactulose began to produce at the same time (**Figure 3.7b**). On the other hand, no lactulose was found until the pH reached above 11 (pH≈11.10) for 15 and 20% lactose solutions. Consequently, the formation of lactulose was observed to begin at different EA times (**Figure 3.7c**), and even no lactulose was found for 300 mA in a 20% lactose solution (**Figure 3.7d**) because the pH never reached 11. It can be seen (**Figure 3.2**) that the pH was observed to be decreased after 30 min of EA at 900 mA for all lactose concentrations. It is interesting to mention that the formation of lactulose was almost stable at that condition (after 30 min of EA at 900 mA) in 5 and 10% lactose solutions even though the pH decreased slightly. In contrast, the formation of lactulose was found to increase in the same condition (after 30 min of EA at 900 mA) for 15 and 20% lactose solutions. It is worth noting that the rate of lactulose formation was always higher for greater current intensities, and this phenomenon was correlated to the pH evolution. Furthermore, the formation of galactose increased linearly with lactulose until the end of the EA process (**Figure 3.9**). The yield of galactose was increased with isomerization time because the lactulose formed by the isomerization reaction was later hydrolyzed into galactose (**Figure A2**) (Aissa and Aïder, 2013c). Nevertheless, the results showed that only galactose was generated as a side product; no other impurities (such as tagatose, epilactose, etc.) were found in the reaction medium (**Figure 3.8**). However, trace amounts of glucose and fructose were found in some cases depending on the experimental conditions (**Figure 3.8** and **Table A1**). Here, it can be mentioned that the glucose and fructose could be isomerized into galactose during the EA process of lactose, whey, or WP (**Figure A2**) (Kareb et al., 2016b, Djouab and Aïder, 2019b).

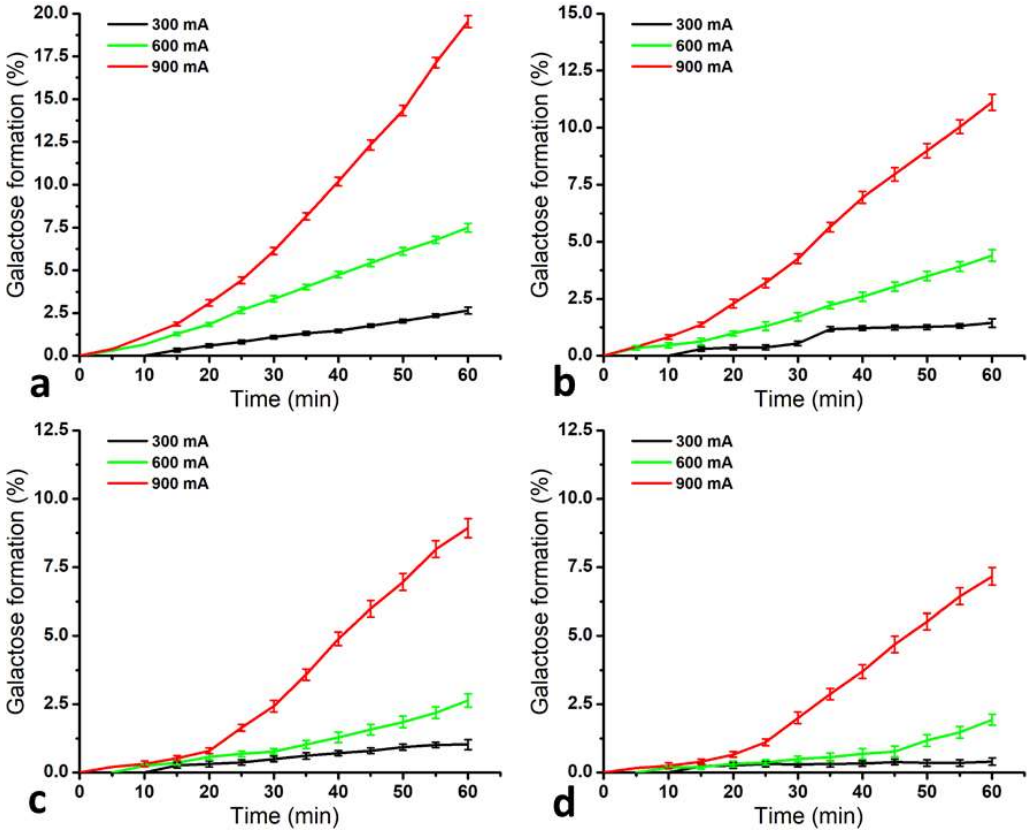

Figure 3.9: Formation of galactose as a function of the EA time for (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions at different current intensities (300, 600, and 900 mA).

As can be seen from **Figure 3.9**, the formation of galactose followed a similar trend for all concentrations. However, the greater current intensity and running time led to the increased formation of galactose (**Figure 3.10**). For instance, the formation of galactose was higher at a 900 mA current intensity, particularly, it increased drastically after 30 min of EA (**Figures 3.9** and **3.10**), while the pH was slightly decreased (**Figure 3.2**). In this case, the longer treatment time was immaterial but energy consuming, since no significant increase in lactulose formation was observed after 30 min but increased the galactose formation to an unacceptable level. This finding of the present study was in good agreement with those previously reported for lactose and WP (Aissa and Aïder, 2014b, Djouab and Aïder, 2019b). Here, it can be noted that the commercial lactulose (as syrup) must not contain more than 12% lactose, 16% galactose, 8% epilactose, and 1% fructose according to United States Pharmacopeia (USP, 2008).

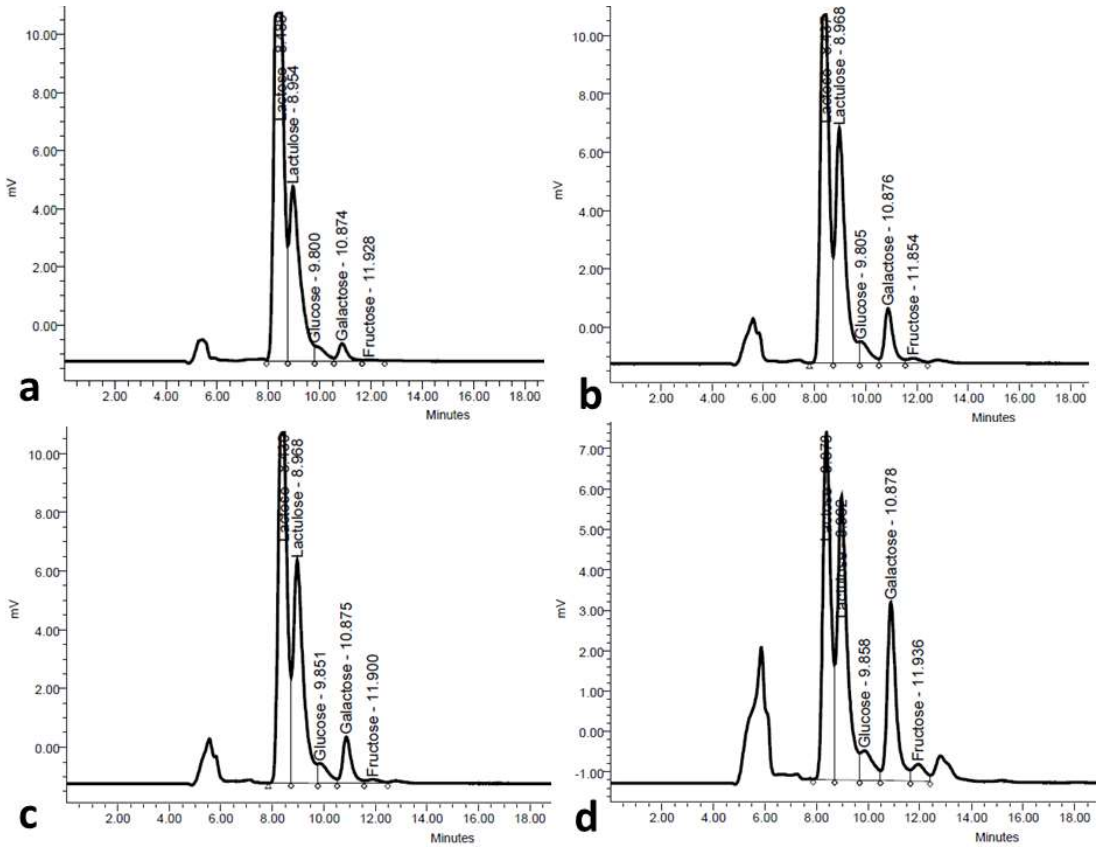

Figure 3.10: HPLC chromatograms of galactose formation as a function of the current intensity and running in EA for a 5% lactose solution; (a) at 300 mA after 60 min (2.65%), (b) at 600 mA after 60 min (7.49%), (c) at 900 mA after 30 min (6.54%), and (d) at 900 mA after 60 min (20%).

3.3.6.2 Effect of lactose solution alkalinity

The isomerization of lactose into lactulose is feasible only under a high alkaline condition because the high alkalinity of the reaction medium is a sine qua non condition for the successful conversion of lactose into lactulose (Aider and Gimenez-Vidal, 2012). Indeed, the molecular rearrangement of lactose into lactulose requires proton acceptors, which was ensured by achieving a high alkaline medium in the cathodic compartment of the EA reactor (Aider and Gimenez-Vidal, 2012, Kareb et al., 2016b). However, different amounts of alkalinity were observed to be required for producing lactulose in different lactose solutions. Lactulose began to form while the alkalinity obtained a value of ~3 and ~5 mmol/L for 5 and 10% lactose solutions, respectively; thereafter, it was increasing with time. It is worth noting that the rate of lactulose formation was hindered but not reduced while the alkalinity was decreasing after 30 min at 900 mA (**Figure 3.4a,b**) rather, it reached a plateau and remained stable (**Figure 3.7a,b**). On the other hand, no lactulose was found to be produced before the

alkalinity reached beyond ~30 mmol/L for 15 and 20% lactose solutions. Consequently, the formation of lactulose was noticed to start at different times of EA (**Figure 3.7c,d**), and even no lactulose was found for 300 mA in 20% lactose solution (**Figure 3.7d**). Unlike 5 and 10% lactose solutions, a different scenario was observed for 15 and 20% lactose solutions at 900 mA, where the lactulose formation was not impeded, even though the alkalinity was decreasing after 30 min (**Figure 3.4c,d**). However, the formation of galactose was seen to be drastically increased at this stage (i.e., after 30 min at 900 mA) of EA for all lactose concentrations (**Figure 3.9**).

3.3.6.3 Effect of temperature

The temperature is one of the most important parameters in the isomerization of lactose into lactulose. During the EA process, the temperature increased throughout the isomerization time, which has already been explained in the previous Section 2.5. As can be seen from **Figure 3.7**, the formation of lactulose was proportional to the temperature increment, i.e., increasing the temperature increases the production of lactulose. However, the formation of byproduct, i.e., galactose, was also increased with increasing the temperature. Similar findings to the present study have been reported in several studies (Martinez-Castro et al., 1986b, Aissa and Aïder, 2013c). This might be due to the increased conductivity of lactose with the increase of temperature because the ions that were present in the solution (i.e., K^+, SO_4^{2-}) moved quickly at the high temperature and at the crossing of the electrical current (Aissa and Aïder, 2013a). The amount of galactose increased with the elevated temperature because greater activation energy formed and which pushed the reaction on the other side pathways (Hashemi and Ashtiani, 2010a, Kareb et al., 2016b). It seems to be important to point out that the synthesis of lactulose without heat is possibly contrary to that already been reported in the literature. The alkaline isomerization of lactose to lactulose via the LA rearrangement was usually carried out at a high temperature in the range of 50 to 130 °C combined with different reaction times (Hicks et al., 1984a, Hashemi and Ashtiani, 2010a, Corzo-Martínez et al., 2013, Song et al., 2013b, Sakkas et al., 2014b). Contrary to these studies, the temperature has never been exceeded 50 °C in the present study using EA. Similar to this study, Aissa and Aïder (2013a) demonstrated that lactulose could be obtained at a low temperature such as 0, 5, and 10 °C in the EA reactor. They achieved a lactulose

yield of 25 ± 1.34% (with a purity of 95 ± 1.34%) at a temperature of 0 °C, and a pH of 10-10.50 at a short duration of 2 min using the EA process (Aissa and Aïder, 2013a).

3.3.7 Conventional chemical lactose isomerization

The conventional isomerization reactions were carried out at ambient temperature using similar lactose concentrations (5, 10, 15, and 20%) and generating equivalent alkalinity as those generated using 300, 600, and 900 mA in the EA treatment. The alkalinity-equivalent tests were conducted to observe the lactulose yield compared to the results obtained by EA under similar solution alkalinity. The obtained results are presented in **Figure 3.11**. As can be observed, the pH, running time, and solution concentration had a significant effect on the conversion rate of lactose into lactulose. For 5 and 10% lactose (**Figure 3.11a,b**), the lactulose was gradually increased until the end of the reactions, although they started to form at a different reaction time. On the other hand, no lactulose was found to be formed for 15 and 20% lactose solutions during the isomerization process, except for the alkalinity equivalent to 900 mA in a 15% lactose feed solution (**Figure 3.11c,d**).

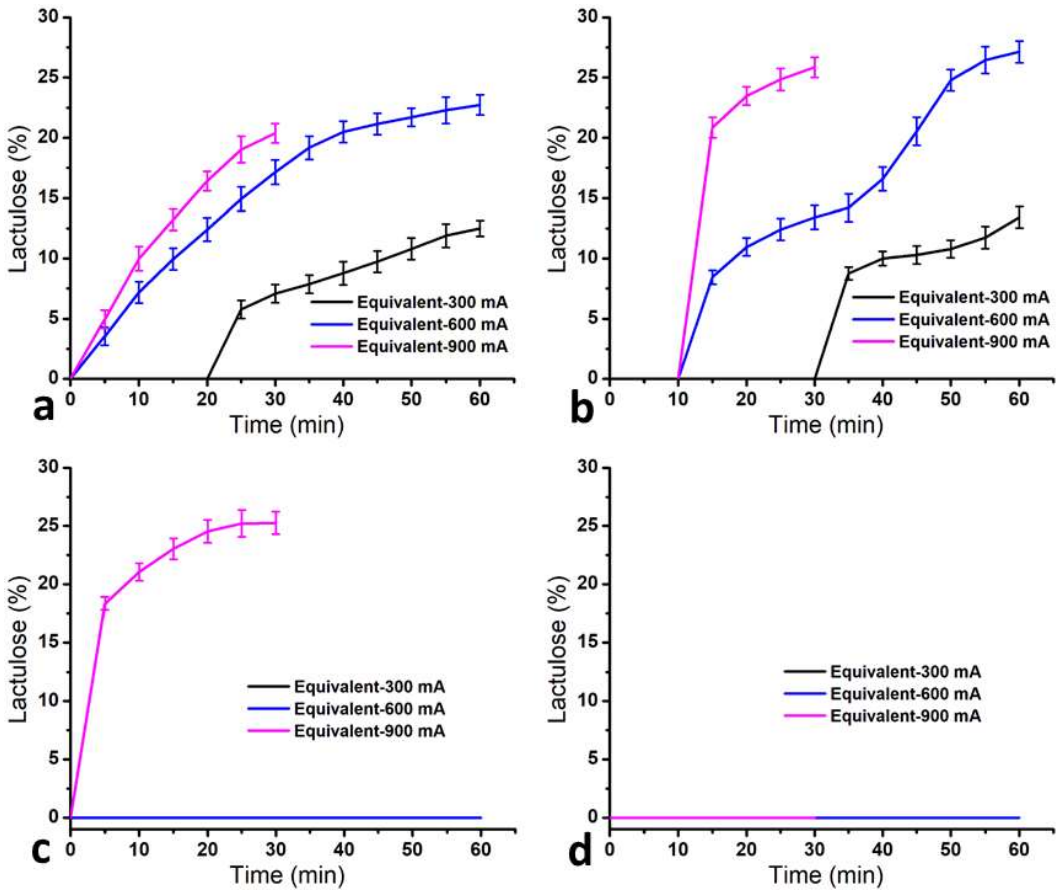

Figure 3.11: Formation of lactulose as a function of isomerization time for (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions.

It can be seen for 5% lactose that approximately ~12%, ~23%, and ~20% lactulose were formed at the end of the reactions for the alkalinity of equivalent to that of the EA treatments at 300, 600, and 900 mA, respectively (**Figure 3.11a**). These results were correlated to the pH rise of 11.31, 11.65, and 11.58 for the equivalent alkalinity to the EA treatments at 300, 600, and 900 mA, respectively (**Figure 3.12a**). Although the increment of pH in a 10% lactose solution (**Figure 3.12b**) was less than that of a 5% lactose solution, however, the lactulose formation was comparatively higher for a 10% lactose solution (**Figure 3.11b**). The yields of lactulose for a 10% lactose solution were ~13%, ~27%, and ~26% for the alkalinity of equivalent to 300, 600, and 900 mA, respectively (**Figures 3.11b** and **3.13**) and the pH values were 11.06, 11.33, and 11.08 for the alkalinity of equivalent to 300, 600, and 900 mA, respectively (**Figure 3.12b**). As can be seen from Figure 10c (for a 15% lactose solution), ~25% lactulose was produced only for equivalent to 900 mA at a pH

of 10.93, but no lactulose was found for equivalent to 300 and 600 mA, even though the pH reached 10.87 and 11.11 at the end of the reaction, respectively (Figure 11c). On the other hand, no lactulose was formed when a 20% lactose solution was used (**Figure 3.11d**), which might be due to the inadequate pH values of 10.72, 10.85, and 10.57 for an equivalent to 300, 600, and 900 mA, respectively (**Figure 3.12d**).

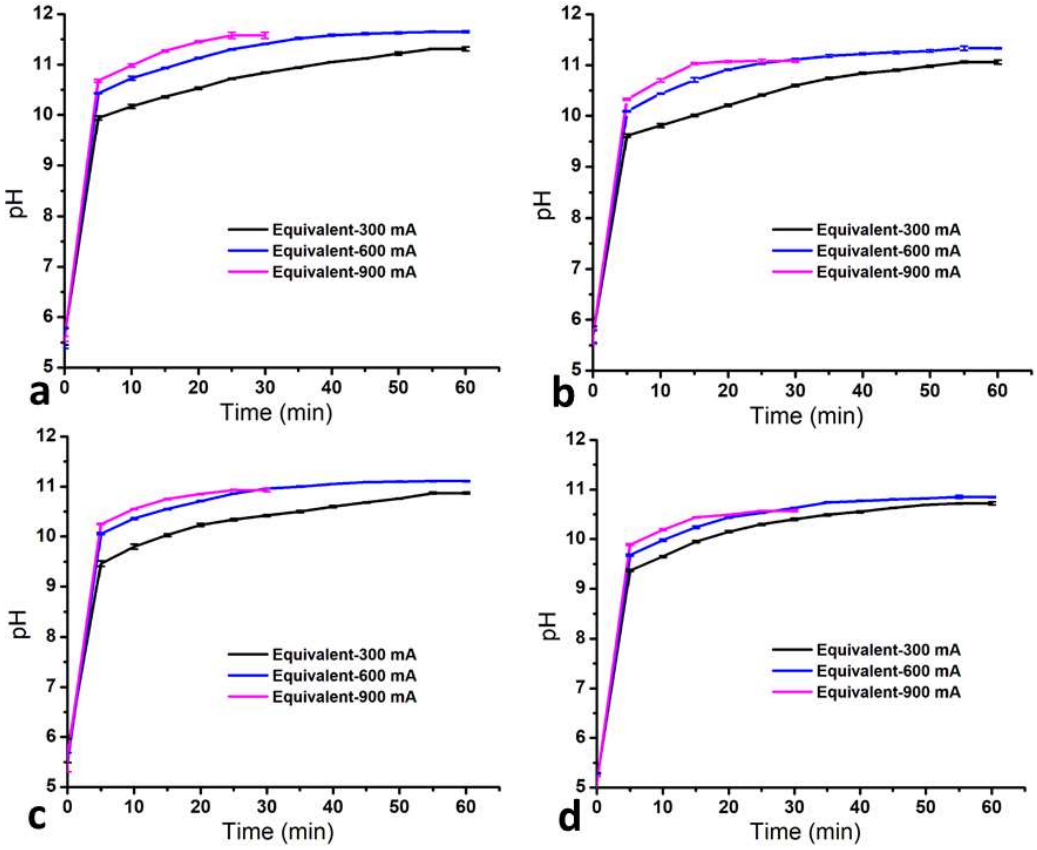

Figure 3.12: Evolution of pH as a function of isomerization time in (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions.

It has been previously reported that the highest isomerization yield in an alkaline medium could be achieved with a pH of 11 (Hicks et al., 1984a, Hashemi and Ashtiani, 2010a) and at temperatures higher than 70 °C (Rendleman Jr and Hodge, 1979, Olano and Calvo, 1989, Aissa and Aïder, 2014b). In a recent study, Seo et al. (2015b) observed that the lactulose yield could be increased from 4% to 29.6% in a 20 min reaction time by increasing the temperature from 60 °C to 90 °C using cheese whey as lactose source and sodium carbonate as catalyst. Hashemi and Ashtiani (2010a) achieved an optimum conversion of 25.40% % (with 5.58% galactose as byproduct) at 70 °C and a pH of 11 for 60 min using

10% lactose in the feed solution. In our study, the same amount of lactulose (i.e., 25.85%) was obtained at ambient temperature and pH 11.08 (alkalinity equivalent to EA at 900 mA in 10% lactose) in 30 min. Moreover, only 0.84% galactose was produced as the byproduct at this condition due to the reduced temperature. It is worth noting from this finding that the lactulose can be produced at ambient temperature using adequate solution alkalinity. This new finding from the present work is essential because it highlighted the fact that not only the pH but also the alkalinity of the feed solution to reach adequate alkalinity are also important. Besides, it has been reported that the conversion of lactose into lactulose was typically followed by a rapid degradation of lactulose into galactose, tagatose, epilactose, and many other byproducts with an acidic character such as isosaccharinic acids and formic acids in the chemical-based processes (Corbett and Kenner, 1953b, Dendene et al., 1994a, Hashemi and Ashtiani, 2010a, Schuster-Wolff-Bühring et al., 2010b, Djouab and Aïder, 2019b), causing the lowering of pH in a medium (Paseephol et al., 2008b, Hashemi and Ashtiani, 2010a). Basically, heating lactose in an alkaline solution causes isomerization and degradation of lactose and lactulose; epilactose and galactose were produced (Martinez-Castro et al., 1986b, Hashemi and Ashtiani, 2010a). However, this phenomenon did not occur in the present study, and consequently, only glucose was found as the reaction byproduct other than that of galactose (**Tables A2** and **A3**).

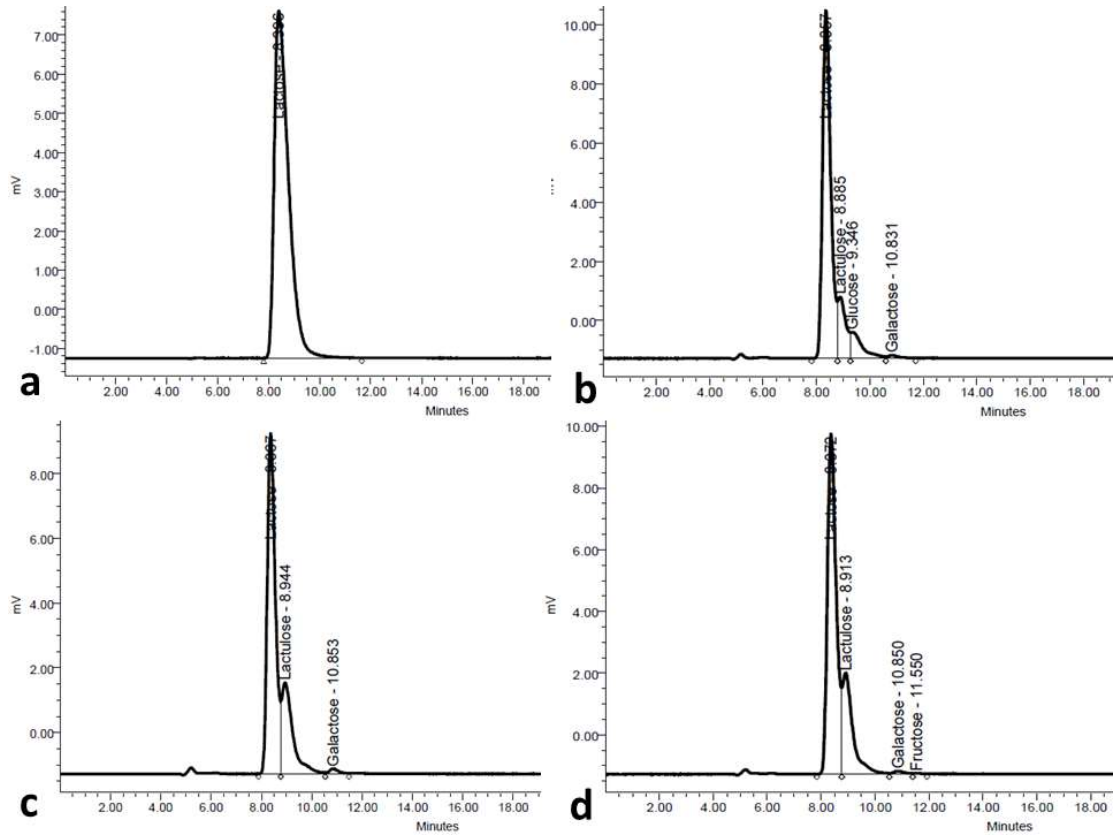

Figure 3.13: HPLC chromatograms of lactose isomerization into lactulose using the chemical method for a 10% lactose solution; (a) initial feed solution, (b) equivalent to 300 mA after 60 min (13%), (c) equivalent to 600 mA after 60 min (27%), and (d) equivalent to 900 mA after 30 min (26%).

Not only the pH and temperature, but the solution alkalinity was also a key factor in lactulose production. For example, lactulose began to form at an alkalinity of 9.07 mmol/L (pH=10.43, 10 min, alkalinity equivalent to 600 mA) in 5% lactose, whereas it was 18.67 mmol/L (pH 10.71, 20 min, alkalinity equivalent to 600 mA) in 10% lactose. Nevertheless, the pH was also varied for the same amount of alkalinity/catalyst depending on the solution concentrations and consequently influenced the lactulose yield. For instance, although the same amount of alkalinity (~43 mmol/L at alkalinity equivalent to 600 mA for 5, 10, and 15% lactose concentrations) was produced at the end of the reactions (60 min), however, the pH values were 11.65, 11.33, and 11.11 and lactulose was 22.73, 27.13, and 0% for 5, 10, and 15% lactose concentrations, respectively. It is also important to note that the concentration of the catalyst and the rate of pH or alkalinity generation, i.e., the dosing pattern of the catalyst might play a pivotal role in the lactulose yield. For example, the alkalinity (concentration of the catalyst) and the pH were almost the same at the end of the reaction for

alkalinity equivalent to 300 mA (at 60 min) and alkalinity equivalent to 900 mA (at 30 min) in a 10% lactose solution. However, the formation of lactulose (i.e., 28.85%) for alkalinity equivalent to 900 mA at 30 min was higher than for the lactulose (i.e., 13.41%) of alkalinity equivalent to 300 mA at 60 min. A similar phenomenon was also found for alkalinity equivalent to 300 mA and alkalinity equivalent to 900 mA in a 15% lactose solution. Thus, from the obtained results of the chemical lactose isomerization into lactulose at alkalinity-equivalent conditions as those formed during EA, it can be observed that the EA technique was far more effective than the chemical method.

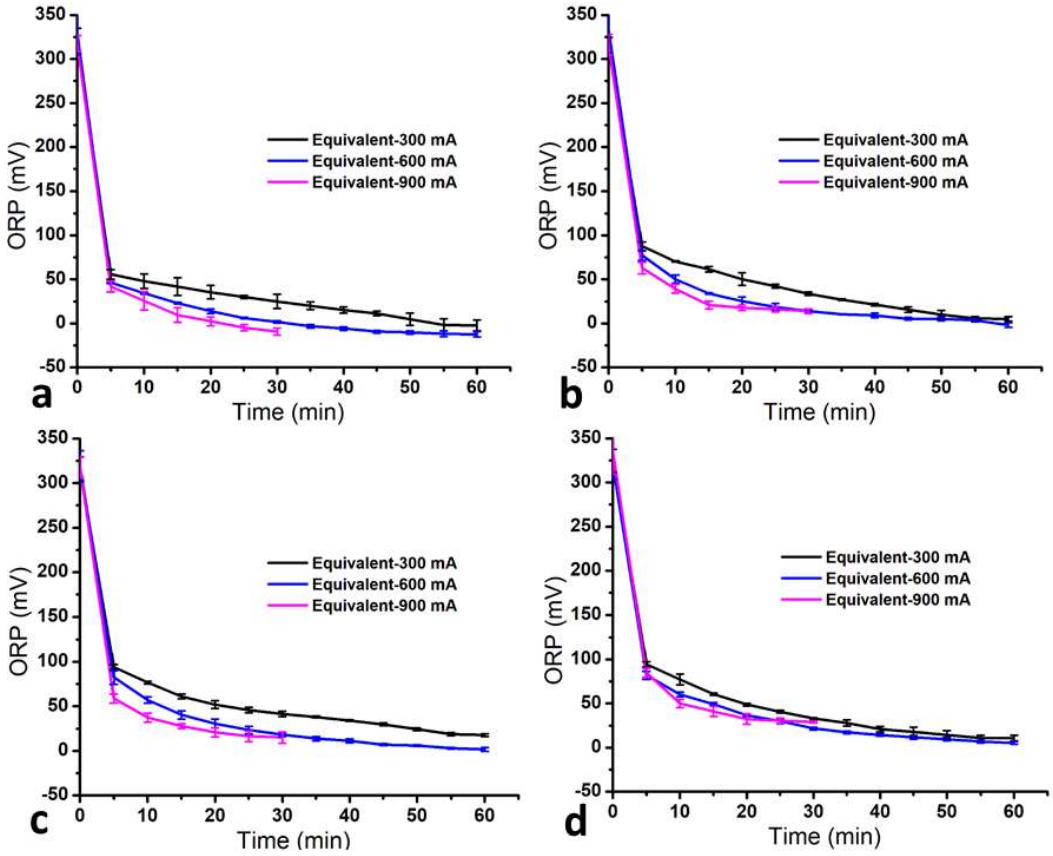

Figure 3.14: Evolution of ORP as a function of isomerization time in (a) 5%, (b) 10%, (c) 15%, and (d) 20% lactose solutions.

It is also worth noting that the ORPs in the chemical isomerization of different lactose solutions (5, 10, 15, and 20%) were reduced only to a value of around +50 to +100 mV within the first 5 min depending on the concentration and alkalinity (equivalent to different current intensities), as shown in **Figure 3.14**. Thereafter, it gradually decreased till the end of the

reaction and then reached a value of around 0 mV. In contrast, the ORP values in EA were decreased drastically to a value of around -800 mV within the first 5 min for all current intensities and concentrations and remained almost steady during the EA process (**Figure 3.5**). The electric field triggers the feed solution to be transformed into a metastable state and made the solution highly activated. Therefore, the reduced the ORP of EA lactose solutions in a metastable state rendered the solutions highly reactive because the reactivity of the electroactivated solutions was significantly increased under this state than a normal state (Aider et al., 2012b). Thus, the excessive internal potential energy of the activated solution possibly intensified the isomerization reaction of lactose into lactulose. Consequently, a higher yield of lactulose was achieved in the EA process compared to that of the chemical isomerization. Therefore, the modified ORP and critical pH of the EA lactose solutions in a metastable state could make the solutions highly reactive and convenient for nonconventional chemical reactions and different applications in the food industry and biotechnology, including food safety (Suzuki et al., 2002, Aider et al., 2012b). Indeed, the ORP of the drinking water/aqueous solutions should be negative to be highly efficient for physiological activities in humans (Petrushanko and Lobyshev, 2004, Aider et al., 2012b).

3.4 Conclusions

In this study, the electroisomerization of lactose into lactulose was successfully carried out using the EA technology. Moreover, lactose isomerization into lactulose by EA was compared with the chemical method using KOH at equivalent solution alkalinity as in the EA method. The obtained results demonstrated that in contrast to the chemical method, the EA process was found to give a higher yield of lactulose in a reduced reaction time for all conditions. The highest lactulose yield was obtained during the electroisomerization process of lactose and was ~38% at 40 min using a 900 mA current intensity in a 10% lactose solution with a solution pH of 11.27 and the alkalinity of 21.07 mmol/L. The highest lactulose yield was obtained during the conventional chemical isomerization process was ~27% at 60 min in a 10% lactose solution, while the pH was 11.33 for the alkalinity of 43.20 mmol/L (equivalent to 600 mA in EA). The correlated lactulose yield with the process parameters suggested that the lactulose can be produced at ambient temperature without additional heating if the required alkaline condition is achieved, although a higher temperature was

positively correlated to the lactulose formation but leading to a higher byproduct formation. A highly alkaline condition was required for the formation of lactulose; however, the lactulose produced in the medium did not reduce during the EA process while the alkalinity has been declined. Furthermore, EA triggers the feed solution to transfer into a metastable state characterized by unusual values of the chemical and physical parameters such as the ORP, pH, and alkalinity. Thus, it can be concluded on the basis of the compared approaches (EA vs chemical) that EA significantly reduced the activation energy required for the isomerization reaction of lactose into lactulose, and consequently, a higher yield of lactulose was achieved within a shorter duration at ambient temperature compared to that of the chemical isomerization using KOH as catalyst. In addition, the electroisomerization process was carried out under complete autocatalytic conditions, i.e., no alkali was added to the reaction medium. Finally, the findings of this study provide an insight into the feasibility of the electroisomerization of lactose into lactulose and its process mechanism of action. In contrast to the chemical method, the EA process was found to offer a higher potential for an economic and environmentally friendly approach to produce lactulose by the isomerization of lactose. However, further research is still required to understand the thermodynamics behind the EA phenomena of aqueous solutions, mainly regarding its action on the reaction activation energy.

ANNEXE A

Global electric resistance of EA reactor as a function of time at different current intensities (Figure A1); the possible mechanism pathway of the electroisomerization of lactose into lactulose and subsequent galactose formation as a reaction byproduct by using the electroactivation process (Figure A2); the formation of glucose and fructose for different lactose solutions at different current intensities of EA (Table A1); the formation of galactose and glucose for 5 and 10% lactose solutions in chemical isomerization (Table A2); the formation of galactose and glucose for 15 and 20% lactose solutions in chemical isomerization (Table A3); residual lactose (%) in different electroactivated lactose solutions (5, 10, 15, and 20%) (Table A4); and residual lactose (%) in different chemically isomerized lactose solutions (5, 10, 15, and 20%) (Table A5).

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Chapitre 4: Sustainable valorization of whey by electroactivation technology for in situ isomerization of lactose into lactulose: Comparison between electroactivation and chemical processes at equivalent solution alkalinity

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RÉSUMÉ

La demande de production de prébiotiques à une échelle commerciale augmente en raison de la sensibilisation croissante des consommateurs à la santé. Le lactosérum, un coproduit de l'industrie laitière, est un milieu approprié pour produire une lactulose, une prébiotique éprouvé, par l'isomérisation du lactose dans des conditions alcalines. Le but de la présente étude était de comparer l'isomérisation du lactose en lactulose *in situ* du lactosérum en utilisant la technologie d'électro-activation par rapport à la méthode d'isomérisation chimique utilisant le KOH comme catalyseur sous une alcalinité de solution équivalente. L'électro-isomérisation du lactose en lactulose a été réalisée en utilisant des solutions de lactosérum de 7, 14 et 21% (w/v) de matière sèche sous des intensités de courant électrique de 300, 600 et 900 mA, respectivement, pendant 60 min avec un intervalle d'échantillonnage de 5 min. La méthode chimique classique a été réalisée en utilisant de la poudre de KOH comme catalyseur à une alcalinité qui correspondait à celle mesurée dans le lactosérum électro-activé à chaque intervalle de 5 min. Les résultats ont montré que la production de lactulose dépendait de la concentration de lactosérum, de l'intensité du courant et du temps d'électro-activation (EA). Le rendement de lactulose le plus élevé de 32 % a été obtenu sous une intensité de courant de 900 mA à 60 min pour une solution de lactosérum à 7 %. Par la suite, les conditions d'EA ont été comparées à celles d'un processus d'isomérisation chimique classique en maintenant une alcalinité similaire dans les solutions testées. Les résultats ont montré que la lactulose n'a pas été produit par le procédé chimique pour l'alcalinité équivalente à celle de la solution électro-activée. Ces résultats ont été corrélés avec le pH de la solution, qui a atteint les valeurs requises dans une solution de lactosérum à 7% avec des valeurs allant jusqu'à pH 11,50; alors que les valeurs de pH maximales obtenues à des concentrations de lactosérum plus élevées étaient d'environ 10-10,50; ce qui n'était pas suffisant pour initier la réaction d'isomérisation du lactose. Les résultats de cette étude suggèrent que l'EA est une technologie efficace pour produire de la lactulose en utilisant du lactosérum.

Mots-clés: Lactosérum; Électroactivation; Lactose; Lactulose; alcalinité; Potentiel d'oxydoréduction; Température; Migration d'ions; Isomérisation chimique.

ABSTRACT

The demand for production of prebiotics at a commercial scale is rising due to the consumers' growing health awareness. Whey, a coproduct of the dairy industries, is a suitable feed medium to produce a prebiotic lactulose through the isomerization of lactose under alkaline conditions. The aim of the present study was to compare the isomerization of lactose into lactulose *in situ* of whey by using electroactivation technology with the chemical isomerization method using KOH as catalysis under equivalent solution alkalinity. Electroisomerization of lactose into lactulose was performed by using whey solutions of 7, 14, and 21% (w/v) dry matter under current intensities of 300, 600, and 900 mA, respectively, during 60 min with a sampling interval of 5 min. The conventional chemical method was carried out using KOH powder as catalyst at the alkalinity that corresponded to that measured in the electroactivated whey at each 5 min interval. The results showed that lactulose production was dependent on the whey concentration, current intensity, and EA time. The highest lactulose yield of 32% was achieved under a 900 mA current intensity at 60 min for a 7% whey solution. Thereafter, the EA conditions were compared to those of a conventional chemical isomerization process by maintaining similar alkalinity in the feed solutions. However, no lactulose was produced by the chemical process for the equivalent solution alkalinity as in the EA technique. These results were correlated with the solution pH, which reached the required values in a 7% whey solution with values of up to pH 11.50, whereas the maximum pH values that were obtained at higher whey concentrations were around 10-10.50, which was not enough to initiate the lactose isomerization reaction. The outcomes of this study suggest that EA is an efficient technology to produce lactulose using whey lactose.
Keywords: Whey; Electroactivation; Lactose; Lactulose; Alkalinity; Oxidation-reduction potential; Temperature; Ion migration; Chemical isomerization.

4.1 Introduction

Whey is a coproduct of cheese or casein production, typically comprising 5-8% (w/w) of dry matter in which 60-80% is represented by lactose and 10-20% by proteins (Seo et al., 2015b, Parashar et al., 2016a). About 9 kg of whey can be generated to make 1 kg of cheese, and it mostly consists of water (93-94%), lactose (4-5%), proteins (0.6-0.8%), and minerals (0.5%) (Djouab and Aïder, 2019a, Schmidt et al., 2020). The worldwide production of whey was estimated at around 180-190 × 10^6 tons/year, of which only 50% is being processed into different food and feed derivatives, and about 50% of total production is mainly discarded as dairy effluent with serious environmental concerns because of its high biochemical and chemical oxygen demands (Ghaly et al., 2007b, Baldasso et al., 2011). Therefore, innovative and sustainable approaches of managing the whey must be addressed for its valorization, more likely, in respect to its significantly high contents of potentially valuable ingredients like lactose.

Lactose is used for producing various value-added derivatives, like lactose, lactitol, lactobionic acid, lactosyl urea, lactosucrose, and galacto-oligosaccharides. Among these, the production of lactulose has received particular interest in recent years due to its proven bifidogenic functionality with many food and pharmaceutical applications (Seo et al., 2015b). Lactulose production is typically carried out by isomerization of lactose, following either a chemical or an enzymatic method (Seo et al., 2016b, Wu et al., 2017). However, recently, the isomerization of lactose into lactulose is successfully performed under autocatalytic conditions using electroactivation (EA) of pure lactose or whey solution by following self-generated alkaline conditions through water electrolysis at the solution/cathode interface (Aissa and Aïder, 2014b, Kareb et al., 2016b). Indeed, the hydroxyl (OH^-) ions generated by water decomposition at the solution/cathode interface create a high alkaline condition needed for this isomerization reaction. The interference of the alkaline condition in the cathodic compartment and the acidic condition in the anodic compartment is usually avoided by using a suitable reactor configuration, in which a cation-exchange membrane (CEM) is placed between the cathodic and the central compartments and an anionic-exchange membrane (AEM) between the anodic and the central compartments (**Figure 4.1**). Besides lactulose production, EA can be effectively used in the food industry and biotechnology to enhance

the antioxidant activity of whey following a formation of Schiff basis known to have a strong antioxidant capacity (Aider et al., 2012b).

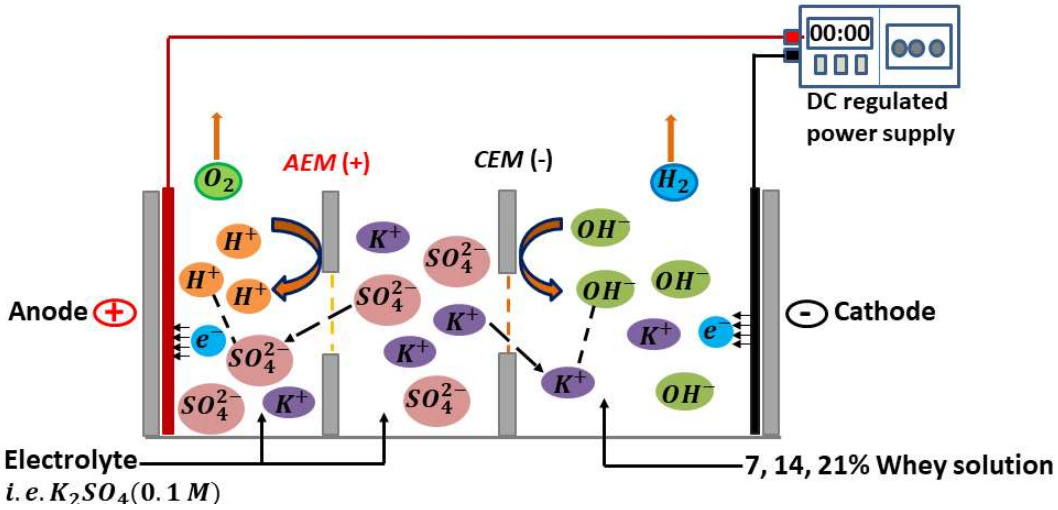

Figure 4.1: Graphical representation of the electroactivation reactor used for the isomerization of lactose into lactulose *in situ* of whey.

In the case of whey valorization, EA could be employed to produce lactulose directly *in situ* of whey by electroisomerization of lactose into lactulose, which is a proven prebiotic. Consequently, a completely new product, lactulose-enriched whey, is produced, which could be used as a high-value-added prebiotic with antioxidant properties (Kareb et al., 2016b, Djouab and Aïder, 2019a). Furthermore, when whey is subjected to the EA, the amino groups of whey proteins or peptides interact with carbonyl functions of the reducing sugars existing in the medium to form intermediate Maillard reaction products, which enhance the antioxidant capacity of the final product (Oh et al., 2013, Kareb et al., 2017c). Nevertheless, more recently, Djouab and Aïder (2019b) achieved a higher yield of lactulose formation (~38%) under a 330 mA current intensity during only 14 min of EA using a 5% lactose solution. However, the type of feed solution (lactose/whey/whey permeate) and the concentration of lactose in the feed solution had a significant effect on the lactulose formation (Aider and Gimenez-Vidal, 2012, Djouab and Aïder, 2019b). The influence of feed composition, especially for whey, would have a substantial impact on lactulose production since it has a broad variation in composition and may exhibit strong buffering capacity (Schmidt et al., 2020). Aider and Gimenez-Vidal (2012) observed that the formation of lactulose was different for whey permeate (8.84 ± 0.19%) compared to that of a pure lactose

solution (25.47 ± 1.18%) even though similar experimental conditions (200 mA, 60 min, 23.1 ± 1 °C) and similar initial lactose concentration (~5%, w/v) of feed solutions were used. Besides the types and lactose concentration of the feed solution, the activity of isomerization reaction in the EA can be affected by current intensity, time, temperature, volume of the feed solution, salt type and concentration used as electrolyte, electrode material, interelectrode-membrane distance, configuration, and geometry of the EA reactor (Aissa and Aïder, 2014b, Aït-Aissa and Aïder, 2014, Djouab and Aïder, 2019b).

Recently, Kareb et al. (2016b) described the effect of feed solution concentration and volume, temperature, and current intensity on whey lactose isomerization into lactulose. They obtained a maximum yield of ~35% using 100 mL of a 7% whey solution during 40 min of EA under a 400 mA current intensity at a 10 °C temperature. However, the evolution of alkalinity, temperature, oxidation-reduction potential (ORP), and ion migration phenomena during the electroisomerization of whey has never been explained to date. In EA, the lactulose formation rate would be significantly influenced by the solution alkalinity in the cathodic compartment because an adequate level of alkalinity was needed to achieve the isomerization reaction (Kareb et al., 2016b, Djouab and Aïder, 2019b). Furthermore, the electrolyte concentration in the central compartment and the migration of ions toward the cathode can affect the solution alkalinity of the catholyte, which in turn may possibly influence the lactulose formation following lactose isomerization (Aissa and Aïder, 2013c). In addition, the feed solution could reach some metastable state due to the increased reactivity of the electroactivated solution while an external electric field is applied. Thus, the highly reactive solution probably intensifies the isomerization reaction of lactose (Aider et al., 2012b). This phenomenon could partly be explained by the evolution of ORP during the EA. Therefore, all of these aspects should be taken into consideration to understand the process mechanism of the action involved behind the isomerization of lactose into lactulose *in situ* of whey by using the EA technique.

Currently, conventional chemical isomerization is used to produce lactulose at the industrial scale following the Lobry de Bruyn-Alberda van Ekenstein (LA) transformation. Isomerization via LA rearrangement requires elevated temperature (50-130 °C) and addition of alkalinizing chemicals as catalysts with different reaction times (Hashemi and Ashtiani, 2010a, Seo et al., 2015b). The efficacy of such isomerization reaction is mostly affected by

the pH, temperature, processing time, and concentration of catalysts (Seo et al., 2015b). Hashemi and Ashtiani (2010a) observed that the increase in temperature and pH could accelerate the conversion rate and shorten the reaction time to obtain the maximum yield. However, higher temperature or pH possibly led to the rapid degradation of lactose and lactulose into higher byproduct formation such as epilactose, galactose, glucose, or other acidic products. In a recent study, Seo et al. (2015b) observed that a higher amount of catalysts (0.51% Na_2CO_3) and temperature (90 °C) were required to achieve a high alkaline condition for efficient lactulose production. However, the yields of chemical isomerization were <30% (Hashemi and Ashtiani, 2010a, Seo et al., 2015b). In contrast to traditional chemical isomerization, the electroisomerization process could be performed without using any alkalinizing chemicals in the medium. In addition, the EA process can be carried out in the mild temperature range; thus, the formation of byproducts could be avoided (Aider and Gimenez-Vidal, 2012). Consequently, the purification step could be simplified, which is an economical advantage for the industrial applications. Furthermore, the EA process may possibly reduce the energy consumption and use of chemicals, and thus it can be considered as an eco-friendly and sustainable technique to produce lactulose through the isomerization of lactose (Kareb et al., 2016b, Djouab and Aïder, 2019b). Regarding this context, it is necessary to compare the efficiency of the EA with a chemical isomerization process under equivalent solution alkalinity. However, no study was found to meet this disparity in the literature; thus, the efficiency of lactulose production must be compared by producing an equivalent alkaline condition in both processes as the solution alkalinity is one of the most critical parameters for the isomerization reaction. This important factor has usually been overlooked in the previous isomerization studies but can be formidable for the development of sustainable and economic processes.

In the present study, several EA process parameters such as solution alkalinity, pH, ion migration, temperature, and ORP during the EA of different whey solutions were studied to explain their impact on the isomerization of lactose contained in the whey into lactulose. In addition, a conventional chemical isomerization was carried out under equivalent solution alkalinity to compare with the EA process.

4.2 Materials and methods

4.2.1 Chemicals and reagents

The high-performance liquid chromatography (HPLC)-grade sugars such as lactose, lactulose, galactose, glucose, and fructose were obtained from Sigma-Aldrich (Ottawa, Ontario, Canada), whereas the high-purity (purity ≥ 95%) chemicals and reagents of analytical grade were procured from different suppliers. The potassium hydroxide (KOH), potassium sulfate (K_2SO_4), hydrochloric acid (HCl), and phenolphthalein ($C_{20}H_{14}O_4$) was purchased from Fisher Chemical (Fair Lawn, NJ, USA), Sigma-Aldrich Co. (St. Louis, MO, USA), Fisher Chemical (Geel, Belgium), and MAT Laboratory Inc. (Laboratoire MAT Inc., Quebec, Canada), respectively. The food-grade whey powder (lactose, 75%; total proteins, 12%; ash, 7%; and moisture content, < 5%) was obtained from Agropur Co-operative (St-Hubert, Quebec, Canada). The cation-exchange membrane (CMI 7000S) and anion-exchange membrane (AMI 7001S) were bought from Membrane International Inc. (Ringwood, NJ); and were directly used in the EA reactor without any pretreatment.

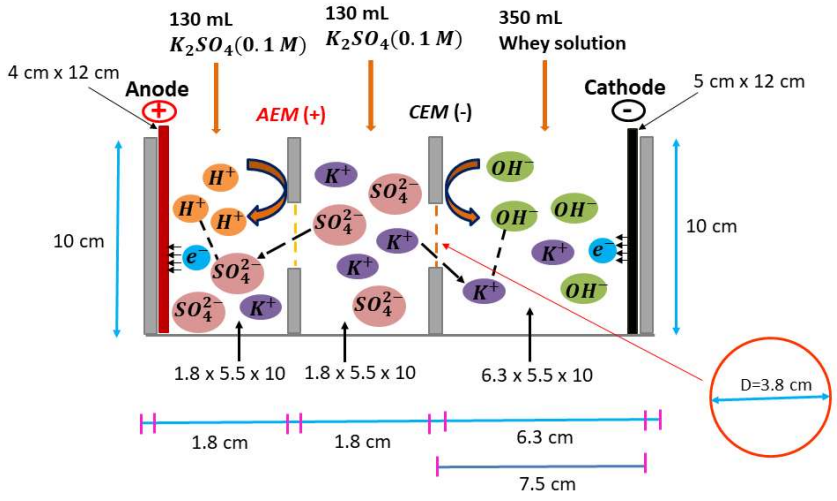

Figure 4.2: Graphical representation and geometrical parameters of the electroactivation reactor used for the isomerization of lactose into lactulose *in situ* of whey.

4.2.2 Electro-activation protocol

An EA reactor made of Plexiglas, comprising three compartments (anodic, central, and cathodic compartments), was used in this study (**Figure 4.2**). The dimensions of the cathodic compartment, in which the EA process for lactose isomerization into lactulose was targeted, have the following geometrical dimensions: 6.5(L)×5.5(W)×10(D) cm^3 for a total volume of 357 cm^3. The anodic and central compartments are similar to the following geometrical dimensions: 5.5(L)×1.8(W)×10(D) cm^3. The anodic compartment was connected to the positive side of a DC-regulated power generator (model: CSI12001X, CircuitSpecialists.com) by a titanium electrode coated with ruthenium-iridium, whereas the cathodic compartment was linked to the negative side through a food-grade stainless steel electrode. However, the cathodic and anodic compartments were separated by the central compartment and were communicating with the cathodic and anodic compartments via a CEM and an AEM, respectively. A freshly prepared whey solution (350 mL) of different concentrations (7, 14, and 21%) was placed in the cathodic compartment, while the anodic and central compartments were filled with 0.1 M K_2SO_4 solution (**Figure 4.2**). The selected whey concentrations are based on the following considerations: the 7% concentration was selected because it corresponds to the whey that is generally obtained as a coproduct of the cheese-making industry. Thus, the EA process can be applied to whey directly after it is generated without further concentrating step. The 21% concentration was chosen on the basis of whey solubility in water so as to avoid any precipitation. The 14% concentration was selected as an intermediate between the whey as obtained following a cheese-making process and a concentrated whey without any precipitation. The potassium sulfate was used as an electrolyte in the anodic side of the EA reactor to avoid chlorine generation following the oxidation reaction that takes place at the anode surface. The experiments were carried out under three current intensities of 300, 600, and 900 mA for a 60 min duration. The samples were obtained from the central and cathodic compartments at regular intervals of 5 min and were stored at 4 °C until further analysis. All experiments were operated at an ambient temperature (22 ± 2 °C). The EA reactor was properly cleaned with DI water prior to each experiment and remained filled with DI water after each batch to maintain high membrane hydration.

4.2.3 Determination of pH, alkalinity, temperature, and oxidation-reduction potential

The pH, alkalinity, temperature, and ORP of whey solution in the cathodic compartment were measured at 5 min intervals during the 60 min EA process. The pH was evaluated using a digital pH meter (Oakton pH 700) outfitted with a pH probe (Oakton, Vernon Hills, IL). The temperature and ORP were assessed by using an ORP meter (Ultrapen, Myron L Company, Carlsbad, CA). A standard titration method was used to determine the total alkalinity of the electroactivated whey solutions (catholytes), as described by Karim and Aider (2020b) . Finally, the total alkalinity was calculated using **Eq. 4.1** and was expressed in mmol/L

$$Total\ Alkalinity_{eq} = \frac{C_{titrant} \times V_{titrant} \times 1000}{V_{sample}} \quad \text{(Eq. 4.1)}$$

$V_{titrant}$ is the total volume of the titrant (0.1 M HCl) needed for titration in mL, $C_{titrant}$ is the concentration of the titrant in mol/L, V_{sample} is the volume of sample in mL, $Total\ Alkalinity_{eq}$ is equivalent concentration of NaOH/KOH in the electroactivated solutions in mmol/L (equiv).

4.2.4 Evaluation of potassium concentration

The atomic absorption spectrometry was used to evaluate the concentration of potassium (K^+) ions in the central compartment. The samples (that were collected from the central compartment) were analyzed following a standard protocol for the atomic absorption spectrometer (PerkinElmer Instruments, model AAnalysts 200, Boston, MA).

4.2.5 Chemical isomerization of lactose in whey

The conventional chemical isomerization was performed by adding the equivalent (to the total alkalinity in the EA) amounts of potassium hydroxide (KOH) to the feed solutions (7, 14, and 21% whey). In brief, the total alkalinity (mmol/L) obtained in the EA-whey solutions was converted to the equivalent amounts of KOH in mg/L. Thereafter, the equivalent amounts of KOH were added to the feed solution at regular intervals of 5 min to maintain a similar alkaline condition as in the EA. The mixture was continuously stirred at an ambient temperature (22 ± 2 ℃). The pH and ORP of the medium were measured at 5 min intervals during the reaction. Finally, the samples were obtained from the reaction medium at regular intervals of 5 min and were preserved at 4 ℃ for further analysis.

4.2.6 Evaluation of sugars composition

A HPLC system (Water, Millipore Corp., Milford, MA) was used to determine the sugar contents in the samples (that were collected from the cathodic compartment and reaction medium of whey solutions during the EA and chemical isomerization, respectively). The HPLC system was equipped with a carbohydrate analysis column (Waters Sugar Pak-I, 300 × 6.5 mm^2, Waters Co.) and a refractive index detector (Hitachi, model: L-7490). The column temperature was set at 90 ℃, and an isocratic mobile phase (a solution of 50 mg/L Ca-ethylenediamine tetraacetic acid) was used at a flow rate of 0.5 mL/min. The analysis was then performed by injecting 50 µL of sample and setting the operating time to 30 min/sample. Finally, the identification and quantification of different sugars (lactose, lactulose, glucose, galactose, and fructose) were achieved by matching their retention times with the standard solutions.

4.2.7 Statistical analysis

A complete randomized factorial design with repeated measurements was used for statistical analysis. The factors for the analysis were whey concentration, current intensity, and reaction time. The pH, alkalinity, K^+ ion migration, temperature, ORP, lactulose yield, and the yield of byproducts (galactose, glucose, and fructose) were considered as dependent variables. Each experiment was performed in triplicate, and the mean values ± standard deviation was used for the analysis. Differences at $p < 0.05$ were considered as significant. Analysis of variance (ANOVA) of the data was carried out using SAS software (V9.3, SAS Institution Inc., Car, NC).

4.3 Results and discussion

4.3.1 Evolution of solution pH and alkalinity

The development of pH in the cathodic compartment was studied during the 60 min of EA under different current intensities of 300, 600, and 900 mA for different whey solutions (7, 14, and 21%, respectively) and is depicted in **Figure 4.3**. It is apparent that the evolution of pH was significantly ($p < 0.001$) influenced by whey concentration, current intensity, and EA time. The higher pH was obtained for a 7% whey solution at 60 min compared to 14 and 21% whey solutions for all current intensities used. For 7 % whey, the pH values were 10.60 ± 0.02, 11.47 ± 0.04, and 11.50 ± 0.06 after 60 min of EA under 300, 600, and 900 mA current intensities, respectively. The maximum pH was observed for a 900 mA current

intensity whatever the solution concentrations. The highest pH values obtained for 900 mA current intensity after 60 min of EA were 11.50 ± 0.06, 10.74 ± 0.04, and 8.90 ± 0.22 for 7, 14, and 21% whey solutions, respectively.

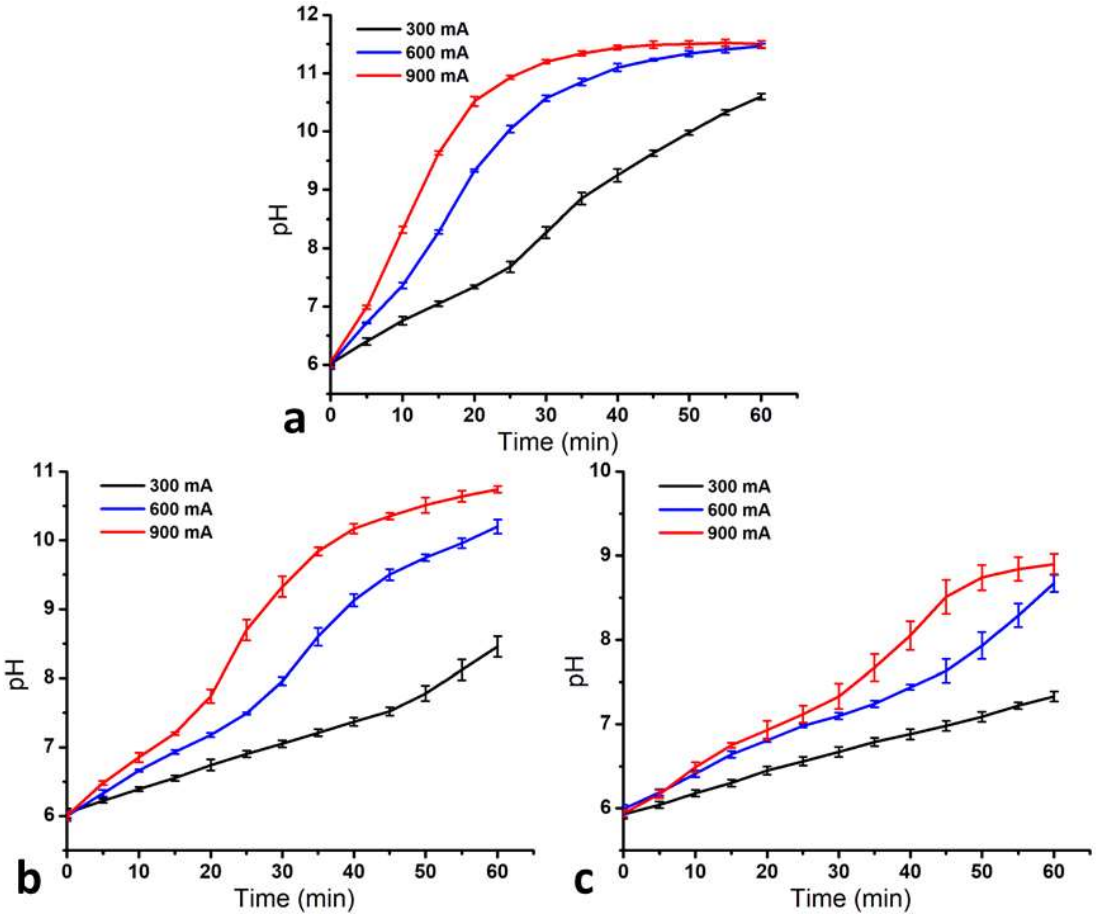

Figure 4.3: Evolution of pH as a function of EA time for (a) 7%, (b) 14%, and (c) 21% whey solutions under different current intensities.

It can be seen from **Figure 4.3** that the rate of pH progression was higher for greater current intensities for all solution concentrations. This could be attributed to the generation of more OH^- ions by intensive water dissociation. In fact, a reduction reaction occurred in the cathodic compartment, which resulted in the production of H_2 and OH^- $[2H_2O(l) + 2e^- \rightarrow H_2(g) + 2OH^-(aq)]$ by water decomposition. The amount of water electrolysis is, indeed, directly proportional to the electric current applied. **Figure 4.3a** implies that the pH was profoundly increased during the first 30 min for both 600 and 900 mA. This was due to the production of more OH^- ions in the medium followed by rigorous water splitting at the start of the reaction to allow the current transfer in the cathode-solution interface. Thereafter,

the pH evolution showed a quasi-steady stage because the solution became saturated with enough OH^- ions. In contrast to that, different phenomena were observed for 14 and 21% whey solutions, as depicted in **Figure 4.3b,c**. This might be due to the increased buffering capacity of the highly concentrated whey solutions, which was resisting the changes in pH by either absorbing or desorbing the OH^- ions. Indeed, the higher concentration of buffering compounds like whey proteins and their respective degradation products, inorganic phosphate, and organic acids present in the highly concentrated whey solutions (14 and 21%) may increase the intensity of the buffering capacity (Salaün et al., 2005a).

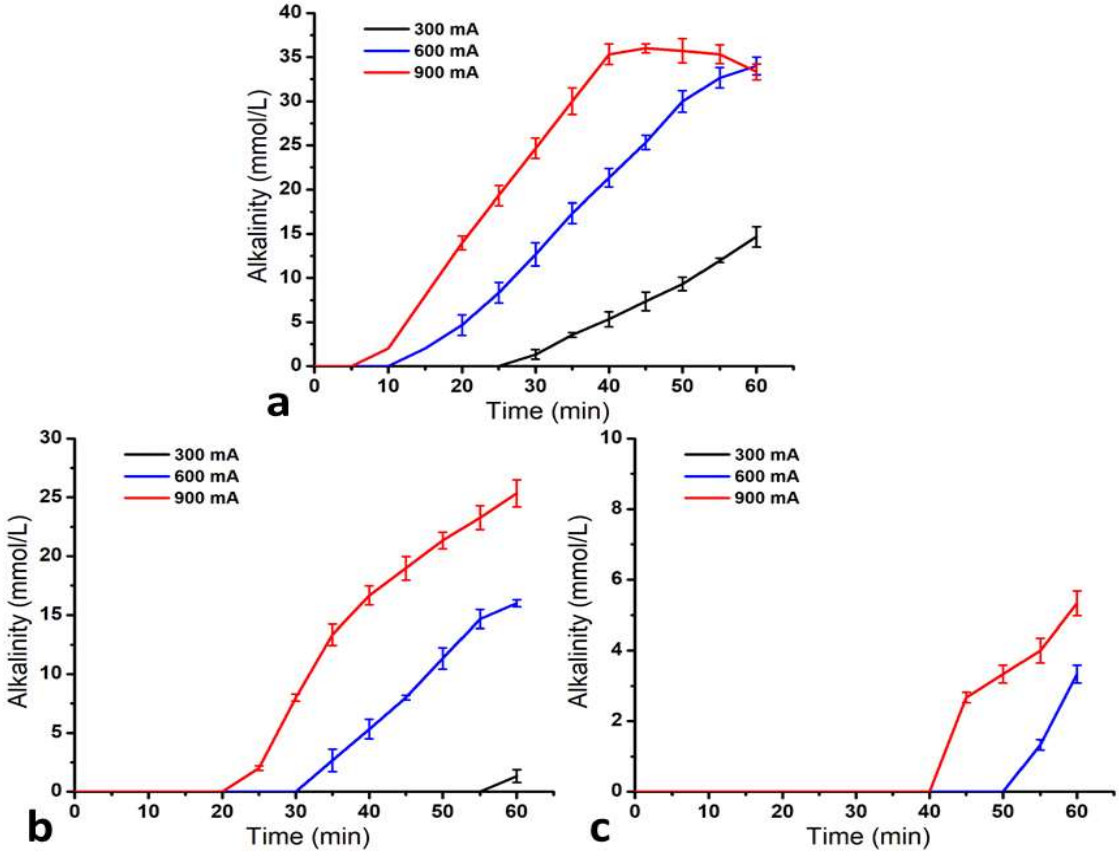

Figure 4.4: Evolution of alkalinity during the EA process for (a) 7%, (b) 14%, and (c) 21% whey solutions under different current intensities.

The solution alkalinity in the cathodic compartment was evaluated at 5 min intervals of the EA process under different current intensities (300, 600, and 900 mA) for different whey solutions (7, 14, and 21%) as presented in **Figure 4.4**. It was observed that the whey concentration, current intensity, and EA time had a significant impact ($p < 0.001$) on the

solution alkalinity. For a 7% whey solution, the solution alkalinity was linearly rising with time and achieved a maximum of 14.67 and 34.00 mmol/L alkalinities under 300 and 600 mA current intensities, respectively. However, it drastically increased to 35.33 mmol/L during the first 40 min and reached a plateau at 45 min (36.00 mmol/L) for a 900 mA current intensity; thereafter, it gradually decreased to 33.33 mmol/L at 60 min. The difference of the alkalinity for the current intensities was correlated to the concentration of OH^- ions formed in the medium. The decrease in the alkalinity after 45 min of EA time could be attributed to the fact that some H^+ ions would have migrated to the cathodic compartment from the central compartment and caused acidification of the solution. In fact, H^+ and OH^- ions might be generated by water decomposition at the CEM interface facing the central compartment once the reaction reached a critical stage, to evade the ion deficiency in the central compartment (Karim and Aider, 2020b). Maximum alkalinities of 1.33, 16.00, and 25.33 mmol/L were obtained at 60 min of EA under 300, 600, and 900 mA current intensities, respectively, when a 14% whey solution was used. On the other hand, only 3.33 and 5.33 mmol/L alkalinities were obtained for 600 and 900 mA current intensities, respectively, and no alkalinity was created for 300 mA in the 21% whey solution. This difference of the alkalinity in the higher concentrations of whey could be correlated to the higher buffering capacity of the solution, as well as higher resistance for more concentrated whey solutions (**Figure A3**). Thus, the electric conductivity of the solutions (14 and 21% whey) was probably less than that of the 7% whey solution. Furthermore, a lesser amount of OH^- ions were generated at the cathodic interface due to the higher concentration of whey (greater solid/water ratio).

4.3.2 Ion migration

The concentration of K^+ ions in the central compartment was evaluated during 60 min of EA under different current intensities of 300, 600, and 900 mA for different whey solutions (7, 14, and 21%, respectively) as demonstrated in **Figure 4.5**. It can be seen from **Figure 4.5** that the concentration of K^+ ions in the central compartment was decreasing with EA time whatever the solution concentrations and current intensities used. The rate of decrease was relatively higher for greater current intensities and higher whey solution concentrations.

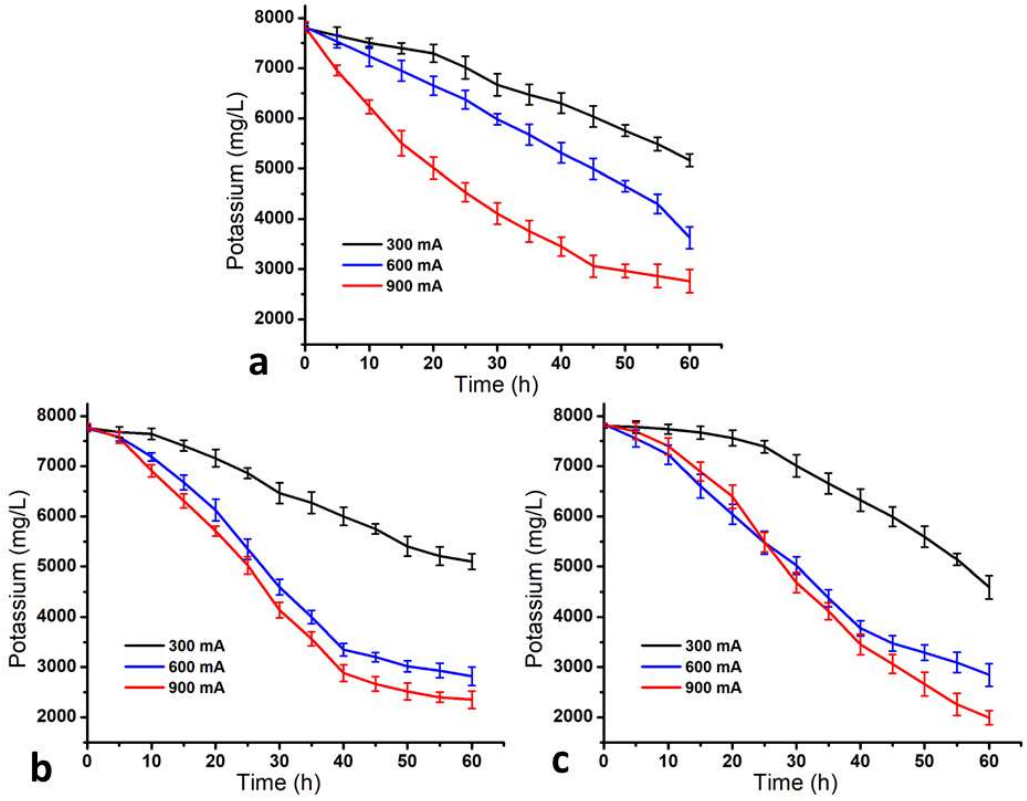

Figure 4.5: Concentration of potassium ions in the central compartment during the 60 min EA time for (a) 7%, (b) 14%, and (c) 21% whey solutions under different current intensities.

Figure 4.5a shows that the K^+ ions were gradually decreasing during the EA process for a 7% whey solution; however, a quasi-static behavior was observed after 45 min under a 900 mA current intensity. This observation of K^+ ion migration from the central compartment can be corroborated with the evolution of pH and alkalinity. The K^+ ions were continuously migrating to the cathodic compartment and reacting with the OH^- ions in the cathodic compartment to create alkalinity during the first 45 min. After 45 min of EA, the pH and solution alkalinity decreased due to the water splitting at the solution-CEM interface to reimburse the lack of current carriers toward the cathode. In fact, more H^+ and OH^- ions were produced through this water splitting, and newly generated H^+ ions competed for electromigration with K^+ ions (Karim and Aider, 2020b). The competition for electromigration from the central compartment towards the cathodic one was favorable for the H^+ ions because H^+ ion has higher electrophoretic mobility than K^+ ion in solution. As a result, the K^+ cation migration toward the catholyte also decreased at the same time. Likewise, as it can be seen from **Figures 4.5b,c**, the K^+ ions were also progressively

decreasing during the EA process of 14 and 21% whey solutions, and a quasi-steady behavior was observed after 40 min at 600 and 900 mA, respectively. However, unlike the 7% whey solution, the pH and alkalinity were not found to be reduced for the 14 and 21% whey solutions. This difference in comparison with the 7% whey solution can be attributed to the absence of a very weak water decomposition at the solution-CEM interface.

4.3.3 Evolution of temperature

The temperature increase in the cathodic compartment was studied during the EA for different whey solutions (7, 14, and 21%) under current intensities of 300, 600, and 900 mA, respectively, and is presented in **Figure 4.6**. It appears that the evolution of temperature is mainly dependent on the current intensity and EA time. The higher temperature was achieved for the 7% whey solution for all current intensities, and the ultimate temperatures were 26.23 ± 0.12, 34.57 ± 0.42, and 43.13 ± 0.40 °C at 60 min of EA under 300, 600, and 900 mA current intensities, respectively. The maximum temperature was noticed at 900 mA current intensity whatever the solution concentrations, and the highest temperatures were 43.13 ± 0.40, 40.63 ± 0.15, and 41.47 ± 1.31 °C for 7, 14, and 21% whey solutions, respectively.

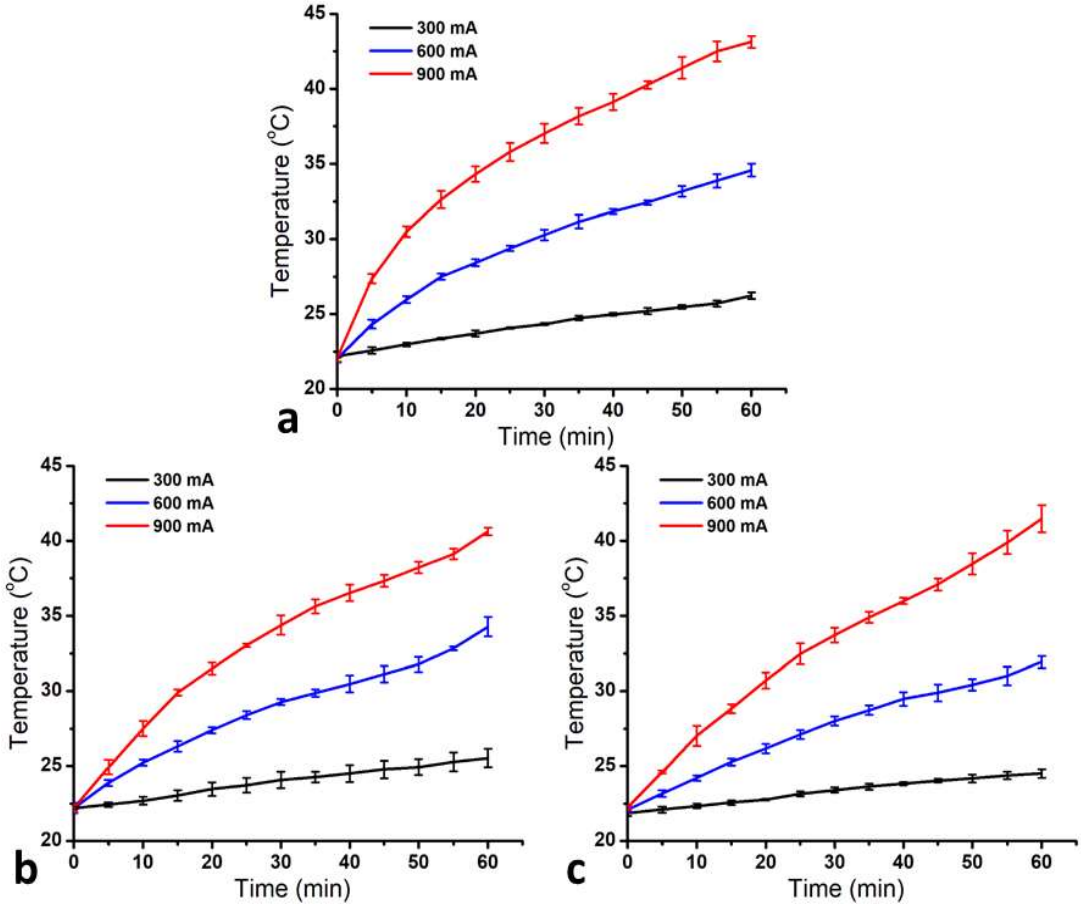

Figure 4.6: Change in temperature during EA for (a) 7%, (b) 14%, and (c) 21% whey solutions under different current intensities.

The temperature increase during the EA was mainly due to the Joule effect in the electrode, and the produced heat was dispersed in the solution (Aissa and Aïder, 2013c). The initial resistance of the ion exchange membranes and resistance of the feed solutions could also, to some extent, contribute to the temperature rise (Cifuentes-Araya et al., 2011b). Another possible reason could be the decreased conductivity in the central compartment because the demineralization due to ion migration may have increased the resistance of the system (Karim and Aider, 2020b). The rate of temperature increase was higher for greater current intensities used because the heated energy dissipation is proportional to the increase in the electric tension and electric current (Joule's law) (Aissa and Aïder, 2013c). Nevertheless, the statistical analysis of the obtained data did not show any correlation between the temperature rise (from 22 to 42 °C) and the formed lactulose. This can be

explained by the fact that this temperature rise was not enough to have any catalyzing effect on lactose isomerization into lactulose.

4.3.4 Evolution of oxidation-reduction potential

The changes of oxidation-reduction potential (ORP) in the cathodic compartment were observed during the EA of different whey solutions (7, 14, and 21%) under the current intensities of 300, 600, and 900 mA, respectively, as presented in **Figure 4.7**. It was observed that the ORP values drastically deceased at the beginning of EA for all solution concentrations and current intensities. The ORP values reached -500 to -600 mV within the first 5 min of EA whatever the current intensities and solution concentrations used. Thereafter, it differently decreased depending on the current intensities and solution concentrations.

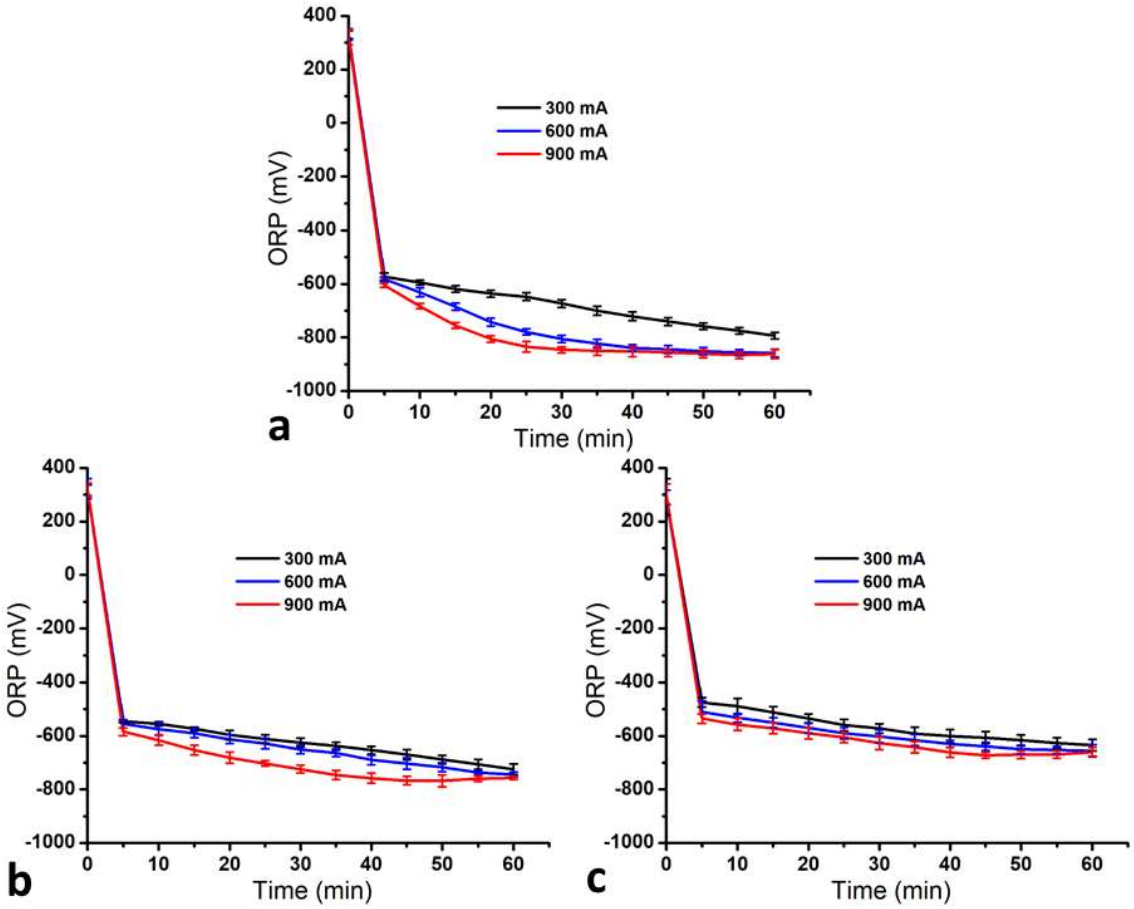

Figure 4.7: Variation of ORP as a function of time during EA for (a) 7%, (b) 14%, and (c) 21% whey solutions under different current intensities.

The negative ORP values of electroactivated solutions in the cathode may probably relate to the training effect of excess electrons and formation of various radicals and ionic species (Podkolzin et al., 2001a). Similarly, Shironosov and Shironosov (1999) explained the fact of highly negative ORPs in the electroactivated solution by generation of the high-energy resonant water microclusters in the solution due to co-vibrating dipoles of water molecules and charged species near-electrode interfaces. Basically, EA caused a vigorous water splitting, which resulted in the enrichment of the negative-charge concentrations by accumulation of OH^- groups in the cathodic compartment (Kareb et al., 2017c). The drastic difference in ORP in the first 5 min of EA may be ascribed to the formation of excessive electrons and generation of other highly active reducers such OH^-, H^-, H_3O^{2-}, $\cdot H$, $\cdot HO$, $\cdot O_2^-$, $\cdot HO_2^-$, $H_2O_2^-$ caused by rigorous electrolysis (Aider and Gimenez-Vidal, 2012). Thereafter, it showed a quasi-steady fashion because the cathodic compartment might be saturated with the charged species. From the practical point of view, ORP is very important for the applications of the electroactivated whey. Indeed, apart from lactulose, which is a well-known prebiotic, the negative (reductive) ORP is very suitable because it characterizes a medium with reducing properties. This means that bacteria that can be grown in this medium will have oxidative protection against stressing factors.

4.3.5 Assessment of lactulose formation during the EA process

The isomerization of whey lactose into lactulose was studied for different whey solutions (7, 14, and 21%) under 300, 600, and 900 mA current intensities, respectively. It was observed that the solution concentration, current intensity, and EA time had a significant effect ($p < 0.001$) on the conversion of whey lactose into lactulose. Lactulose was produced only for the 7% whey solution, as presented in **Figure 4.8**. However, no lactulose was noticed for the 14 and 21% whey solutions.

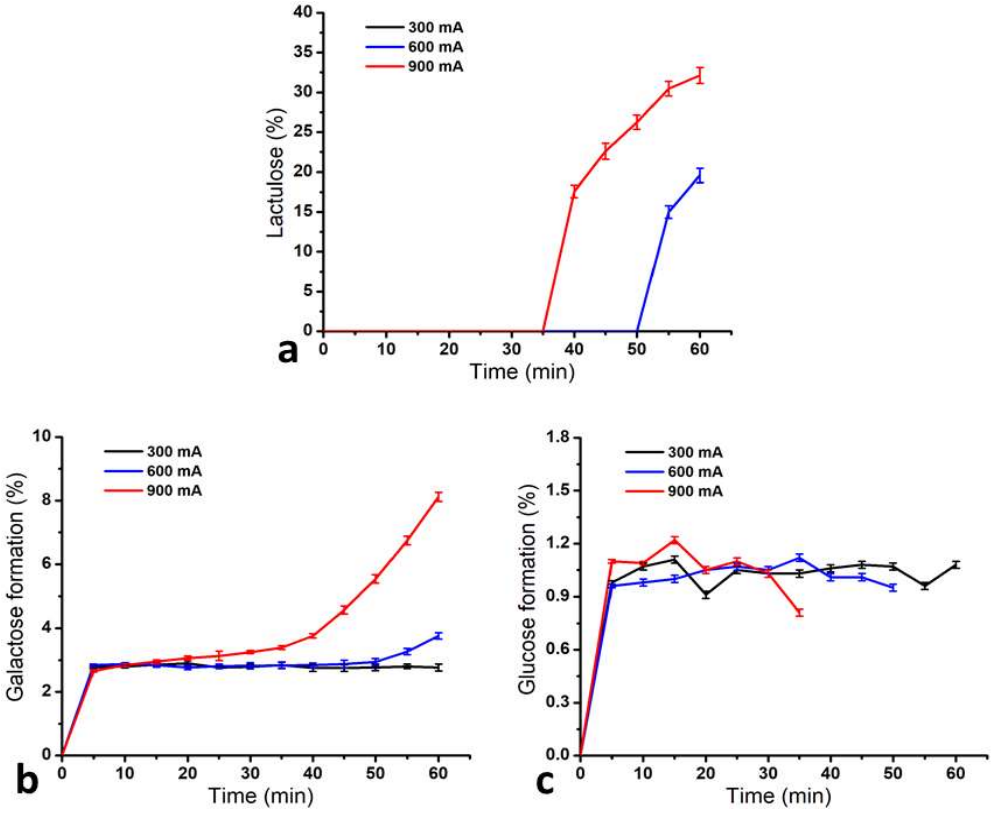

Figure 4.8: Formation of (a) lactulose and other byproducts like (b) galactose and (c) glucose during the EA for the 7% whey solution under different current intensities.

It can be seen from **Figures 4.8a** and **4.9** that the lactulose formation was started at 40 min (17.51%), and then gradually increased until the end (32.13% at 60 min) of EA under 900 mA for the 7% whey solution. The pH and alkalinity significantly influenced ($p < 0.001$) the production of lactulose. Lactulose formation started at 40 min while the pH and alkalinity reached 11.44 ± 0.04 and 35.33 ± 1.15 mmol/L, respectively, and thereafter, the yield increased until the end of EA under a 900 mA current intensity. On the other hand, only 19.58% lactulose was produced at the end of reaction under a 600 mA current intensity while pH and alkalinity attained 11.47 ± 0.04 and 34.00 ± 2.00 mmol/L, respectively. Higher alkalinity is the sinequanon for lactulose formation because the molecular rearrangement of lactose isomerization into lactulose needs proton acceptors. The higher current intensities (600 and 900 mA) might produce enough OH^- ions by intensive water splitting, which ensured the high alkaline condition in the cathodic compartment of the EA reactor (Aider and Gimenez-Vidal, 2012, Kareb et al., 2016b). However, no lactulose was detected for a 300 mA current intensity. This might be due to the lack of adequate alkalinity. It is generally

believed that a high pH (>10.00) is required for lactose isomerization (Hashemi and Ashtiani, 2010a, Kareb et al., 2016b, Djouab and Aïder, 2019b). Kareb et al. (2016b) stated that a solution pH of 11 ± 0.3 is more suitable for lactulose synthesis from whey using EA. In this study, it was observed that the lactulose could not be formed even if the pH reached around 11 in several cases. Thus, it can be argued that not only pH but also the adequate alkalinity of the feed solution is crucial to achieve isomerization of lactose. Moreover, the required alkalinity could be different depending on the type and concentration of the feed solutions.

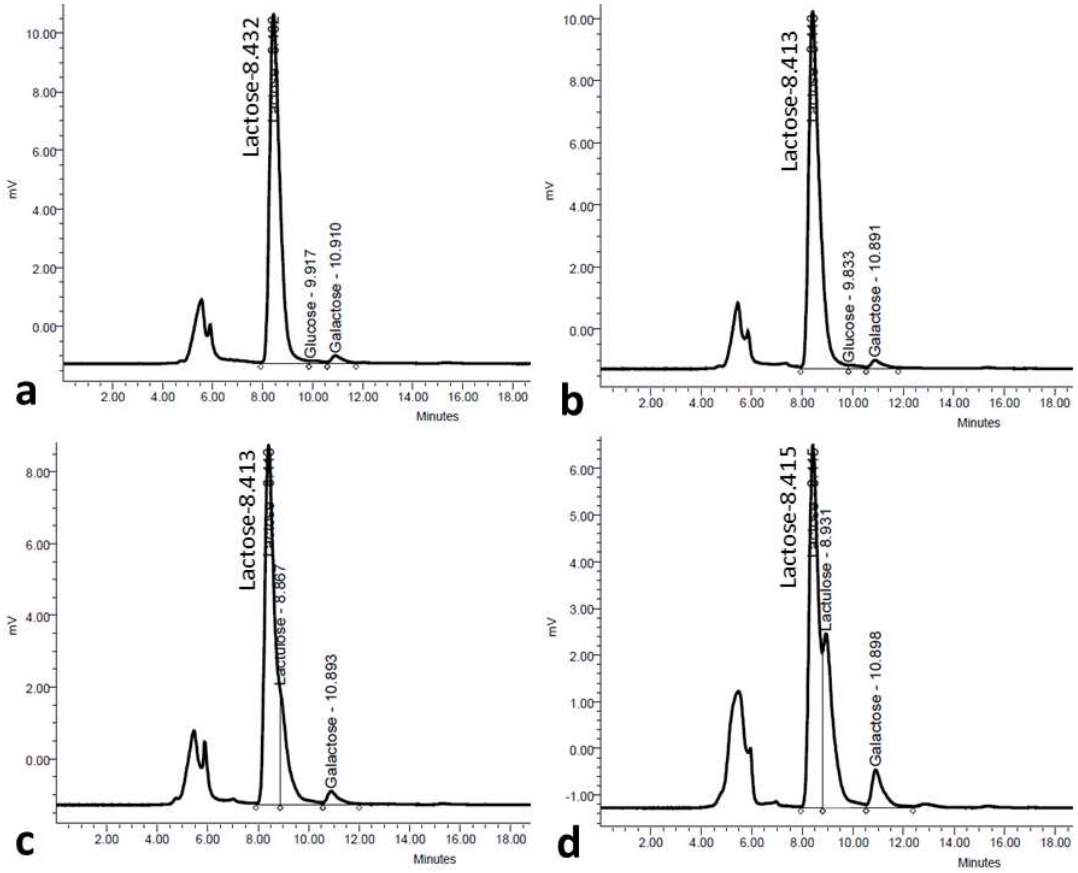

Figure 4.9: High-performance liquid chromatography (HPLC) chromatograms of lactulose formation for the 7% whey solution during EA. (a) initial feed solution, (b) at 60 min under 300 mA (glucose: 1.08%, galactose: 2.77%), (c) at 60 min under 600 mA (lactulose: 19.58%, galactose: 3.76%), and (d) at 60 min under 900 mA (lactulose: 32.13%, galactose: 8.12%).

As it can be seen from **Figures 4.8b** and **4.10**, the formation of galactose significantly increased with running time during lactulose production using the EA process, because some part of the lactose and lactulose was later hydrolyzed into galactose with increased isomerization time. Moreover, galactose formation was intensified by higher current intensity. This could be due to the higher temperature rise, pushing the reaction on the other side pathways due to higher activation energy (Hashemi and Ashtiani, 2010a, Kareb et al., 2016b). However, the maximum 8.12% of galactose was formed at 60 min of EA under 900 mA, while the acceptable limit for the commercial lactulose syrup can be up to 16% (according to the United States Pharmacopeia) (USP, 2008). It is worth noting that only galactose was produced as a byproduct, and no other impurities such as glucose, tagatose, epilactose, etc. were detected in the EA reactor (**Figures 4.9** and **4.10**). Therefore, it can be postulated that the glucose and fructose moieties from lactose and lactulose hydrolysis might be isomerized to galactose under the EA conditions. This observation was well corroborated with those previously reported (Kareb et al., 2016b, Djouab and Aïder, 2019b). In contrast to EA, the lactulose production via chemical isomerization was usually followed by a rapid degradation of lactulose into tagatose, epilactose, galactose, and other acidic byproducts such as isosaccharinic acids and formic acids. This is because an isomerization reaction via LA transformation was typically performed with a higher concentration of alkalinizing chemicals at elevated temperature (50 to 130 °C) combined with a long reaction time (Hashemi and Ashtiani, 2010a, Corzo-Martínez et al., 2013, Song et al., 2013b, Sakkas et al., 2014b). Thus, the purification steps could be more complex and costly (Hashemi and Ashtiani, 2010a, Schuster-Wolff-Bühring et al., 2010b, Djouab and Aïder, 2019b). However, in this study using EA, the temperature has never exceeded 43 °C and no alkalinizing chemical was required.

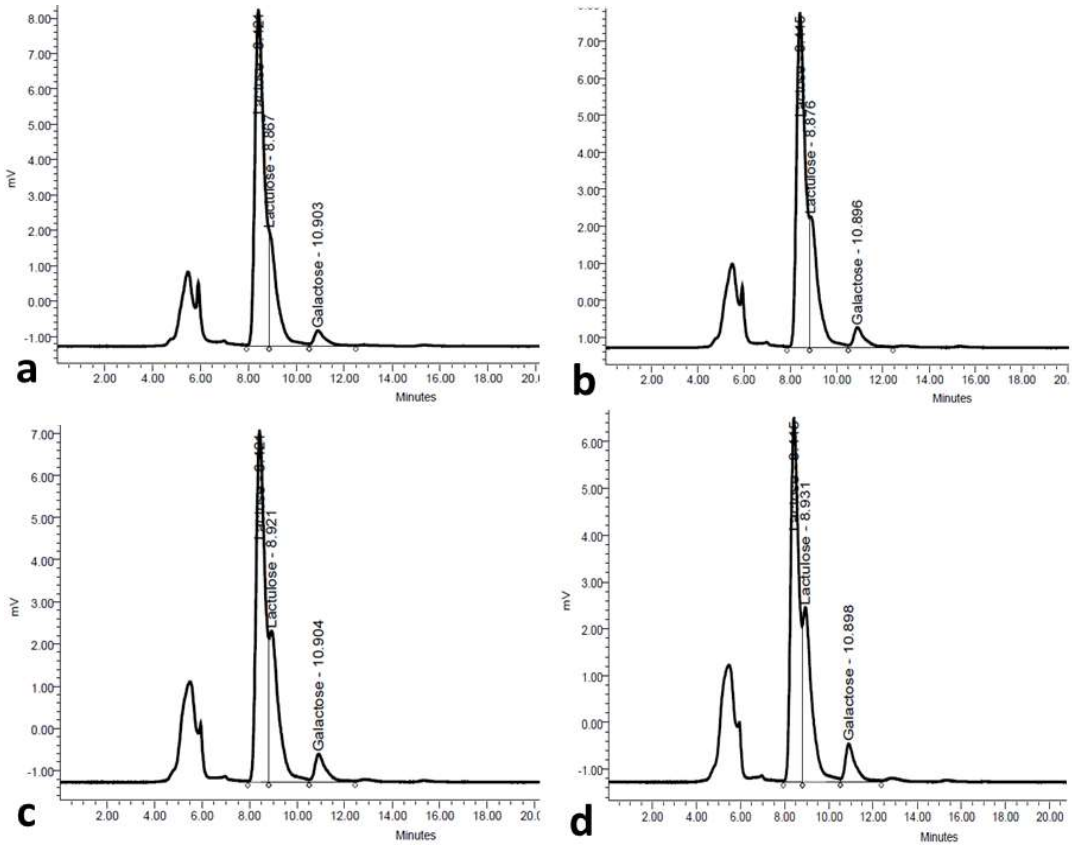

Figure 4.10: HPLC chromatograms of galactose formation as a function of EA time for 7% whey under a 900 mA current intensity: (a) at 45 min (lactulose: 21.59%, galactose: 4.57%), (b) at 50 min (lactulose: 26.24%, galactose: 5.54%), (c) at 55 min (lactulose: 30.47%, galactose: 6.75%), and (d) at 60 min (lactulose: 32.13%, galactose: 8.12%).

Regarding 14 and 21% whey solutions, no lactulose was produced but only galactose (**Figure A4**). This is probably because of the retarding pH and low alkalinity development in these two feed solutions due to the higher buffering capacity and resistance of the solutions. For 14% whey, the pH reached 10.20 ± 0.10 and 10.74 ± 0.04 at the end of the EA under the current intensities of 600 and 900 mA, respectively, but the solution alkalinity was probably inadequate (16.00 ± 2.00 and 25.33 ± 1.55 mmol/L) for the isomerization reaction to occur. It seems that the findings in the present study are perhaps contrary to what has been reported in the earlier literature by Kareb et al. (2016b). They reported that the 7 and 14% whey solutions were suitable, whereas the 28% whey solution was less effective for lactulose production using EA. However, the geometrical parameters in their study were quite different from those used in the present study, mainly the volume of the cathodic compartment and the distance between the CEM and the cathode. A volume of 100 mL feed solution was used in

their study, whereas it was 350 mL in the present study. In fact, the volume of the feed solution significantly influenced the formation of lactulose. Kareb et al. (2016b) observed that the lactulose yield decreased from 34.57 ± 0.79 to 7.08 ± 1.07% when the volume was increased from 100 to 300 mL under similar EA conditions (400 mA, 40 min, 10 °C). This is because an increase in the volume of feed solution under a constant current intensity and for the same electrode surface results in lower pH evolution due to the similar amount of OH^- ions produced at the solution/cathode interface. Nevertheless, the lactulose yield is directly related to the amount of OH^- ions formed in the medium because they act as proton acceptors and ensure the optimum alkalinity for lactose isomerization into lactulose (Aït-Aissa and Aïder, 2014, Kareb et al., 2016b). The lower the solution volume, the higher was the alkalinity in the cathodic compartment.

Besides the volume of the cathodic compartment, the distance between the cathode and the CEM could have a significant effect on lactulose formation. In the present study, the distance (7.5 cm) was about 3 times higher than that of Kareb et al. (2016b) which they used in their study. In a study, Aït-Aissa and Aïder (2014) demonstrated that the interelectrode-membrane distance in the cathodic compartment significantly ($p < 0.001$) influenced the production of lactulose, as well as galactose using lactose as feed solution. The highest lactulose formation (32.50%) was obtained by using the shortest interelectrode-membrane distance (~2.5 cm). They observed that lactulose production was decreased when the distance was increased from 2.5 to 5 cm. This is because the internal resistance of the cell, which depends on the electrode surface area and the distance between the electrodes, could be increased if the distance is extended. Obviously, with the higher distance, the moving electrical charges/ions encounter more collisions and, therefore, the resistance might be increased (Aït-Aissa and Aïder, 2014). Consequently, the current intensities used for higher concentrations (14 and 21%) and volume (350 mL) of whey were not adequate to create enough alkalinity for isomerization in the present study. In addition, the concentration of the electrolyte (0.5 M Na_2SO_4) in the central compartment used by Kareb et al. (2016b) was higher compared to that of the present study (0.1 M K_2SO_4), which could be another reason for anomalies in lactulose production. Therefore, it can be deduced that the higher volume, higher distance between the CEM and cathode, and other geometrical factors affected the lactulose production by using EA for 14 and 21% whey as feed solutions.

4.3.6 Conventional chemical isomerization using KOH as catalyst

The conventional chemical isomerization reactions were performed at an ambient temperature by producing equivalent alkalinity as those obtained in the EA using similar whey concentrations (7, 14, and 21%). The evolutions of pH and ORP were studied during the chemical isomerization and are presented in **Figures 4.11** and **4.12**. A significant difference between chemical isomerization and electroisomerization was observed regarding the pH and ORP evolution. In chemical isomerization, the pH never reached 8 (pH < 8), except for the equivalent alkalinity as for EA under the current intensities of 600 and 900 mA for the 7% whey solution. For equivalent alkalinities of 600 and 900 mA, the maximum pH values of 10.26 ± 0.02 and 10.48 ± 0.01 were achieved after 50 and 40 min, respectively. However, it is noteworthy that no lactulose but only some galactose and glucose (in some cases) was produced by chemical isomerization reactions (**Table A6** and **Figure A5**).

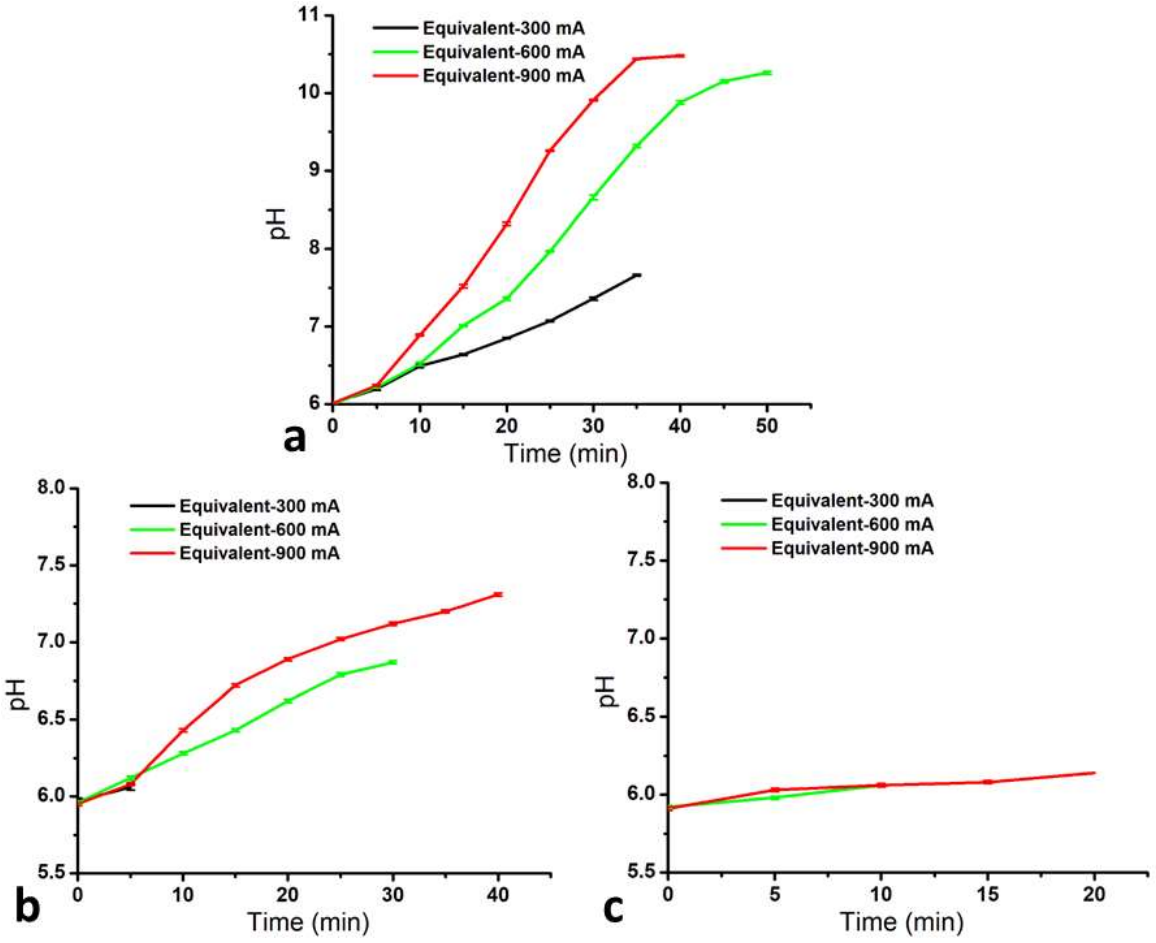

Figure 4.11: Evolution of pH as a function of reaction time in the chemical isomerization for (a) 7%, (b) 14%, and (c) 21% whey solutions.

In chemical isomerization, a higher temperature and time were typically required to achieve lactulose. Hashemi and Ashtiani (2010a) reported that a maximum lactulose of 11.8 ± 5.1% could be obtained with a pH of 10 and 50 ℃ temperature using a 10% lactose solution and 1.0 M NaOH as catalyst; however, a long reaction time of 300 min was required. They observed that by increasing the pH and temperature to 11 and 70 ℃, an optimum production of 25.4 ± 0.4% could be achieved in 60 min; then again, the higher pH and temperature led to the degradation of lactose and lactulose into many byproducts like epilactose, glucose, galactose, and other acidic products. In the present study, the chemical isomerization was carried out in the ambient temperature for a short duration, which seems to be a reason why lactulose was not produced. However, in our previous study, it was found that lactulose can be produced without external heating with a high pH (>10.00) (Karim and Aider, 2020b). It is worth mentioning that all of the above-mentioned reports used lactose as feed solution, whereas whey was used in our study. Thus, it is obvious that higher activation energy was required for the isomerization reaction for whey than for lactose because it contains proteins, peptides, and other components with high buffering capacity. Consequently, a higher alkalinity was needed to induce an isomerization reaction. Moreover, a higher amount of catalysts was required to produce an adequate level of alkalinity when whey was used as feed solution. In a recent study, Seo et al. (2015b) produced a high alkalinity of 48.11 mmol/L in the cheese whey solution by using high amounts of 5.1 g/L or 0.51% Na_2CO_3 as catalyst to obtain only 3-4% lactulose at 60 ℃. However, they achieved an optimum lactulose yield of 29.6% by increasing the temperature up to 90 ℃. In another study, Seo et al. (2016b) used 7.6 g/L or 0.76% $(NH_4)_2CO_3$ as catalyst to produce an alkalinity of 79.09 mmol/L, using cheese whey and obtained a maximum yield of 29.6% lactulose at 97 ℃ and 28 min. In contrast, in the present study, only 1.91 and 2.02 g/L KOHs was required to produce the maximum alkalinities of 34 and 36 mmol/L as corresponding to EA under 600 and 900 mA for the 7% whey solution. This could be one of the main reasons why lactulose was not formed in this study using chemical isomerization.

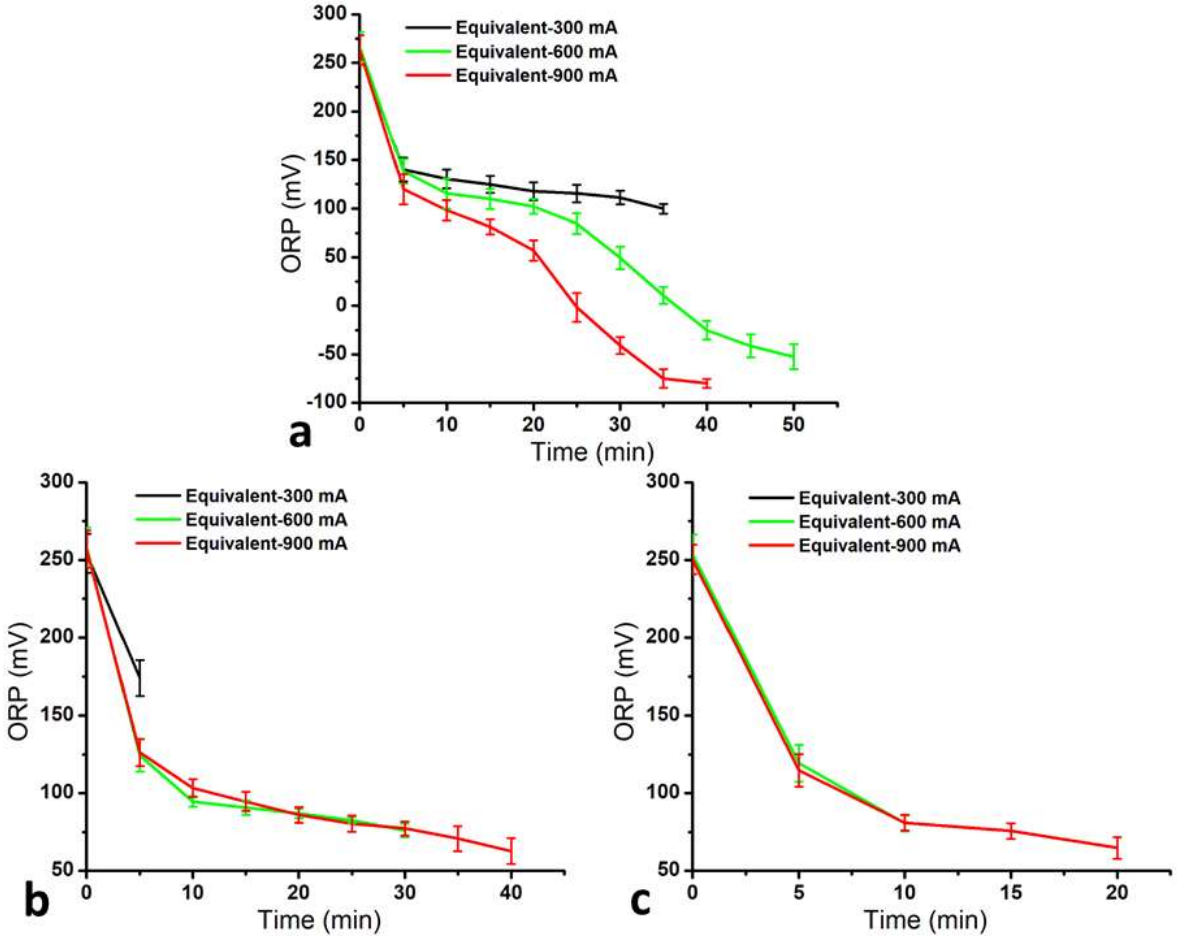

Figure 4.12: Variation of ORP as a function of reaction time in the chemical isomerization process for (a) 7%, (b) 14%, and (c) 21% whey solutions.

It is important to mention here that the ORPs in the chemical isomerization were reduced only to a value of around +150 to +100 mV within the first 5 min and reached a maximum value of around +50 to −100 mV at the end of reactions depending on different reaction conditions (**Figure 4.12**). Contrary to chemical isomerization, the ORP values reached at around −500 to −600 mV whatever the solution concentration and current intensity used; thereafter, they achieved a value of −650 to −850 mV depending on the solution concentration and current intensities and remained almost steady during the EA process (**Figure 4.7**). The highly reduced ORPs in the EA process rendered the whey solutions highly reactive because the electric field triggered the feed solutions to a metastable state (Aider et al., 2012b, Karim and Aider, 2020b). Consequently, the isomerization reactions in the EA were probably intensified by a higher internal potential energy of the activated solution. This could be another reason why an isomerization reaction could not

occur in the chemical isomerization, although the same alkaline conditions were generated by KOH as catalyst. Besides ORP, other physical and chemical factors such as solution alkalinity, pH, temperature, and ion migration in the EA technique may possibly facilitate the feed solution to achieve such conditions, in which the activation energy required for the isomerization of whey lactose into lactulose would significantly be reduced. Thus, in contrast to the conventional chemical method, the EA technique offers a high potential to produce lactulose by using whey as a lactose source. Moreover, the difference between the ORP values of the chemical method *versus* the EA method can be explained as follows: during the electroactivation (EA) process, water electrolysis at the cathode interface generates two main components: OH^- which is responsible for the medium alkalinization, and hydrogen (H_2) gas, which is a strong reducing agent. Thus, the ORP in the EA process was highly reduced. In the case of the chemical method, the addition of KOH had only an effect on solution pH. Thus, even at equivalent solution alkalinity, the ORP in the EA process was very much higher than that of the chemically alkalinized whey solutions.

4.4 Conclusions

To sum up, the EA process could be an environmentally friendly and sustainable method for producing prebiotic lactulose through the isomerization of whey lactose because it can be performed under complete autocatalytic conditions, meaning that alkalinizing chemicals and external heating are not required. In this present study, a maximum lactulose yield of ~32% was achieved under a 900 mA current intensity at 60 min of EA for the 7% whey solution. The results suggest that the formation of lactulose was dependent on the whey concentration, current intensity, and EA time. Furthermore, no lactulose was produced in the chemical isomerization although equivalent alkalinity was created as in the EA. Thus, it is obvious that other process mechanisms of action were involved with the EA technique to achieve the required alkaline conditions. However, despite some meaningful achievements of EA, an ideal optimized condition must be developed that is more economical in terms of the geometry and configuration of the reactor and the type and concentration of the feed solutions. Moreover, the electrical conductivity of the used whey was 5.27 ± 0.12 mS/cm, which is enough to allow the passage of the electric current in the EA reactor. This means that whey can be used in all of the compartments of the EA reactor instead of electrolytic solutions. However, because whey contains some chlorine-containing salts, supplementary

protecting conditions must be considered to avoid chlorine accumulation in the working environment. This can be ensured by connecting the EA reactor to an adequate ventilation system.

ANNEXE B

Variation of voltage during the EA process under different current intensities using different whey solutions (**Figure A3)**; HPLC chromatograms for 14 and 21% whey solutions (**Figure A4)**; HPLC chromatograms for chemical isomerization in a 7% whey solution (**Figure A5)**; and the formation of sugars in chemical isomerization for a 7% whey solution using equivalent solution alkalinity as in the EA of different current intensities (**Table A6**).

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Chapitre 5: Contribution to the process development for lactulose production through complete valorization of whey permeate by using electro-activation technology versus a chemical isomerization process

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RÉSUMÉ

Le perméat de lactosérum (WP) est un co-produit des procédés de fabrication de fromages ou de caséine et qui est issu du lactosérum à la suite de la séparation des fractions protéiques majeures par des procédés membranaires. Il est considéré comme un polluant environnemental en raison de sa charge organique élevée et crée un problème majeur d'élimination pour l'industrie laitière. Cependant, il peut être utilisé comme substrat approprié pour répondre à la demande croissante de production de lactulose, qui est un prébiotique, par l'isomérisation du lactose présent dans le WP dans des conditions alcalines adéquates. Le but de cette étude était de produire du lactulose *in situ* de WP en utilisant la technologie d'électro-activation (EA) et de comparer l'efficacité de l'EA par rapport à l'isomérisation chimique conventionnelle en conditions équivalentes d'alcalinité en KOH titrable des solutions traitées. L'électro-isomérisation a été réalisée sous différentes intensités de courant électrique de 300, 600 et 900 mA pendant 60 min d'EA avec un intervalle d'échantillonnage de 5 min en utilisant des solutions de WP à 6, 12 et 18 % (p/v). L'isomérisation chimique a été réalisée à une alcalinité de la solution équivalente au KOH par rapport à celle mesurée dans la solution soumise à l'EA à chaque intervalle de 5 min en utilisant de la poudre de KOH comme agent alcalinisant (catalyseur). Les résultats de cette étude ont révélé que la production de lactulose à l'aide de l'approche par EA dépendait de l'intensité du courant électrique, de la concentration de WP et du temps de réaction. Cela a donné un rendement de lactulose le plus élevé de 36,98 % après 50 min de temps d'EA sous une intensité de courant de 900 mA en utilisant une solution de 6 % de WP comme solution initiale, alors qu'un rendement de lactulose maximal de 25,47 % a été obtenu par l'isomérisation chimique à alcalinité équivalente de la solution correspondant à celle de l'EA sous 900 mA d'intensité de courant après 50 min en utilisant une solution de 6% WP. De plus, un rendement supérieur en lactulose a été obtenu en utilisant la technique d'EA pour toutes les conditions de réaction par rapport au procédé chimique à l'alcalinité équivalente dans la solution. Par conséquent, les résultats de ce travail suggèrent que l'EA peut être une technologie durable émergente pour atteindre le double objectif de production de lactulose en valorisation intégrale du perméat de lactosérum.

Mots-clés: Valorisation du perméat de lactosérum; Électro-isomérisation; Isomérisation chimique; Lactulose; Alcalinité de la solution; Potentiel d'oxydoréduction; Migration d'ions; Température.

ABSTRACT

Whey permeate (WP) is a co-product of a cheese or casein production process that is regarded as an environmental pollutant because of its high organic load and is creating a major disposal problem for the dairy industry. However, it can be used as a suitable substrate to meet the increasing demand of producing a prebiotic lactulose through the isomerization of lactose present in the WP under adequate alkaline conditions. The goal of this study was to produce lactulose *in situ* of WP using electro-activation (EA) technology and compare the productivity of EA with conventional chemical isomerization at potassium hydroxide (KOH)-equivalent solution alkalinity in the feed medium. Electro-isomerization was conducted under different current intensities of 300, 600, and 900 mA for 60 min of EA with a 5 min sampling interval using 6, 12, and 18% (w/v) WP solutions. Chemical isomerization was carried out at the KOH-equivalent solution alkalinity to that measured in the EA solution at each 5 min interval using KOH powder as a catalyst. The outcomes of this study revealed that the production of lactulose using the EA approach was current intensity-, WP concentration-, and reaction time-dependent; and produced the highest lactulose yield of 36.98% at 50 min of EA-time under 900 mA current intensity using 6% WP as a feed solution, whereas a maximum lactulose yield of 25.47% was achieved by the chemical isomerization at the solution alkalinity corresponding to that of the EA under 900 mA current intensity at 50 min in the 6% WP solution. Furthermore, a greater yield of lactulose was obtained using the EA technique for all reaction conditions compared to the chemical process at the equivalent solution alkalinity. Therefore, the results of this work suggest that the EA can be an emergent sustainable technology for achieving dual objectives of prebiotic lactulose production and concurrent valorization of WP using it as a feed medium.

Keywords: Whey permeate valorization; electro-isomerization; chemical isomerization; lactulose; solution alkalinity; oxidation-reduction potential; ion migration; temperature.

5.1 Introduction

Whey permeate (WP) is the secondary co-product of the cheese and casein making industry. It is the residual material after proteins are extracted from whey by membrane filtration of precipitation (Sabater et al., 2017a, Djouab and Aïder, 2019c). Casein, which is the main protein of milk, can be precipitated by either pH lowering of heat denaturation. At pH close to 4.6, which corresponds to the isoelectric point of casein, this molecule (micelles) is least soluble and can easily precipitate as agglomerates. This precipitation by pH lowering can be achieved by adding an acid (*e.g.*, HCl or lactic acid). Casein can also be precipitated following heat treatment combined with some minerals such as calcium. The high temperature can induce casein denaturation leading to significant loss of its stability. From the point of view of its proximate composition, WP is a poor raw material and has low commercial and nutritional value. In its liquid form, it is primarily comprised water (~93%), lactose (~5%), minerals (~0.53%), and traces of nitrogen-containing molecules such as free amino acids and peptides (Chandan et al., 1982, Djouab and Aider, 2019). From environmental considerations, WP constitutes a serious concern because of its high biological (BOD ≈ 30,000-50,000 mg/L O_2) and chemical (COD ≈ 60,000-80,000 mg/L O_2) oxygen demand. This particularity can easily cause eutrophication of different ecosystems because of its impact on oxygen depletion of water (Parashar et al., 2016b, Sabater et al., 2017a). Thus, WP is still a huge challenge for researchers and industries to find rational, efficient, and economical ways of valorization.

WP is mainly dried and sold as a powder for some applications as a feed additive or used as a raw material for lactose production by cold crystallization of saturated solutions after being purified from minerals and N-containing molecules. The processes of drying, demineralization, and purification are costly, which make the end-product of low commercial value. Lactose is then used in its turn as a raw material to produce high costly derivatives such as sorbitol, mannitol, ethanol, lactobionic acid, and other derivatives. Thus, it can be seen that the industry is multiplying different processes to achieve a product with relatively good commercial value but which is highly expensive if one considers the overall product life cycle (Yadav et al., 2015b).

Analysis of the current situation of WP pointed out a necessity to investigate other nontraditional ways of valorization. In this context, a sustainable approach was highlighted

in the present study with a possibility of valorizing WP as a whole component by adding a net positive added value to this material from both the nutritional and the environmental and economic points of view (Yadav et al., 2015b). Thus, by following this innovative way, it would be possible to produce a highly valuable food grade ingredient by the whole valorization of WP by targeting specific modifications without any need of fractionation of the initial material. The target is the effective conversion of lactose (present in the WP) into lactulose, a recognized prebiotic with many applications in the pharmaceutical and food industries (Panesar and Kumari, 2011a, Djouab and Aïder, 2019c). Thus, it will be possible to convert WP into value-added ingredient rich of lactulose and possibly other valued molecules and minerals.

Lactulose is classically produced following the chemical isomerization of lactose under strong alkaline conditions requiring heat and catalysts (Montgomery and Hudson, 1930a, Speck Jr, 1958b). This process is generally costly because of the low process efficiency caused by the necessity of different purifying and concentrating steps (Aider et al., 2012b, Seo et al., 2015a). To overcome the inconveniencies of the chemical isomerization process of lactose into lactulose, recently electro-activation (EA) technology has been shown to be highly effective to convert lactose into lactulose by exploiting water electrolysis at the cathode-solution interface in an adequately designed EA reactor. The reaction of water electrolysis combined with adequate ion exchange membrane disposition permits to create strong alkaline conditions without using chemical alkalinizing agents and catalysts (Djouab and Aider, 2019, Karim and Aider, 2020a). Right now, the reported studies showed a conversion of lactose into lactulose to be in the range of 35-45% which is considered to be very good and highly promising for large scaling of the EA process (Aissa and Aïder, 2014a, Djouab and Aider, 2019, Karim and Aider, 2020a). However, because of the fundamental and process design differences between the chemical and EA methods of lactose isomerization into lactulose, it is difficult to state which of these processes is the most efficient. Thus, a structured and objective comparison of the isomerization of lactose into lactulose using a conventional chemical process versus the method based on the EA technology (electro-isomerization) for WP integral valorization is necessary, which is the key objective of the present study.

Specifically, this study was intended to compare the efficiency of lactose isomerization into lactulose between the EA process and the chemical method based on the equivalent solution alkalinity using potassium hydroxide (KOH) as a catalyst and WP as a substrate of the isomerization process.

5.2 Materials and methods

5.2.1 Reagents and chemicals

Highly pure (purity ≥ 95%) reagents and chemicals were purchased from various suppliers. Lactulose, lactose, galactose, glucose, and fructose of high-performance liquid chromatography (HPLC)-grade were acquired from Sigma-Aldrich (Ottawa, Ontario, Canada). The analytical grade hydrochloric acid (HCl), potassium sulfate (K_2SO_4), phenolphthalein ($C_{20}H_{14}O_4$), and potassium hydroxide (KOH) were purchased from Fisher Chemical (Geel, Belgium), Sigma-Aldrich Co. (St. Louis, MO, USA), Fisher Chemical (Fair Lawn, NJ, USA), MAT Laboratory Inc. (Quebec, Canada), respectively. The WP powder was procured from Agropur Co-operative (Longueil, Quebec, Canada). The major components (%) of WP are as follows: total sugars: 85 ± 0.12; total proteins: 1.93 ± 0.24; ash: 6.5 ± 0.11; residual humidity: 6.4 ± 0.13; other components: 0.07 ± 0.01. The AMI-7001S anion-exchange membrane (AEM) and the CMI-7000S cation-exchange membrane (CEM) were obtained from Membrane International Inc. (Ringwood, NJ, USA).

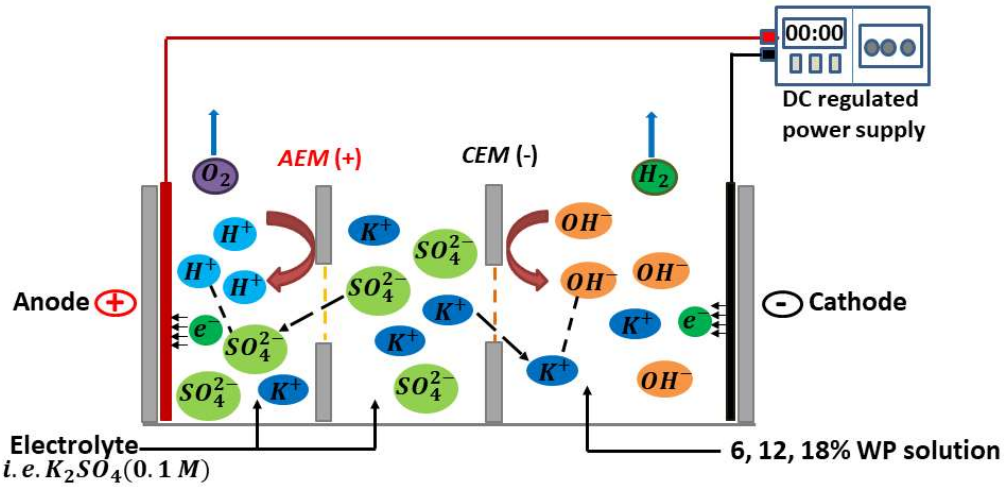

Figure 5.1: Schematic of the EA reactor used in this study for electro-isomerization of lactose into lactulose *in situ* of WP.

5.2.2 Protocol for electro-activation

The EA reactor used in this work consists of three chambers, namely, anodic (5.5 × 2.2 × 10 cm), central (5.5 × 2.2 × 10 cm), and cathodic (6.5 × 5.5 × 10 cm) compartments (**Figure A6**). The cathodic compartment was connected to the negative side of a direct current (DC) CSI12001X power generator (Circuit Specialists, Tempe, AZ, USA) through a stainless steel (food grade) electrode (12 × 5 cm), while the anodic compartment was tied to the positive side using a ruthenium-iridium-coated titanium electrode (12 × 4 cm). The central compartment divided the cathodic and anodic compartments, and it was communicating with them through the CEM and AEM, respectively. The 350 mL of WP solutions (6, 12, and 18% w/v) was introduced in a cathodic chamber, while the central and anodic chambers were loaded with the solution of 0.1 M K_2SO_4 (**Figure 5.1**). The solution of K_2SO_4 was used as an anolyte in the EA reactor to avoid chlorine formation at the anode surface following the oxidation reaction. The EA experiments were performed under different current intensities (*i.e.*, 300, 600, and 900 mA) for a reaction time of 60 min at ambient temperature (22 ± 2 °C). Aliquot samples were collected from the cathodic and central chambers in a regular interval (*i.e.*, 5 min) and preserved at 4 °C for further analysis.

5.2.3 Assessment of pH, alkalinity, potassium ion, temperature, and oxidation-reduction potential

The WP solution pH, total alkalinity, oxidation-reduction potential (ORP), and temperature prior to and during the EA process, were evaluated at 5-min intervals during the 60-min of EA. An Oakton pH 700 pH meter equipped with a standard pH probe (Oakton, Vernon Hills, IL, USA) was used to determine the medium pH while an Ultrapen ORP meter (Myron L Company, Carlsbad, CA, USA) was used to measure ORP and temperature. The concentration of the potassium ion (K^+) was measured in a central chamber in 5-min intervals using an AAnalyst 200 atomic absorption spectrometer (PerkinElmer Instrument, Boston, MA, USA). The total alkalinity of the electro-activated WP solutions *(i.e.*, catholytes) was evaluated using a standard titration method with phenolphthalein as an indicator according to Karim and Aider (2020a). The total alkalinity of the solutions was determined following **Eq. 5.1** and was expressed in mmol/L.

$$Total\ Alkalinity_{eq} = \frac{C_{titrant} \times V_{titrant} \times 1000}{V_{sample}} \quad \text{(Eq. 5.1)}$$

where, $C_{titrant}$ is the concentration (in mol/L) of the titrant (*i.e.*, HCl); $V_{titrant}$ is the total volume (in mL) of the titrant, that is, 0.1 M HCl required for titration; V_{sample} is the total volume (in mL) of the sample; and the total alkalinity $_{eq}$ is the equivalent concentration of KOH/NaOH in mmol/L.

5.2.4 Chemical isomerization by KOH

The conventional chemical isomerization process was conducted using similar WP solutions (6, 12, and 18%; w/v) as a feed medium. The isomerization reactions were carried out by creating an equivalent solution alkalinity corresponding to that obtained in the EA process by adding KOH as a catalyst to the WP solution. Briefly, the equivalent amount of KOH (mg/L) corresponding to the total solution alkalinity using the EA process was determined each 5 min interval. Thereafter, the KOH powder was added in 5-min intervals to achieve an equivalent solution alkalinity as the one formed during the EA process. The feed medium was constantly mixed at an ambient temperature and the medium pH and ORP were measured during the isomerization reaction. The samples were collected at regular 5-min intervals and maintained at 4 ℃ for further analysis.

5.2.5 Analysis of carbohydrates composition

The analysis of the carbohydrate composition of electro-activated WP was performed by using a Hitachi L-7000 HPLC system series (Hitachi High-Tech, Japan). A Waters refractive index detector and a Sugar Pak-I 300 × 6.5 mm carbohydrate analysis column (Waters Corp., Milford, MA, USA) were used in the HPLC system. A solution of 50 mg/L Ca-ethylenediamine tetra-acetic acid (50 mg/L) was used as a mobile phase at a flow rate of 0.5 mL/min. The column temperature was maintained at 80 ℃. The running time and injection volume were set to 30 min and 50 µL per sample, respectively. The composition of the targeted carbohydrates (lactose, lactulose, glucose, galactose, and fructose) were identified and quantified comparing their retention times with the corresponding standard solutions.

5.2.6 Statistical analysis

Statistical analysis was carried out following a complete randomized factorial design with repeated measurements. The studied independent variables were WP concentration (6, 12, and 18%), electric current intensity (300, 600, and 900 mA), and reaction time (5-60 min at 5 min interval). The dependent variables were the electro-activated WP solution pH, total

alkalinity, ORP, temperature, and migration of K^+ ions from the central chamber to the catholyte, the yield of lactulose and other carbohydrates (residual lactose, glucose, galactose, and fructose). The experiments were repeated three times and the mean values ± standard deviations were considered for the analyses. The differences at $p < 0.05$ were termed as significant. Analysis of variance (ANOVA) of the obtained data was conducted by using SAS software V9.3 (SAS Institute Inc., Cary, NC, USA).

5.3 Results and discussion

5.3.1 Evaluation of medium pH and alkalinity

The evolution of medium pH in the cathodic compartment was assessed during the 60-min EA process under several current intensities (*i.e.*, 300, 600, and 900 mA) using WP solutions (*i.e.*, 6, 12, and 18%) and presented in **Figure 5.2**. It appeared that the current intensity, solution concentration, and EA time significantly affected ($p < 0.001$) the pH evolution during electro-isomerization of WP. The higher pH was achieved for 6% WP solution than for the 12 and 18% WP solutions and reached the maximum values of 10.86 ± 0.04 and 11.36 ± 0.02 at 60 min under 300 and 600 mA current intensity, respectively. However, it reached a plateau (11.37 ± 0.06) at 45 min, and then it decreased to 11.22 ± 0.06 at 60 min when 900 mA current intensity was used. Likewise, the maximum pH of 9.92 ± 0.04 and 10.90 ± 0.03 was obtained under 300 and 600 mA for 12% WP solution, respectively; while it reached plateau (10.90 ± 0.02) at 50 min before slightly deceased to 10.86 ± 0.02 at the end (*i.e.*, 60 min) of the EA process under 900 mA current intensity. However, this phenomenon was not observed for 18% WP solution rather a pH reached maxima of 8.33 ± 0.05, 10.47 ± 0.08, and 10.70 ± 0.02 at the end of the reaction under 300, 600, and 900 mA current intensity, respectively.

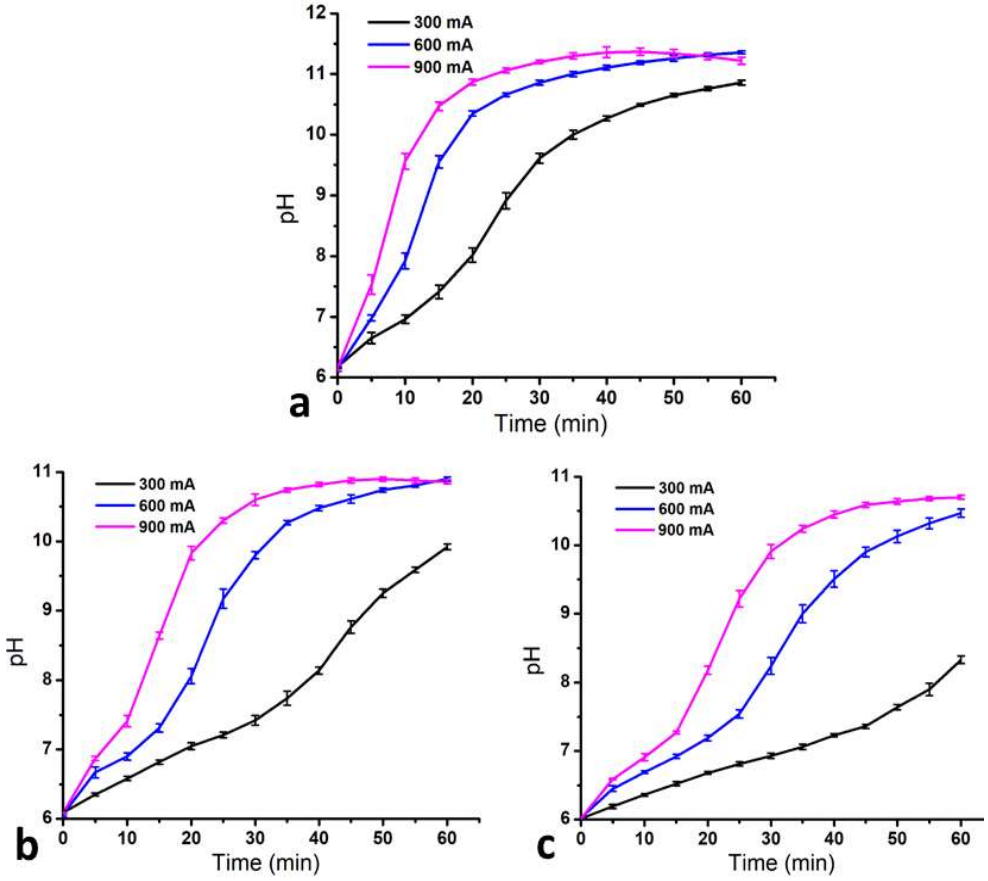

Figure 5.2: Evolution of pH in the cathodic chamber during the EA under various current intensities for (a) 6, (b) 12, and (c) 18% WP solutions.

The evolution of solution alkalinity expressed as KOH-equivalent was also measured during the EA process under 300, 600, and 900 mA current intensities using various WP solutions (6, 12, and 18%), as shown in **Figure 5.3**. Like solution pH, the development of solution alkalinity was significantly ($p < 0.001$) affected by the solution concentration, current intensity, and EA time and showed a strong correlation with pH evolution. Alkalinity was observed to increase with rising current intensity and reaction time, while it was seen to decrease for the higher solution concentrations. The maximum alkalinity was achieved for 6% WP solution, and it reached 20.67 ± 1.15 and 38.00 ± 2.00 mmol/L at a reaction time of 60 min under current intensities of 300 and 600 mA, respectively. Nevertheless, the solution alkalinity achieved a plateau of 40.00 ± 1.46 mmol/L at 45 min under 900 mA, and then it decreased to 36.67 ± 1.15 mmol/L at 60 min. For 12% WP solution, the utmost alkalinity of 7.33 ± 1.15 and 30.00 ± 0.00 mmol/L were obtained at the end, while it reached a plateau (32.67 ± 1.15 mmol/L) at 50 min and remained unchanged until the end. A maximum of 2.67

± 1.15, 21.33 ± 1.15, and 32.00 ± 2.00 mmol/L alkalinity were observed under 300, 600, and 900 mA at the end of EA for 18% WP solution, respectively.

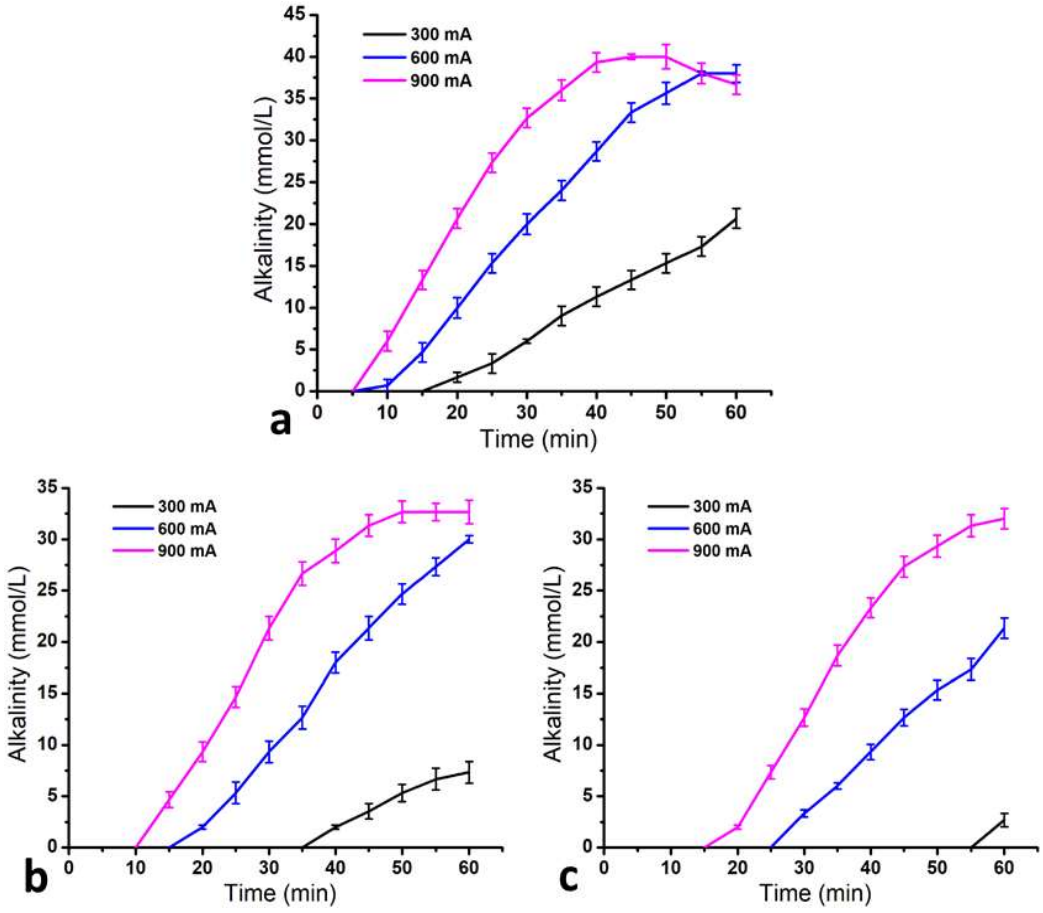

Figure 5.3: Development of solution alkalinity in the cathodic chamber during the EA of (a) 6, (b) 12, and (c) 18% WP solutions.

The higher pH and alkalinity were achieved for greater current intensity whatever the solution concentration was used. The concentration of KOH increased as the EA process progressed because of the continuous formation of OH^- ions in the cathodic compartment and the electro-migration of K^+ ions from the central compartment to the cathodic compartment of the EA reactor. Thus, the simultaneous increase of OH^- and K^+ ions yielded an increase of the KOH concentration in the cathodic side as the EA process progressed. Moreover, this phenomenon was dependent on the applied electric current intensity. Also, this is because the movement of electrons (e^-) through the electro-chemical system was enhanced because of the application of higher current intensity (Fiegenbaum et al., 2013a). As a result, a greater

splitting of water molecules was instigated at the solution-electrode interface which resulted in the generation of more hydroxyl ions (OH^-) in the cathodic chamber. Indeed, a reduction reaction transpired in the cathodic chamber allowing the generation of hydrogen gas (H_2) and OH^- ions through intensive water dissociation [$2H_2O$ (l) + $2e^- \rightarrow H_2$ (g) +$2OH^-$ (aq.)]. The rate of water electrolysis is directly proportional to the applied electric field. Subsequently, the OH^- ions were increased in the medium that led to the increase of the solution pH and alkalinity (Karim and Aider, 2020a). At the very beginning of the EA, the rate of pH increment was sluggish, and it was more apparent for higher WP concentrations. This is probably due to the initial resistance of the feed solutions and the resistance of the system for its intrinsic resistance of the ion-exchange membranes used (Cifuentes-Araya et al., 2011a). Thereafter, pH and alkalinity increased exponentially because of the continuous production of the high amounts of OH^- ions following rigorous water splitting for permitting the current transmission in the solution-cathode interface (Djouab and Aïder, 2019c). In the third stage, the rate of pH increase showed an incurring behavior with a lower rate of pH increase, indicating that it reached a plateau because the solution become saturated with enough OH^- ions (Karim and Aider, 2020a).

The lower rise of solution pH and alkalinity for higher solution concentrations (6 > 12 > 18% WP solution) ascribed to the higher buffering capacity of the more concentrated WP solutions that was retarding the pH evolution in the medium by absorbing/desorbing the OH^- ions (Kareb et al., 2016a). Furthermore, the low rate of water electrolysis for higher WP solutions might be owing to the greater concentration (*i.e.*, greater solid/water ratio) of WP, thus a lower quantity of OH^- ions were created at the solution-cathode interface. In some cases, particularly, for 6% WP solution under 900 mA, pH and alkalinity were found to decrease after a duration of 45 min of EA attributed to the migration of some H^+ ions to the cathodic chamber from the central chamber, and perhaps inducing acidification of the feed medium. Indeed, more H^+ and OH^- ions would have been produced through water dissociation at the solution-CEM interfaces, especially, at the interface that is facing the central chamber when the reaction reached a limiting current density, to compensate ion depletion in the central chamber (Karim and Aider, 2020a).

5.3.2 Evaluation of potassium ion migration

During the EA, the concentration of K^+ ion in the central chamber was studied under several current intensities (*i.e.*, 300, 600, and 900 mA) using 6, 12, and 18% WP solutions and is demonstrated in **Figure 5.4**. The concentration of the K^+ ion was declining over running time no matter what the current intensities and solution concentrations used. The decreasing rate was comparatively higher for greater current intensities and slightly lower for more concentrated WP solutions.

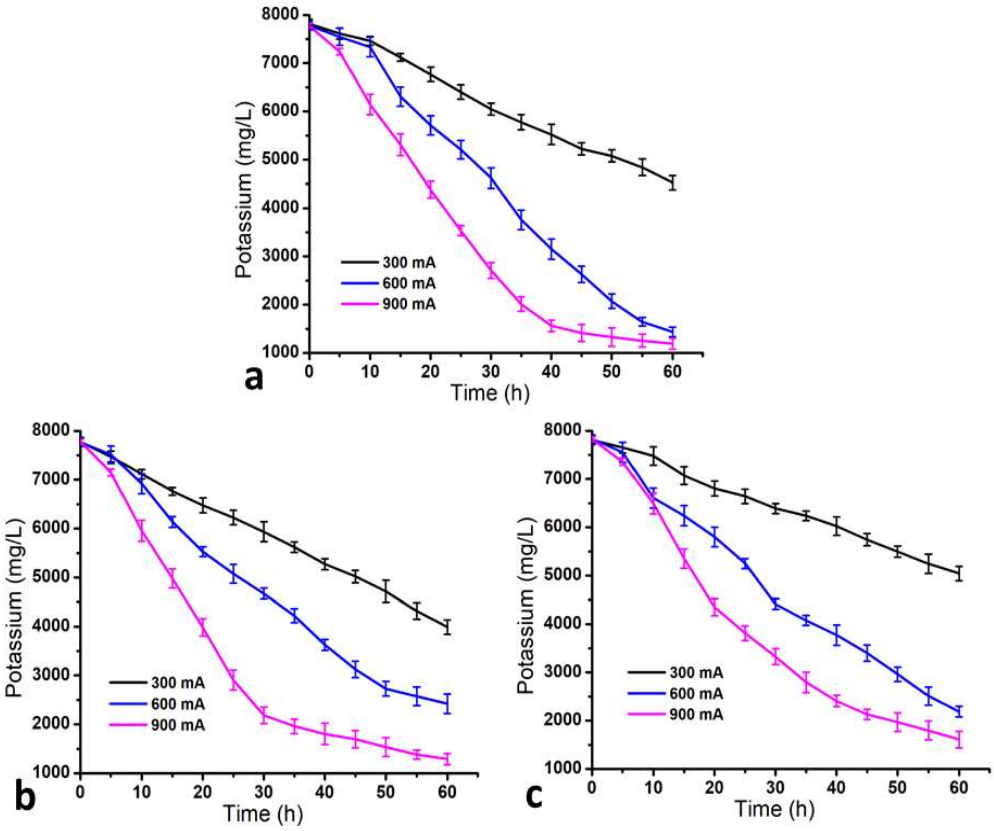

Figure 5.4: Evolution of potassium ion concentration in the central chamber during the EA of (a) 6, (b) 12, and (c) 18% WP solutions.

The concentration of K^+ ions decreased in the central chamber over running time because of the migration of positively charged K^+ (cations) toward the negatively charged cathode through the CEM. The migration rate was more rigorous at the beginning of the EA and slowed down at the end which could be ascribed to the concentration polarization phenomena. Indeed, the concentration polarization phenomena was generated because of a variation between ion transfer numbers in the membrane and solution, leading to a change in

the electrolyte concentration near the membrane surface and a significant potential drop in the polarized region (*i.e.*, Nernst layer), consequently, diminishing migration of ions (Cifuentes-Araya et al., 2011a). At this stage, the global system resistance of the reactor would have increased (**Figure A7**), and water splitting might be ensued at the CEM-solution interfaces to recompense the deficiency of current exporters toward the cathode. As a result, more OH^- and H^+ ions would have been generated, and H^+ ions competed with the K^+ ions for electro-migration toward the cathodic side because of the higher electrophoretic mobility of H^+ ions in solution (Karim and Aider, 2020a). Thus, the migration of K^+ ions was decelerated at the end point of the EA technique, particularly, for the 900-mA current intensity.

5.3.3 Temperature evolution in the EA reactor

The evolution of temperature in the cathodic chamber was monitored throughout the EA process using several WP solutions (6, 12, and 18%), as shown in **Figure 5.5**. The increase in the temperature was mainly reliant on the current intensity and EA-time throughout the 60 min of EA, and it steadily rose till the end for all current intensities and WP concentrations used.

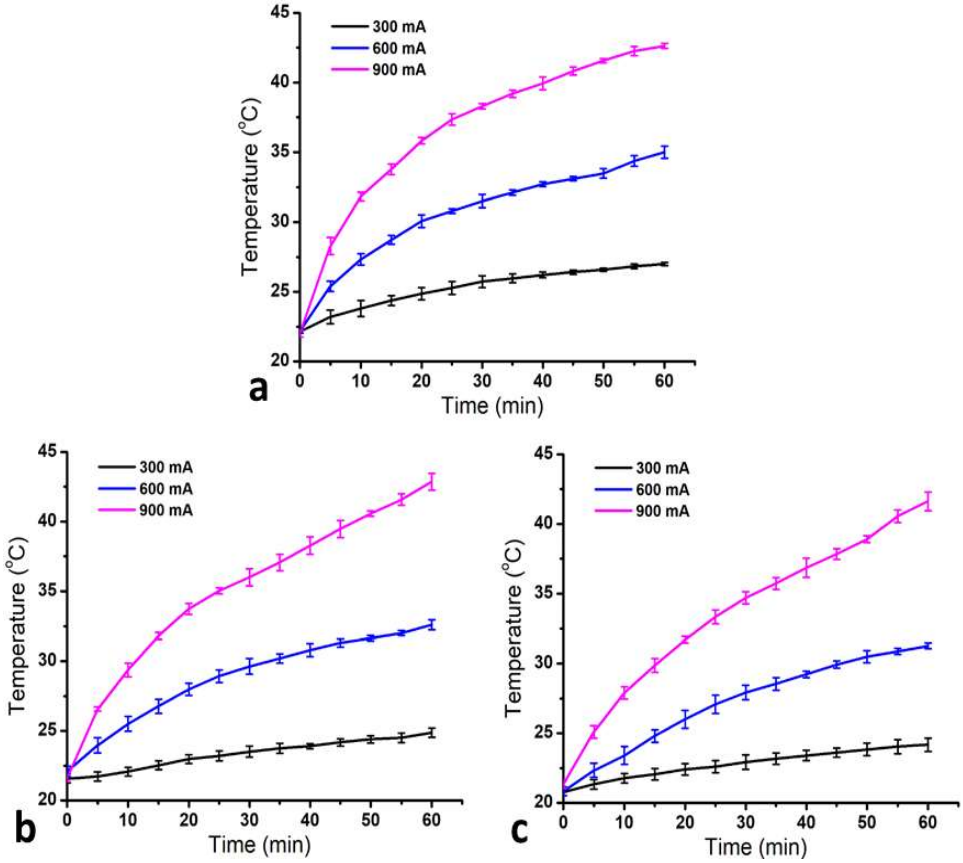

Figure 5.5: Variation of temperature in the cathodic chamber during the EA under different current intensities for (a) 6, (b) 12, and (c) 18% WP solutions.

A relatively higher temperature was observed for 6% WP solution whatever the current intensities used. The maximum values of temperature were 27.00 ± 0.10, 35.00 ± 0.44, and 42.63 ± 0.15 °C for 6% WP at the reaction time of 60 min for 300, 600, and 900 mA current intensities, respectively. The highest temperature was achieved under a current intensity of 900 mA no matter what solution concentrations were used and reached the maximum values of 42.63 ± 0.15, 42.87 ± 0.60, and 41.63 ± 0.67 °C at 60 min for 6, 12, and 18% WP solutions, respectively. The increase in temperature was considerably sharp for higher current intensities for the reason that the greater intensities produced increased heat in the medium and the heated energy dissipated in the solution. In fact, the rise in the temperature during the EA process is mostly due to the Joule effect in the electrodes, and based on the Joule's law: "the rate of increment is directly proportional to the electric current and electric tension" (Aissa and Aïder, 2013b). The Joule heating at the electrodes occurs in any electrochemical device. Indeed, applying electric current to an electrode (anode and

cathode) dissipates heat to the surrounding solution according to Joule's law, leading to an interfacial temperature that is much higher than that of the bulk solution. This phenomenon is known as the interfacial Joule heating effect (Pei et al., 2019). Furthermore, the demineralization phenomena as a result of ion migration resulted in the intensified resistance of the system that could be another possible reason for the temperature rise in the EA (Karim and Aider, 2020a). Additionally, the initial resistance of the feed solutions and ion-exchange membranes may have, to some extent, contributed to the temperature increase (Cifuentes-Araya et al., 2011a).

5.3.4 Evolution of oxidation-reduction potential

The change of ORP in the cathodic chamber was evaluated throughout the EA process using 6, 12, and 18% WP solutions and is presented in **Figure 5.6**. The ORP values were decreased drastically at the beginning of EA and reached -450 to -650 mV in the first 5 min of EA process no matter what the current intensities and WP concentration used. Later, they reduced in a different way depending on the current intensities and WP concentrations, and then achieved a quasi-steady state.

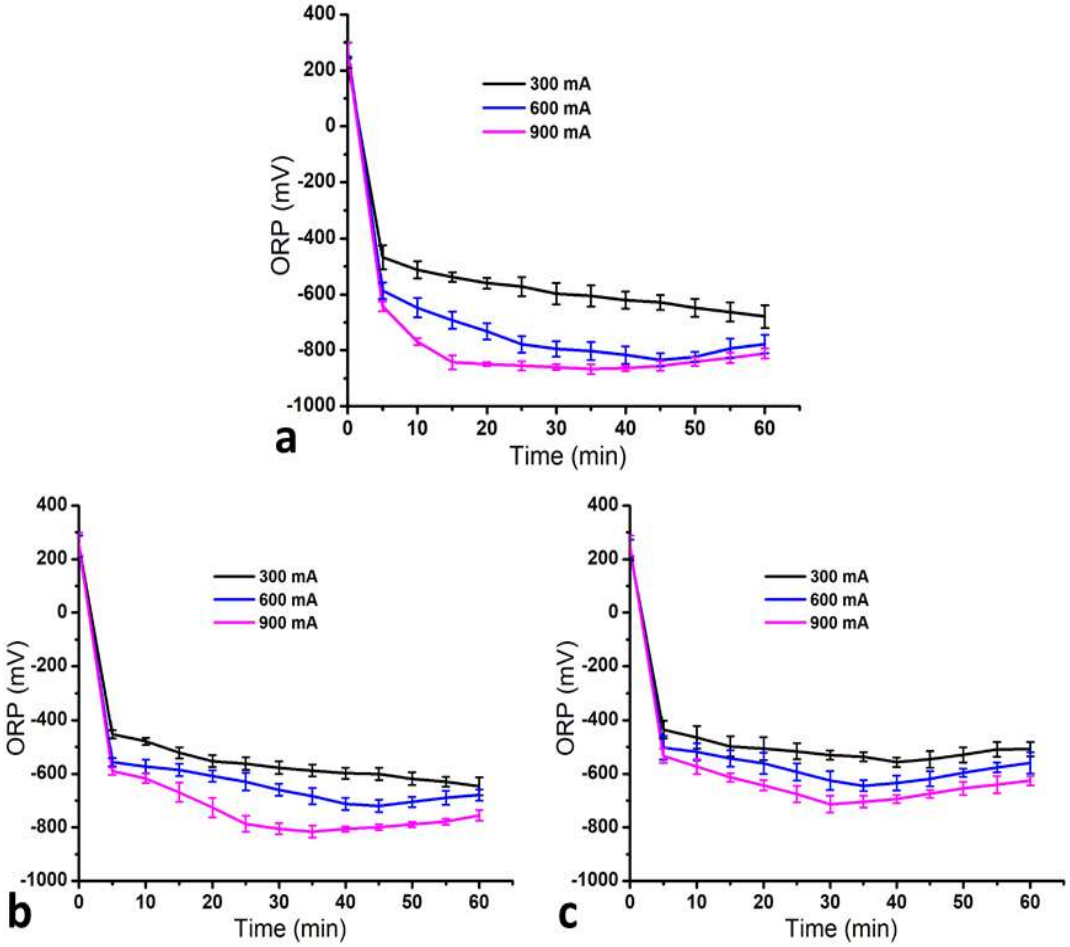

Figure 5.6: Variation of ORP in the cathodic chamber under different current intensities during the EA of (a) 6, (b) 12, and (c) 18% WP solutions.

The ORP drastically reduced within the first 5 min ascribed to the excessive e^- generation and other active reducer formation resulted from rigorous electrolysis of the solution (Karim and Aider, 2020a). Indeed, a negative ORP in the cathodic electro-activated solutions perhaps correlated to the training effect of excess e^- that produced after the electro-chemical activation of the WP solutions (Podkolzin et al., 2001b). Besides, being a dynamic electrochemical process, EA caused the production of many radicals and ionic species such as the generation of extremely active reducers including OH^-, H^-, $\cdot OH$, $\cdot H$, $\cdot O^-$, $\cdot O_2^-$, $H_2O_2^-$, and $\cdot HO_2^-$, leading to the high reduction potentials (Aider et al., 2012b). The ORP values were greater (more reduced) for higher current intensities as the amount of water electrolysis was more vigorous for greater current intensities, and consequently, the number of negative charge concentration was increased through the accumulation of OH^- groups in the electro-

activated feed solution (Kareb et al., 2017d). After a certain period of EA, the ORP achieved a quasi-steady stage as the reaction medium turned out to be saturated with the OH^- ions and other charged species. At the end of the EA, a minor upsurge in the ORP values means decrease in reactivity, attributed to the electro-migration of H^+, H_3O^+, $H_3SO_4^+$, and so forth, toward the cathodic side from the central chamber (Karim and Aider, 2020a).

5.3.5 Formation of sugars in the electro-isomerization

The isomerization of the lactose *in situ* of WP into lactulose was determined throughout the EA process using WP solutions of 6, 12, and 18% as a feed and is demonstrated in **Figures 5.7** and **5.8**. It was noted that the formation of lactulose in the EA was substantially influenced ($p < 0.001$) by the current intensity, WP concentration, and reaction time. The production of lactulose was steadily intensified until the end in 6% WP solution for 300 and 600 mA current and achieved 23.80 ± 0.91 and 32.59 ± 1.01% lactulose at 60 min of the EA, respectively (**Figure 5.7a**). Whereas, the lactulose formation was until 50 min under 900 mA current intensity and reached a plateau (36.98 ± 1.13%), thereafter, slightly decreasing at the end of the process (35.87 ± 0.94%). The higher current intensity could generate more OH^- ions through rigorous water splitting and resulted in a high alkaline condition, which led to greater lactulose formation in the cathodic chamber during the electro-isomerization process (Kareb et al., 2016a, Karim and Aider, 2020a). It is worth saying that the lactulose production was impeded at 50 min for 900 mA current intensity because of the acidification of the solution caused by ion migration phenomena, as discussed in the Sections 3.1 and 3.2.

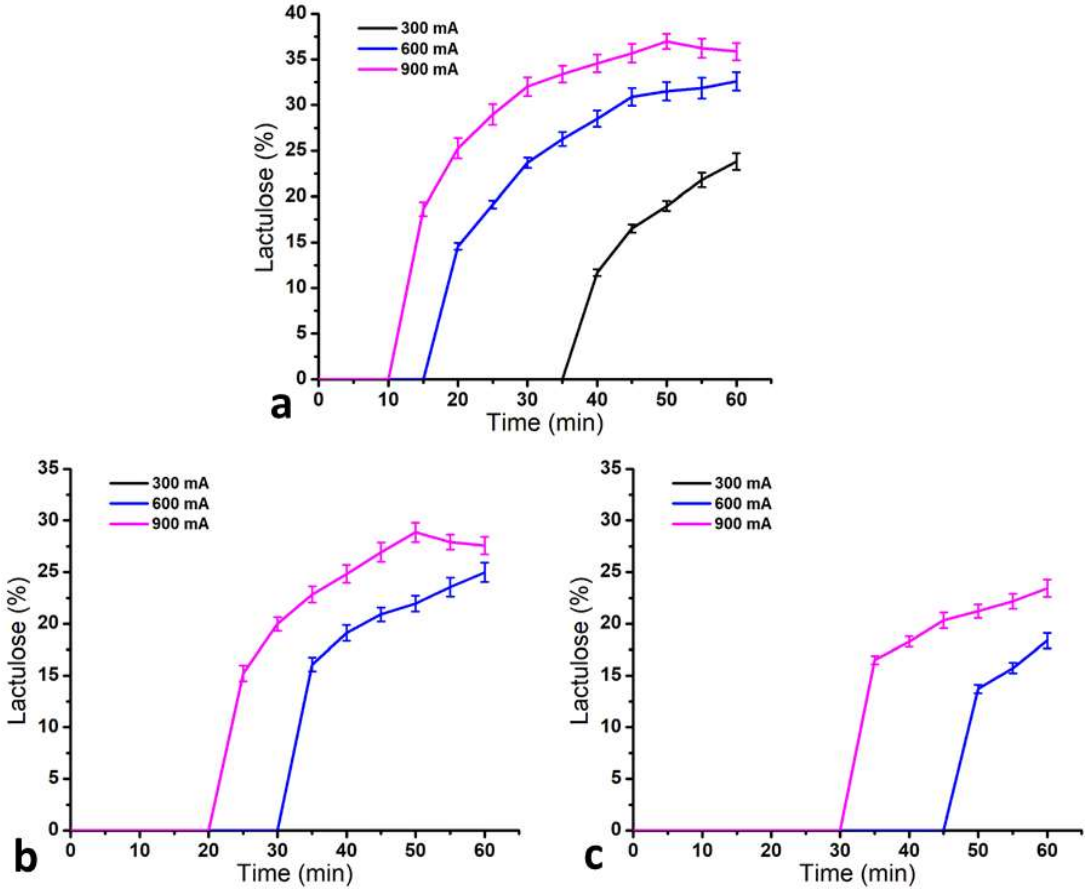

Figure 5.7: Formation of lactulose in the EA process during 60 min of EA time for (a) 6, (b) 12, and (c) 18% WP as feed solutions.

Likewise, a maximum of 28.85 ± 0.95% lactulose was produced for 12% WP solution at 50 min under 900 mA current intensity (**Figure 5.7b**) and then somewhat reduced at the end of EA (27.57 ± 0.84%). While a maximum lactulose of only 24.98 ± 0.95% was produced at the end of the EA (*i.e.*, at 60 min) under 600 mA current intensity. It can be observed that the lactulose yield of the higher WP solution (*i.e.*, 12% WP) was less than that obtained for 6% WP solution under a similar current intensity and reaction time. This might be due to the retarding pH evolution and low alkalinity in a feed solution with higher solid concentration because of a higher resistance and buffering capacity of the solution (Karim and Aider, 2020d). Indeed, the solution alkalinity and pH significantly affected ($p < 0.001$) the formation of lactulose in the EA. For 18% WP solution, the lactulose formation was started at 50 (13.72 ± 0.40%) and 35 min (16.50 ± 0.51%), afterward gradually increased to 18.37 ± 0.76 and 23.42 ± 0.84% at the end for 300 and 600 mA, respectively (**Figure 5.7c**). However, no

lactulose was produced for 300 mA in the 12 and 18% WP solutions. This could be attributed to the lack of sufficient solution alkalinity (Karim and Aider, 2020d). Adequate alkalinity is usually essential for lactulose formation because the rearrangement of the molecules for lactose isomerization into lactulose demands proton acceptors, that is, OH^-, that can be obtained by reaching a higher alkaline condition in the feed solution (Karim and Aider, 2020a).

The results achieved in the present study were corroborated with those obtained from a study by Kareb et al. (2016a), where they produced a maximum lactulose yield of 35% at 40 min of EA under a current intensity of 400 mA using 100 mL of 7% (w/v) whey solution. In another recent study, Djouab and Aïder (2019c) achieved a lactulose yield of 39.78% after 35 min of EA under 330 mA using a solution of 6% (w/v) WP in both cathodic and central compartments. However, the configuration and geometrical parameters of the reactors and the reaction conditions were quite different than those used in the present study.

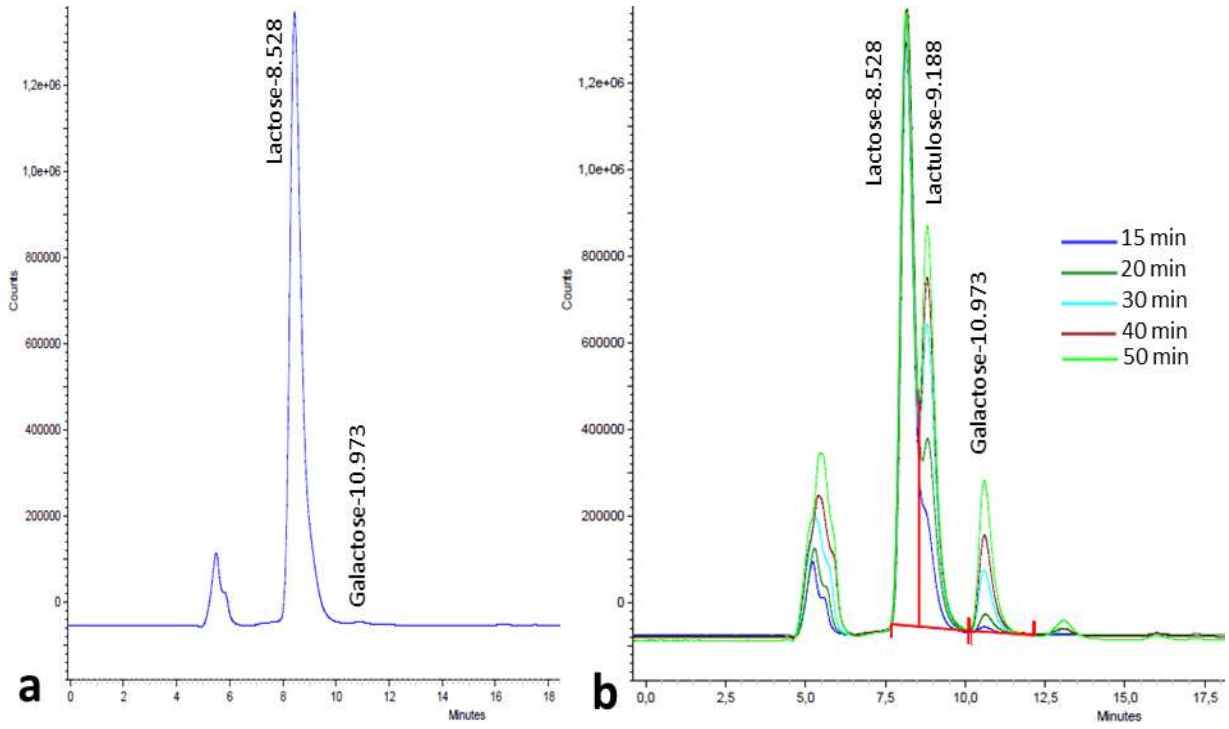

Figure 5.8: HPLC chromatograms of lactose electro-isomerization into lactulose in situ of the 6% WP solution during the EA: (a) initial feed solution and (b) feed solution under 900 mA current intensity.

Beside the lactulose production, the production of galactose was linearly augmented over running time in the electro-isomerization process because the lactulose that was

produced via electro-isomerization of lactose was later hydrolyzed into galactose. It can be noted from **Figures 5.8** and **5.9** that the maximum galactose of 1.73 ± 0.12, 7.04 ± 0.37, and 13.38 ± 0.41% was produced in the 6% WP solution for 300, 600, and 900 mA current intensities, respectively. Higher amount of galactose was formed for a greater current intensity, which might be due to the higher temperature increase in the reactor resulted from global system resistance, as discussed in the Section 5.3.3. The temperature rise in the EA reactor may probably be forcing the reaction to the other side pathways because of the formation of greater activation energy (Hashemi and Ashtiani, 2010b, Kareb et al., 2016a). However, the maximum galactose of 10.41 ± 0.37% was produced at 50 min under a current intensity of 900 mA in the 6% WP solution, while the highest lactulose of ~37% was obtained. Whereas, the commercial lactulose syrup is accepting galactose of up to 16% as per United States Pharmacopeia (USP, 2008).

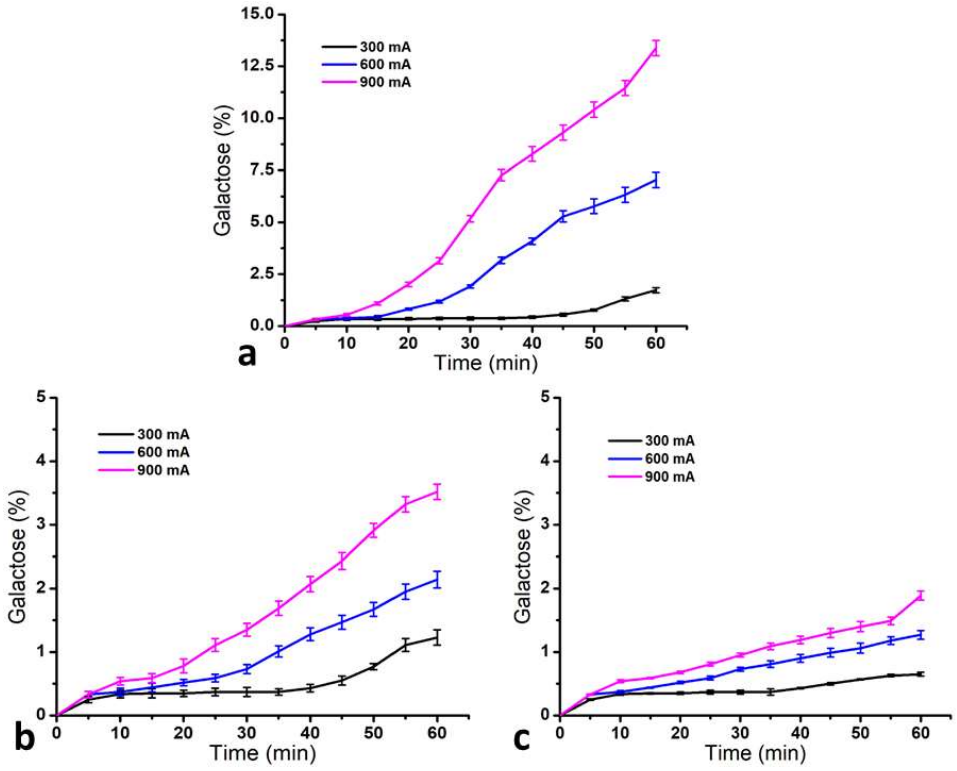

Figure 5.9: Formation of galactose in the EA process during 60 min of reaction time for (a) 6, (b) 12, and (c) 18% WP as feed solutions.

Nevertheless, only galactose was produced as a by-product in the EA reactor, and no other impurities such as tagatose, glucose, epilactose, and so forth, were observed. This finding was comparable to those obtained by Djouab and Aïder (2019c) and Karim and Aider (2020a). Djouab and Aïder (2019b) and Aissa and Aïder (2013b) argued that the only by-product during the EA of whey/lactose was definitely galactose unlike various acidic compounds and impurities in chemical isomerization. Because fructose and glucose were not observed in the medium (only trace amounts fructose in some cases), they might be isomerized into galactose as a final product as explained in a recent study by Djouab and Aïder (2019b). After all, being a more stable carbohydrate, galactose remains intact in the EA reactor (**Figure 5.10**). Therefore, the purification step in the electro-isomerization process could be simplified, which is an economical and environmental benefit for industrial application. Indeed, this is one of the most important advantages of the EA, which it is more selective compared to the conventional chemical isomerization process.

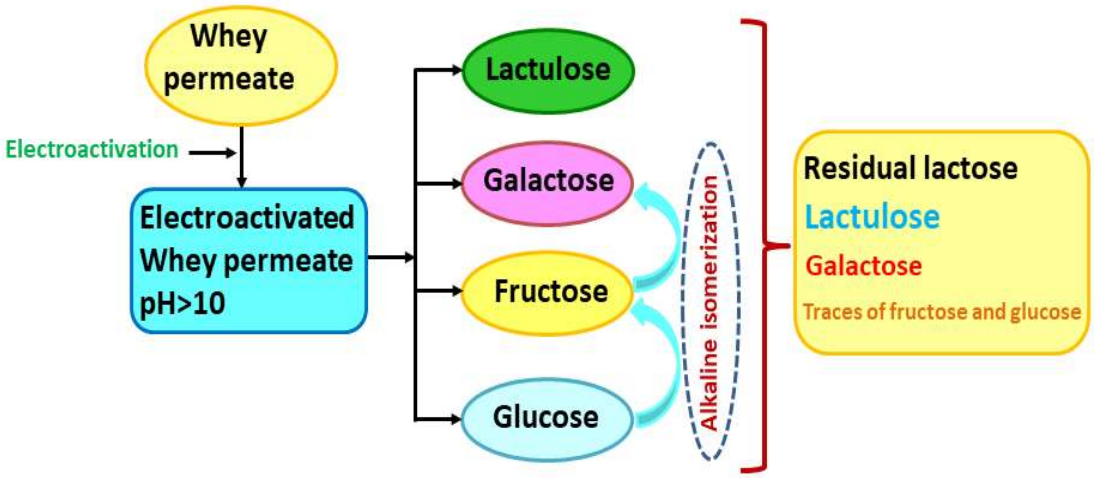

Figure 5.10: Possible pathway of lactulose production and subsequent galactose formation using the EA technique.

5.3.6 Formation of sugars in the chemical isomerization

The conventional chemical isomerization process was conducted using similar feed solutions of 6, 12, and 18% WP and maintaining equivalent alkalinity in the feed solutions corresponding to those of the EA process, and the lactulose yields are presented in **Figures 5.11** and **5.12**. As it can be seen from **Figure 5.11**, the solution alkalinity, WP concentration,

and isomerization time significantly affected ($p < 0.001$) the transformation rate of lactose into lactulose.

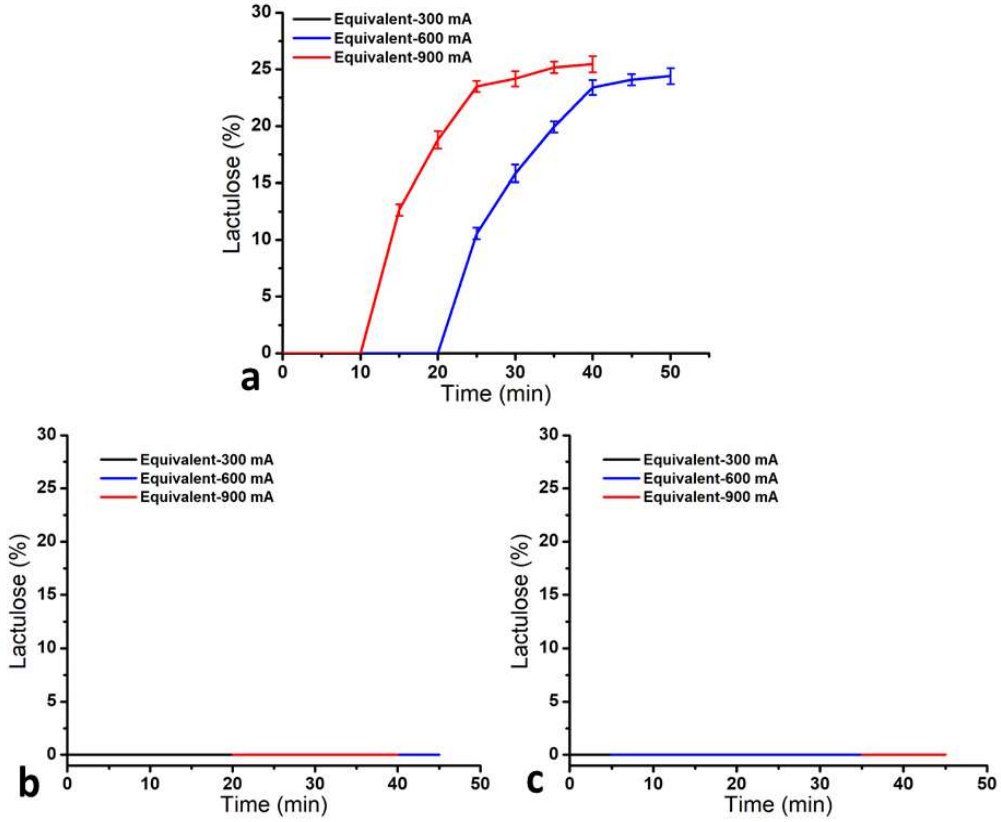

Figure 5.11: Formation of lactulose in the chemical isomerization process at the solution alkalinity equivalent to that of the EA for (a) 6, (b) 12, and (c) 18% WP solutions.

It can be seen from **Figure 5.11a** that the maximum lactulose yields of 24.31 ± 0.67 (with 3.99% galactose) and 25.47 ± 0.71% (with 5.28% galactose) were produced at the end of the conventional isomerization for the solution alkalinity of 38.00 ± 2.00 and 40.00 ± 3.46 mmol/L using 6% WP as a feed solution, which are equivalent to that of the EA process under 600 and 900 mA, respectively. Similarly, an optimum yield of 25.40% lactulose (with 5.58% galactose as a by-product) was achieved by Hashemi and Ashtiani (2010b) at a pH of 11.00 and 70 °C temperature after 60 min reaction time using 10% lactose as a feed solution. No lactulose was detected at the solution alkalinity equivalent to that of the EA for 300 mA current intensity (20.67 ± 1.15 mmol/L) in the 6% WP solution, although pH reached 10.43 ± 0.02 at the end of the reaction (**Figure 5.13**). However, it is commonly assumed that a high pH (>10.00) is needed for the isomerization reaction to occur (Hashemi and Ashtiani, 2010b,

Kareb et al., 2016a, Djouab and Aïder, 2019b). This might be due to inadequate alkalinity in the solution which was essential for an isomerization reaction to occur.

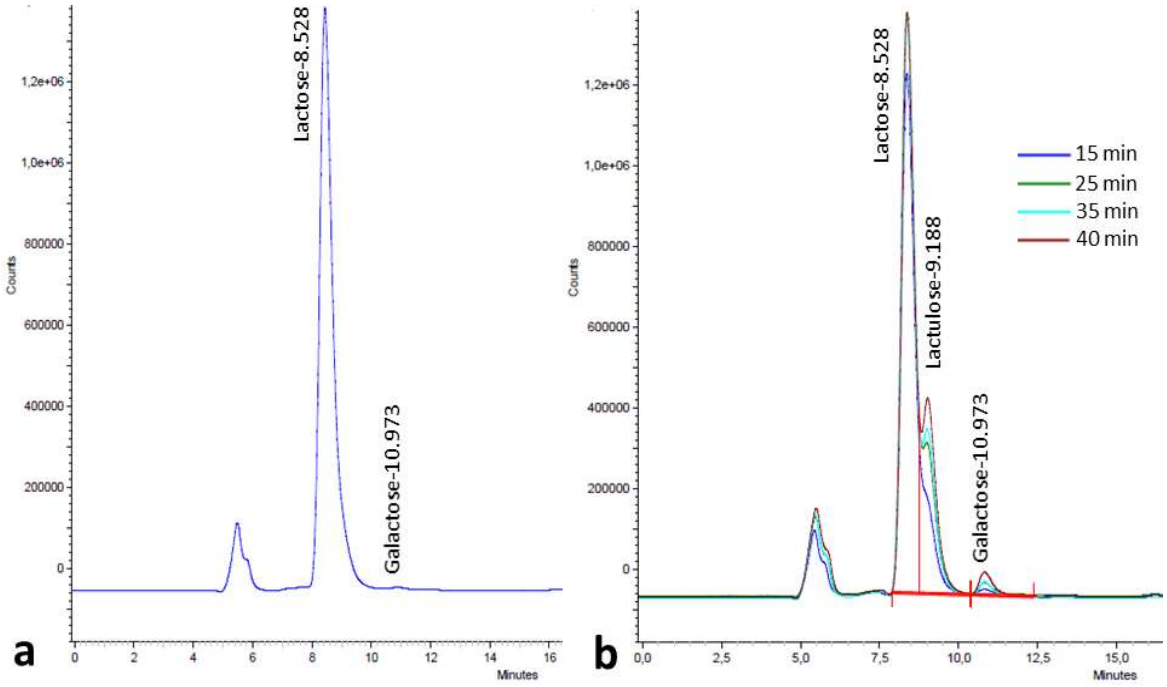

Figure 5.12: HPLC chromatograms for the sugar composition during the chemical isomerization using 6% WP solution: (a) initial feed solution and (b) feed solution at equivalent solution alkalinity as in the electro-isomerization under 900 mA current intensity.

Regarding the pH evolution, a significant variation between electro- and chemical isomerization was noticed. In chemical isomerization, the maximum pH of 10.43 ± 0.02, 11.52 ± 0.02, and 11.60 ± 0.01 were achieved for 6% WP solution after the completion of reactions for similar solution alkalinity corresponding to the EA process for 300, 600, and 900 mA current intensities, respectively. However, medium pH never reached 10 (pH < 10) for 12 and 18% WP solutions at the equivalent alkalinity corresponding to the EA (**Figure 5.13**). Consequently, no lactulose was observed to form for 12 and 18% WP solutions in chemical isomerization (**Figure 5.11b**, **c**). This is obvious that higher solution concentrations were retarding the changes in pH because of the higher buffering capacity of the greater WP concentrations. In fact, greater activation energy was needed for an isomerization reaction to occur in the chemical method. Thus, a higher dosage of catalysts was obliged for producing and adequate degree of alkalinity, while the more concentrated solutions of WP were used as feed solutions (Karim and Aider, 2020d). Indeed, the catalyst concentration is one of the

critical factors to be considered during the conventional lactose isomerization process. In a study by Seo et al. (2016a), they achieved a lactulose yield of 29.60% at a solution alkalinity of 79.00 mmol/L by adding 7.6 g/L of $(NH_4)_2CO_3$ as the catalyst under 97 °C using sweet whey as the feed solution. In contrast, only 1.83 and 1.80 g/L of KOH were used to achieve the alkalinity of 32.67 ± 1.15 and 32.00 ± 2.00 mmol/L in the 12 and 18% WP solutions as equivalent to the EA under 900 mA current intensity, respectively.

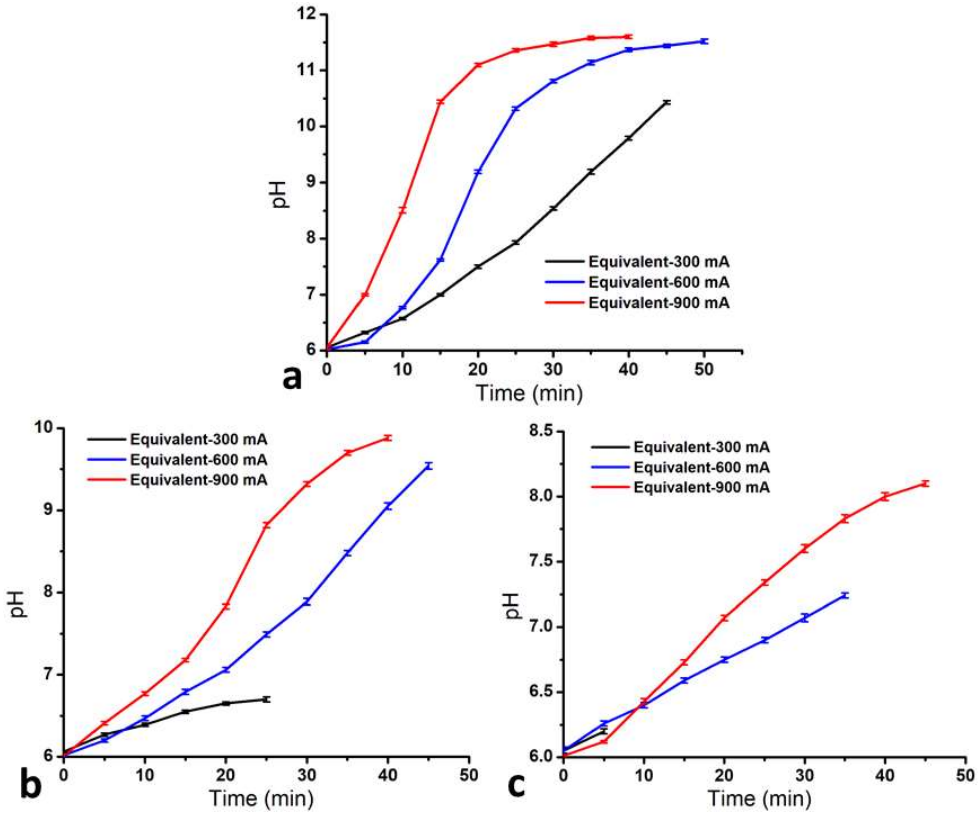

Figure 5.13: pH evolution during the chemical isomerization process at equivalent solution alkalinity to that of the electro-isomerization for (a) 6, (b) 12, and (c) 18% WP solutions.

Beside the pH and catalyst concentration, a higher temperature of more than 50 °C (typically, 50 to 130 °C) combined with various reaction times was used for conventional chemical isomerization in most literature studies (Hicks et al., 1984b, Hashemi and Ashtiani, 2010b, Song et al., 2013a, Sakkas et al., 2014a). In the present study, we performed the chemical isomerization without external heating because the EA process was conducted at an ambient temperature. The temperature was increased in the EA process was self generated in the system because of global electric resistance. Furthermore, the statistical analysis of the

acquired data did not indicate a relationship between the temperature increase and lactulose formation. This can be explained by the fact that the temperature raised in the EA (from 22 to 43 °C) was not enough to have any catalyzing effect. It is important to mention here from this result that the lactulose can be generated at an ambient temperature by creating enough solution alkalinity in the feed medium.

Apart from this, the transformation of lactose into lactulose through the chemical-based processes typically resulted to a quick degradation of lactulose into galactose and tagatose along with some other acidic by-products including isosaccharinic and formic acids (Corbett and Kenner, 1953a, Dendene et al., 1994b, Hashemi and Ashtiani, 2010b, Schuster-Wolff-Bühring et al., 2010a, Djouab and Aïder, 2019b), resulting in the dropping of pH in a feed medium (Paseephol et al., 2008a, Hashemi and Ashtiani, 2010b). In essence, in the conventional isomerization, the use of a high temperature in an alkaline medium triggers isomerization and degradation of lactose and lactulose into epilactose and galactose (Martinez-Castro et al., 1986a, Hashemi and Ashtiani, 2010b). However, in this study, this phenomenon was not observed and, subsequently, only galactose was produced as a reaction by-product.

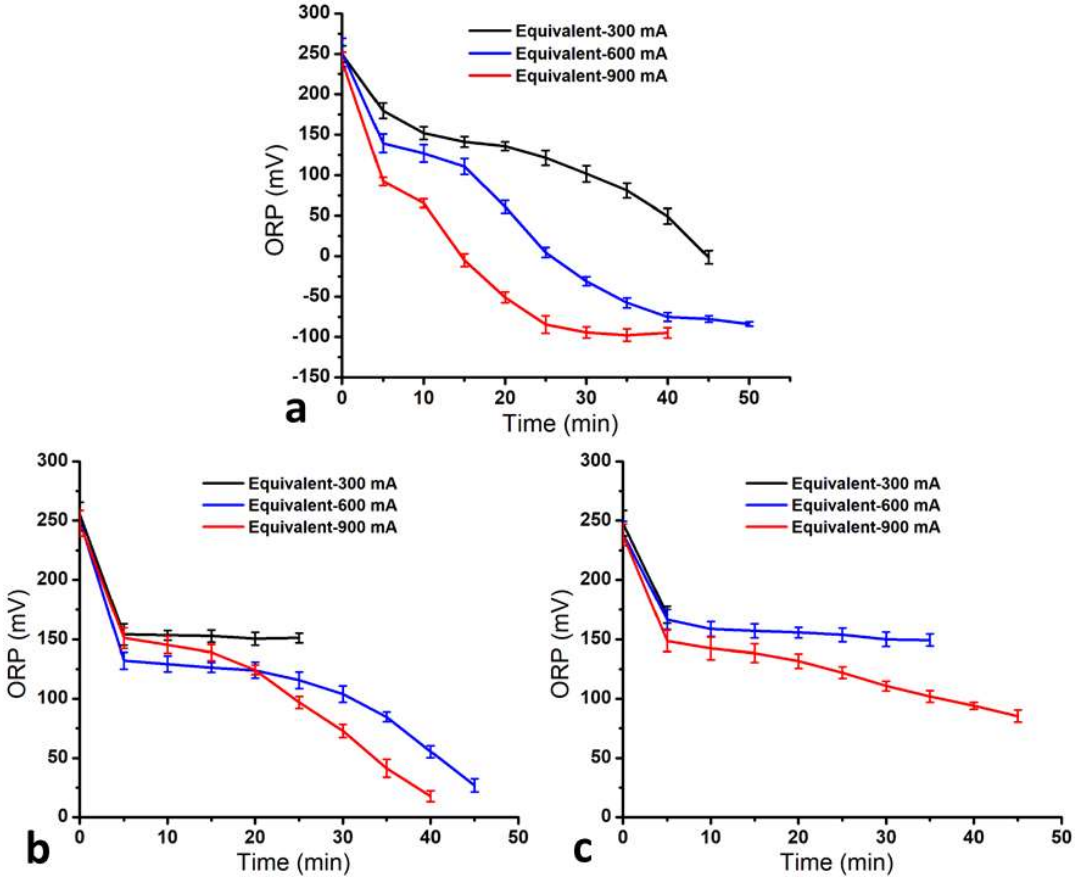

Figure 5.14: Variation of ORP during the conventional chemical isomerization reactions for (a) 6, (b) 12, and (c) 18% WP solutions.

As can be seen from **Figure 5.14** that the ORP values were decreased to around +200 to +100 mV within the first 5 min in the chemical isomerization. Thereafter, depending on the different reaction conditions, they obtained the highest values of around +200 to -100 mV at the end of the isomerization reaction. It appears that the ORP reached a negative value only for 6% solution, and the highest ORP of -95 ± 4.36 mV was reached at the end of the isomerization reaction at an equivalent solution alkalinity corresponding to 900 mA current intensity in the EA. In contrast, in the EA technique, the ORP values were reduced to around -450 to -650 mV with 5 min of electro-isomerization no matter what solution concentrations and current intensities used. After that, they reached maximum values of -570 to -870 mV depending on the different reaction conditions (**Figure 5.6**). The considerable variation between the ORP values of the electro-isomerization versus the chemical isomerization processes attributed to the fact that: during the EA process, the electrolysis of water produces two key components, that is, OH^- ions, which are responsible for the alkalization of the

reaction medium and H_2 gas, which is a powerful reducing agent. As a result, the ORPs were highly reducing in the EA process. However, in the chemical method, the addition of the alkalinizing catalyst, that is, KOH, had an effect only in the pH of the feed medium. Consequently, the ORPs in the electro-isomerization process were considerably higher compared to those in the chemical isomerization method, even using the equivalent solution alkalinity.

In the EA system, the highly reduced ORPs made the WP solutions extremely reactive because the applied electric field activates the feed medium to transform into a metastable state. As a result, the reactivity of the medium was substantially enhanced under the EA conditions compared to the normal state (Aider et al., 2012a), therefore, the activity of the isomerization reactions might be increased because of the high internal potential energy of the activated solution (Karim and Aider, 2020a). Apart from high ORP, other physico-chemical parameters including high medium pH, critical solution alkalinity, ion migration, and moderate temperature rise in the EA process may probably have intensified the feed medium to reach such conditions, in which the required activation energy for an isomerization reaction to occur for transforming lactose into lactulose would have been considerably reduced. Hence, by decreasing the required activation energy, electro-isomerization showed better efficiency for conversion of lactose into lactulose. Subsequently, a higher yield of lactulose was produced within a short reaction time in the electro-isomerization than the conventional isomerization process, although the equivalent alkaline conditions were maintained. To sum up, it can be hypothesized that the main factor, which can explain the difference between the EA process and the chemical one, is that the required activation energy for an isomerization reaction to transform lactose into lactulose *in situ* of WP solutions to occur is much lower using the EA technique than the chemical method.

5.4 Conclusion

The results of the present study suggest that, in contrast to the conventional chemical isomerization process, the EA technology was more efficient in producing a greater amount of lactulose within a short time whatever the reaction conditions. The highest lactulose yield of ~37% was achieved in the EA technique at 50 min under a 900-mA current intensity using a 6% WP solution with a medium pH and alkalinity of 11.34 and 40.00 mmol/L, respectively. Whereas, a maximum of 25% lactulose was obtained in the conventional chemical isomerization for the 6% WP solution as the feed, while the medium pH was 11.60 for an alkalinity of 40.00 mmol/L (equivalent solution alkalinity to 900 mA current intensity as in the EA process). Therefore, the EA technique offers higher potential than the conventional chemical method to produce the prebiotic lactulose through the valorization of the WP, using it as a lactose source. Furthermore, the EA process was carried out under complete auto-catalytic conditions, implying that the alkalinizing catalysts and external heating are not required in the EA technology. To sum up, the EA could be an attractive, green, and clean technique to achieve the combined target of prebiotic (lactulose) production and sustainable valorization of WP for environmental resilience.

ANNEXE C

Schematic of the EA reactor with geometrical parameters used for electro-isomerization (Figure A6) and evolution of voltage in the EA process using different current intensities and WP solutions (Figure A7)

ACKNOWLEDGEMENTS

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Chapitre 6: Comprehensive utilization of electro-activated whey-based media in the cell growth, metabolites production and aroma compounds synthesis using a starter culture originated from kefir grain

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RÉSUMÉ

Le lactosérum et le perméat de lactosérum (WP) sont les principaux co-produits du processus de fabrication de fromage ou de la caséine. Il existe une demande croissante de développer une approche durable pour leur utilisation afin d'éviter la pollution de l'environnement. L'objectif de cette étude était d'explorer la faisabilité d'utiliser des substrats à base de lactosérum électro-activé (EA-Whey), de perméat de lactosérum électro-activé (EA-WP) et du lactose électro-activé (EA-lactose) comme sources de carbone et de lactulose pour produire des métabolites avec une valeur ajoutée significative, y compris des acides organiques (lactique, acétique, citrique et propionique), de l'exopolysaccharide kéfiran et des composés aromatiques volatils. Pour cela, le bioprocédé consistait en une fermentation contrôlée utilisant les grains de kéfir entiers comme culture de fermentation en condition d'incubation statique pendant 96 h à 30 °C. Les résultats ont montré que les substrats électro-activés ont permis d'atteindre une croissance de biomasse plus élevée en un temps de fermentation réduit que leurs milieux correspondants qui non pas été soumis à l'EA (lactosérum, perméat WP, lactose). La croissance cellulaire la plus élevée (6,04 g/L) a été obtenue dans le lactosérum EA (EA-Whey) après 72 h et elle était 1,7 fois supérieure à ce qui était obtenu dans bouillon de nutrition standard; soit le milieu renforcé pour Clostridies (*Reinforced Clostridial Medium* RCM). De plus, le lactosérum EA a permis de produire un maximum de 8,46, 3,97, 0,60 et 1,02 g/L d'acide lactique, acétique, citrique et propionique, respectivement. De plus, la fermentation dans du lactosérum EA a permis la production de kéfiran la plus élevée d'une valeur de 2,99 g/L, suivi par le lactosérum (2,67 g/L), EA-WP (2,31 g/L), WP (1,88 g/L), bouillon RCM (1,42 g /L), EA-lactose (1,37 g/L) et lactose (0,91 g/L). Les résultats ont également démontré que divers composés aromatiques volatils étaient produits au cours de la fermentation du lactosérum EA (EA-Whey), ce qui peut augmenter les caractéristiques organoleptiques et la qualité sensorielle des produits fermentés. Ainsi, les résultats de la présente étude fournissent une preuve du concept que les substrats EA, en particulier le lactosérum EA (EA-Whey), pourraient être une matière première pour développer un bioprocédé efficace pour produire des métabolites de haute valeur commerciale en utilisant des grains de kéfir comme culture de fermentation.

Mots-clés: Substrats à base de lactosérum; Électro-activation; Microbiote de kéfir; Production de biomasse; Acides organiques; Kéfiran; Substances aromatiques volatiles.

ABSTRACT

Whey and whey permeate (WP) are the main by-products from cheese or casein manufacturing process and there is a serious demand of developing a sustainable approach for their utilization to evade environmental pollution. The objective of this study was to explore the feasibility of using electro-activated (EA) whey-based substrates (whey, WP, lactose) as carbon sources to produce valuable metabolites including organic acids (i.e., lactic, acetic, citric, and propionic acids), exopolysaccharides (kefiran), and volatile flavour compounds using the whole kefir grains as a starter culture. Fermentation was performed by inoculating a mixed microbiota from kefir grains in EA and non-EA solutions of lactose, whey, and WP and were statically incubated for 96 h at 30 ℃. The results showed that the EA substrates achieved a higher biomass growth in a reduced fermentation time than their non-EA mediums. The highest cell growth (6.04 g/L) was obtained for EA-whey after 72 h which was even 1.7-fold higher than a standard nutrition broth, the reinforced clostridial medium (RCM). Furthermore, EA-whey produced a maximum of 8.46, 3.97, 0.60, and 1.02 g/L of lactic, acetic, citric, and propionic acid, respectively. Moreover, EA-whey achieved the highest kefiran production of 2.99 g/L, followed by the whey (2.67 g/L), EA-WP (2.31 g/L), WP (1.88 g/L), RCM broth (1.42 g/L), EA-lactose (1.37 g/L), and lactose (0.91 g/L). The results also demonstrated that various aromatic volatile compounds were produced during the fermentation of EA-whey, which may increase the organoleptic characteristic/sensory quality of the fermented products. Thus, the results of the present study provide strong evidence that the EA-substrates, especially EA-whey, could be potential feedstock to develop an efficient bioprocess for producing valuable metabolites using kefir grains as a suitable starter.

Keywords: Whey-based substrates; Electro-activation; Kefir microbiota; Biomass production; Organic acids; Kefiran; Volatile flavour substances.

6.1 Introduction

Whey is a liquid by-product remaining after milk has been curdled and strained (removal of caseins) in the cheese or casein production process (Xiao et al., 2019, Karim and Aider, 2020d).The secondary co-product of this process is called whey permeate (WP), also known as deproteinized whey, that is remained after recovering main whey-proteins such as α-lactalbumin and β-lactoglobulin from liquid whey through ultrafiltration-diafiltration. It also contains lactose (and its derivatives), minerals, and other small molecules such as peptides and free amino acids (Zall, 1992, Xiao et al., 2019). The major constituent of whey/WP is lactose, which is a disaccharide composed of glucose and galactose subunits (Karim and Aider, 2020a). Discarding raw cheese whey or/and WP create huge disposal problems because they have high chemical oxygen demand (COD) and biochemical oxygen demand (BOD) with a value of 60,000-80,000 and 30,000-50,000 mg/L O_2, respectively, due to their high lactose concentration (Djouab and Aïder, 2019c, Karim and Aïder, 2020). A beneficial practice is using of whey onto farming land as a manure, however, it seems to have an adverse impact on soil, leading to the decreased yields of crop and resulting in severe groundwater pollution problems (Ben-Hassan and Ghaly, 1994, Seo et al., 2014b). Therefore, this remains a huge challenge for the dairy industries and researchers to develop economical and sustainable approaches of valorization of these industrial co-products and that is why industries are multiplying different processes to produce various products with good commercial value utilizing these low cost co-products from dairy industries (Yadav et al., 2015a).

Recently, biological routes are gaining attention for valuable metabolites production by utilizing the low-cost by-products as carbon and nitrogen sources (Puyol et al., 2017, Xiao et al., 2019). From nutritional point of view, whey is composed of water (~93%), lactose (4.5-5.0%), soluble proteins (0.60-0.85%), minerals (~0.53%), and fat (~0.36%) (Tesfaye et al., 2019). The accessibility of basic nutrients including lactose and proteins for microbial growth render the whey-based media as potential feedstocks for producing various beneficial metabolites. The microbial conversion of whey to valuable fermented products such as organic acids, exopolysaccharides, volatile flavour substances, and single-cell proteins could be a sustainable approach for whey management and valorization (Xiao et al., 2019, Karim et al., 2020a). However, raw whey/WP is a tricky-type of waste by-product to handle, which

can be quickly acidified because of its high organic content with inadequate bicarbonate alkalinity (Seo et al., 2014b). Another main problem is low total solid content and high lactose to glucose ratio, that makes fermented beverage watery and unpleasant taste (Tesfaye et al., 2019). Furthermore, many microorganisms including probiotic bacteria showed poor growth in the milk-based substrates because of their limited lactose assimilation ability, weak proteolytic activity, and inadequate oxidation-reduction potential of the substrates (Corcoran et al., 2004, Kareb et al., 2018a). Hence, there is still an immense need to explore the efficient substrates or suitable supplements to incorporate with substrates for promoting the growth of microorganisms. A recent study by Kareb et al. (2018a) demonstrated that the electro-activated whey (EA-whey) could promote the growth of different pure cultures such as *Lactobacillus*, *Bifidobacterium*, and *Streptococcus* strains due to its prebiotic lactulose content and high antioxidant capacity. Therefore, it is hypothesized that the challenges of raw whey utilization could be defeated by using electro-activated substrates (EA-substrates) to produce value-added metabolites via fermentation of whey-based media. Furthermore, the activity of whey-based growth media would be increased by electro-activation and the excessive internal potential energy of the highly reactive EA solutions could possibly escalate the fermentation process by promoting the growth of microorganisms by providing easily accessible nutrients in non-stressing conditions.

In the fermentation process, different microorganisms including pure cultures and mixed culture consortia were used to produce various fermented products using whey-based substrates (Yadav et al., 2015a, Carota et al., 2017a, Kareb et al., 2018a). The mixed cultures are regarded robust inocula because they are able to utilize wide range of substrates, more tolerant to the environmental fluctuations, easy to obtain in nature, applicable in large scale, and less susceptible to contamination (Karim et al., 2018a, Islam et al., 2020). All those features make mixed culture processes cheaper than using pure cultures. Kefir grain, a mixed microbial consortium, produces a complex microflora that is containing more than hundred of yeasts and bacteria securely embedded (Cheirsilp and Radchabut, 2011). This mixed-microbiota is a decent example of a mutually beneficial community where lactic acid bacteria (LAB) including *Lactococcus*, *Lactobacillus*, *Streptococcus*, and *Leuconostoc* are the main population (10^8-10^9 CFU/gram of grain) followed by yeast such as *Kluyveromyces*, *Candida*, and *Saccharomyces* (10^7-10^8 CFU/gram of grain) and acetic acid bacteria (AAB) like

Acetobacter and *Gluconobacter* (10^5-10^6 CFU/gram of grain) (Dong et al., 2018, Bengoa et al., 2019b). Due to its' excellent microbial diversity composition, a whole kefir culture is capable of adapting to various food/waste substrates, which can henceforth be used to manufacture innovative beverages with probiotic function (Tu et al., 2019). For instance, the LAB present in the consortium improve the conservation period of the products by producing acetic acid, lactic acid or other antimicrobial compounds (John and Deeseenthum, 2015). They also support to enhance organoleptic properties through the formation of numerous volatile compounds (e.g., ethyl acetate, acetaldehyde, isobutyl alcohol, etc.), functional exopolysaccharides (e.g., kefiran) or free amino acids (Dertli and Çon, 2017, Bengoa et al., 2019b). Moreover, the yeasts produce carbon dioxide and alcohol during the fermentation, which could increase mouthfeel and taste of the fermented products (Rosa et al., 2017).

Specifically, this work was intended to explore the feasibility and viability of a novel fermentation process by utilizing electro-activated (EA) whey-based substrates including EA-lactose, EA-whey, and EA-WP as the potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids (lactic, acetic, citric, and propionic acids), exopolysaccharides (kefiran), and volatile compounds by using kefir grain as a starter culture.

6.2 Materials and methods

6.2.1 Microorganism

A starter culture of microorganisms originated from kefir grains was used in the present study. The lyophilized kefir grains were kindly provided by the Department of Dairy Technology, Odessa State Academy of Food Technologies. The kefir grains were activated in the 5% glucose solution by using the following procedure: Briefly, the 0.1 g of kefir grains were rehydrated in a 50 mL falcon tubes containing 40 mL of 5% glucose solution for 24 h. After that, the grains were aseptically separated using a stainless-steel sieve and the supernatant was discarded. This step (procedure) was repeated five times to obtain activated mixed microorganisms. On six subsequent days of activation, the supernatant was collected and used to inoculate a reinforced clostridial medium (RCM) (CM0149, Oxoid Ltd., Basingstoke, Hants, UK) consisting of yeast extract (13 g/L), peptone (10 g/L), soluble starch (1 g/L), glucose (5 g/L), cysteine hydrochloride (0.5 g/L), sodium chloride (5 g/L), sodium acetate (3 g/L), and agar (0.5 g/L) at a ratio of 1/10. The culture was grown in an incubator

shaker (Innova®44, NJ, USA) at 30 ℃ and 100 rpm overnight and subsequently, lyophilized to maintain the working cell culture in the exponential growth phase. Notably, glucose was used instead of milk/lactose to obtain a homogenous mix culture of all microbes including lactose assimilating and non-assimilating population.

6.2.2 Inoculum preparation

The lyophilized mixed culture, originated from kefir grains was reactivated in the RCM medium overnight prior to the subculture onto Petri plates by spreading 1 mL of broth culture to grow fresh cells. Thereafter, the inoculated plates were nurtured in an incubator (Boekel Scientific, model-165000, PA, USA) at 30 ℃ for 24 h. A working cell bank culture was then produced by dissolving 10 loops of microorganisms from the subcultured cells in 10 mL of sterile water. Thereafter, a primary inoculum was prepared in 250 mL Erlenmeyer flask having 100 mL of broth and injecting 1 mL of working cell bank culture. The inoculated flask was then incubated at 30 ℃ overnight. Finally, the cultivation media used in this study (electro-activated lactose, whey and whey permeate and their corresponding non-electro-activated samples) were inoculated with 5% (v/v) of this inoculum (optical density, OD_{600} = 1.2).

6.2.3 Preparation of culture media for cell growth and metabolites production

To evaluate the most efficient medium, the mixed culture inoculum was cultivated on seven different cultivation media for biomass growth and metabolites production. The composition of various media used in the present study is presented in **Table 6.1**. The selected cultivation media comprised of several carbon sources, such as lactose, lactulose, galactose, etc. The 7% whey or 6% WP were selected based on equivalent concentration of 5% lactose, which is commonly attained as a co-product of cheese manufacturing industry. Thus, they can be used directly after their production from industry without further concentrating step. Medium pH was adjusted to 7.0 ± 0.1 by adding acidic solution that was produced in the anodic compartment during electro-activation process (Karim and Aïder, 2020) or 0.1 N NaOH for all samples. It is worth noting that the natural whey-based substrates without addition of supplements were used in this study.

Table 6.1: The description of seven cultivation media used for cell growth and metabolites production.

No.	Medium Name	Characteristics	Composition
1	RCM broth	Reinforced clostridial medium	Yeast extract, peptone, soluble starch, glucose, cysteine hydrochloride, sodium chloride, sodium acetate, agar: 13, 10, 1, 5, 0.50, 5, 3, 0.50 g/L, respectively.
2	Lactose	5% lactose solution	Lactose (50 g/L)
3	EA-lactose	5% lactose solution after electroactivation with 300 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~4%, and traces of glucose and fructose (Karim and Aider, 2020a)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~1 g/L)
4	WP	6% whey permeate solution (as equivalent to 5% lactose solution)	Lactose (50 g/L) and traces of glucose and galactose
5	EA-WP	6% whey permeate solution after electroactivation with 600 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~6%, and traces of glucose and fructose (Karim and Aïder, 2020)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~1.52 g/L)
6	Whey	7% whey solution (as equivalent to 5% lactose solution)	Lactose (50 g/L) and traces of glucose and galactose
7	EA-whey	7% whey solution after electroactivation with 900 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~7%, and traces of glucose and fructose (Karim and Aider, 2020d)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~2.55 g/L)

6.2.4 Growth profile and cell concentration determination

The growth profile of microbial cells in different media were studied for 96 h of cultivation. In brief, 100 mL of each cultivation media was prepared in the sterilized Erlenmeyer flasks (250 mL) and was injected with 5% (v/v) of primary inoculum. The inoculated flasks were statically and micro-aerobically nurtured in the incubator at 30 ℃ for 96 h. The medium pH was monitored during the cultivation by a pH meter (model: Oakton pH 700) equipped with an Oakton pH probe (Vernon Hills, IL, USA). Samples were collected at 12 h intervals during the cell enrichment to measure the growth pattern based on optical density at 600 nm (OD_{600}) using a Biotek power wave spectrophotometer and Gen5 2.09 software (Biotek Instruments, model-XS2, VT, USA).

6.2.5 Biomass collection and cell dry weight

The weight of biomass (dry weight basis) was measured in triplicate using a gravimetric method. Briefly, the fermented broths were taken in a centrifuge tube (50 mL falcon tubes) at different time of cell growth and centrifuged at 10,000 × g at 4 ℃ during 15 min (Beckman Coulter Avanti® J-E centrifuge, Indianapolis, IN, USA). The supernatant was then transferred to tube for further analysis and the precipitated cells were centrifuged again using 0.9% sodium chloride solution followed by deionized water. Thereafter, the supernatant was discarded immediately, and the cells were dried at 65 ℃ for 48 h in an oven (JEIO Tech, model: OF-02G, Korea).

6.2.6 Biomass composition analysis

The proximate composition was analyzed for the biomass that was obtained from whey and EA-whey after 72 h of cultivation. Briefly, the biomass was freeze-dried for 24 h using a laboratory scale freeze dryer (Lyph-lock®4.5, Labconco corp., Kansas City, MO, USA). Thereafter, the freeze-dried biomass was characterized by following different methods for protein, lipid, ash, carbohydrate, and moisture content. Protein analysis was carried out by following the combustion method using an Elementar rapid MICRO N Cube Nitrogen analyzer (Elementar Analysen systeme GmbH, Langenselbold, Germany). A conversion factor of 6.38 was used to calculate protein content (Mariotti et al., 2008b). Lipid content was determined using ANKOM^{XT10} Extractor (ANKOM Technology, Macedon NY, USA) as described by Am (2005). Ash, carbohydrate, and moisture contents were evaluated following the method described by Laurens (2016).

6.2.7 Evaluation of sugars composition and organic acids

The composition of sugars in the fermented broths were evaluated by high performance liquid chromatography (HPLC) using a Hitachi L-7000 (Hitachi High-Tech, Japan) system equipped with a refractive index detector (Waters, model: 2487) and a Sugar Pak-I 300 × 6.5 mm^2 carbohydrate analysis column (Waters Corp., Milford, MA, USA). The column temperature was set to 80 ℃. A solution of Ca-ethylenediamine tetra-acetic acid (50 ppm) was used as an isocratic mobile phase maintaining a flow rate of 0.5 mL/min. A sample volume of 50 µL was injected and analyzed for 30 min. The composition of several sugars (lactose, lactulose, glucose, galactose, and fructose) was identified by matching their retention time with the corresponding standard solutions. The lactose and lactulose were quantified using standard calibration curves (**Figure A8**).

The organic acids were analyzed using a HPLC system (Hitachi L-7000) outfitted to a UV detector @220 nm and a Phenomenex Kinetex® 2.6 µm PS C18, 100 Å LC Column (100 × 4.6 mm). The column temperature was set to 30 ℃. A solution of 20 mM potassium phosphate KH_2PO_4 (pH 1.59) was used as the eluent with a flow rate of 0.3 mL/min. A volume of 50 µL of samples was injected and analyzed for 30 min. The composition of various organic acids ((lactic, acetic, citric, and propionic) was analyzed using EZChrom Elite software and recognized by comparing their retention time to their corresponding standards. Finally, they were quantified using the standard calibration curves (**Figure A9**).

6.2.8 Determination of kefiran

The exopolysaccharide in the supernatant i.e., extracellular kefiran was recovered and determined according to the method depicted by Piermaria et al. (2009). Briefly, kefiran was precipitated by addition of equal volume of cold absolute ethanol to the fermented broth and remained at -20 ℃ overnight. The resulting precipitate was then amassed following a centrifugation at 10, 000xg at 4 ℃ for 20 min. Pellets were dispersed in hot distilled water and centrifuged to eliminate any undissolved materials. The clear supernatant was then precipitated again in the same way to obtain pure kefiran. Finally, the kefiran precipitate was redissolved in hot distilled water and centrifuged. The supernatant was discarded and the kefiran was dried at 65 ℃ in the oven (JEIO Tech, model: OF-02G, Korea) for 48 h (Mechmeche et al., 2018).

6.2.9 Volatile flavour substances

The samples were collected from fermented broths at different time intervals and were analyzed directly without any pre-treatment according to Magalhães et al. (2011a) with slight modification to detect the volatile compounds in the fermented media using gas chromatography (GC). The experiment was performed by using a G1530A Agilent GC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent 5973N quadrupole mass spectrometer (MS) and was connected to a Split⁄Split-less automatic MPS injector (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). A DB-WAX capillary column with dimensions 60 m × 0.250 mm and 0.25 µm film thickness (Agilent, USA) was used. The carrier gas was helium maintaining a flow rate of 1 mL/min. The temperature of oven was programmed as the following: the temperature was set to 50 ℃ for 5 min and then increased to 220 ℃ at 3 ℃/min (held for 15 min). The quadrupole, source, and transfer line temperatures were retained at 150, 230, and 250 ℃, respectively. The injector inlet temperature was maintained at 250 ℃. A volume of 0.5 µL of samples were injected in the split mode (split ratio, 1:10). The mass spectra of electron ionization were recorded at 70 eV in a full scan mode with the electron energy ranging from 35 to 325 amu. Finally, the compounds were identified in respect to mass spectra matching using a standard NIST05 library and retention index of authentic reference standard by Enhanced ChemStation software (version: MSD ChemStation E.02.02.1431).

6.2.10 Statistical analysis

The experiments were repeated three times and the mean values ± standard deviations were considered for the analyses. The differences at $p < 0.05$ were termed as significant. Analysis of variance (ANOVA) of the obtained data was conducted by using the SAS software V9.3 (SAS Institute Inc., Cary, NC, USA).

6.3 Results

6.3.1 Cell growth and biomass production

The growth profiles of microbial cell and biomass production by a whole kefir culture were studied using several substrates and is shown in **Figure 6.1**. The results shown in **Figure 6.1a** demonstrated that the cell growth kinetics followed similar trends for all non-EA substrates (lactose, WP, and whey), where they reached a plateau at 72 h and then started to decay after 96 h. However, EA-substrates (EA-lactose, EA-WP, EA-Whey) reached the

maxima at 48 h and achieved a significantly ($p < 0.05$) higher growth than their corresponding non-electro-activated media. The highest cell growth was obtained for EA-whey after 72 h which was even 1.7-fold higher compared to a standard nutrition medium (RCM broth).

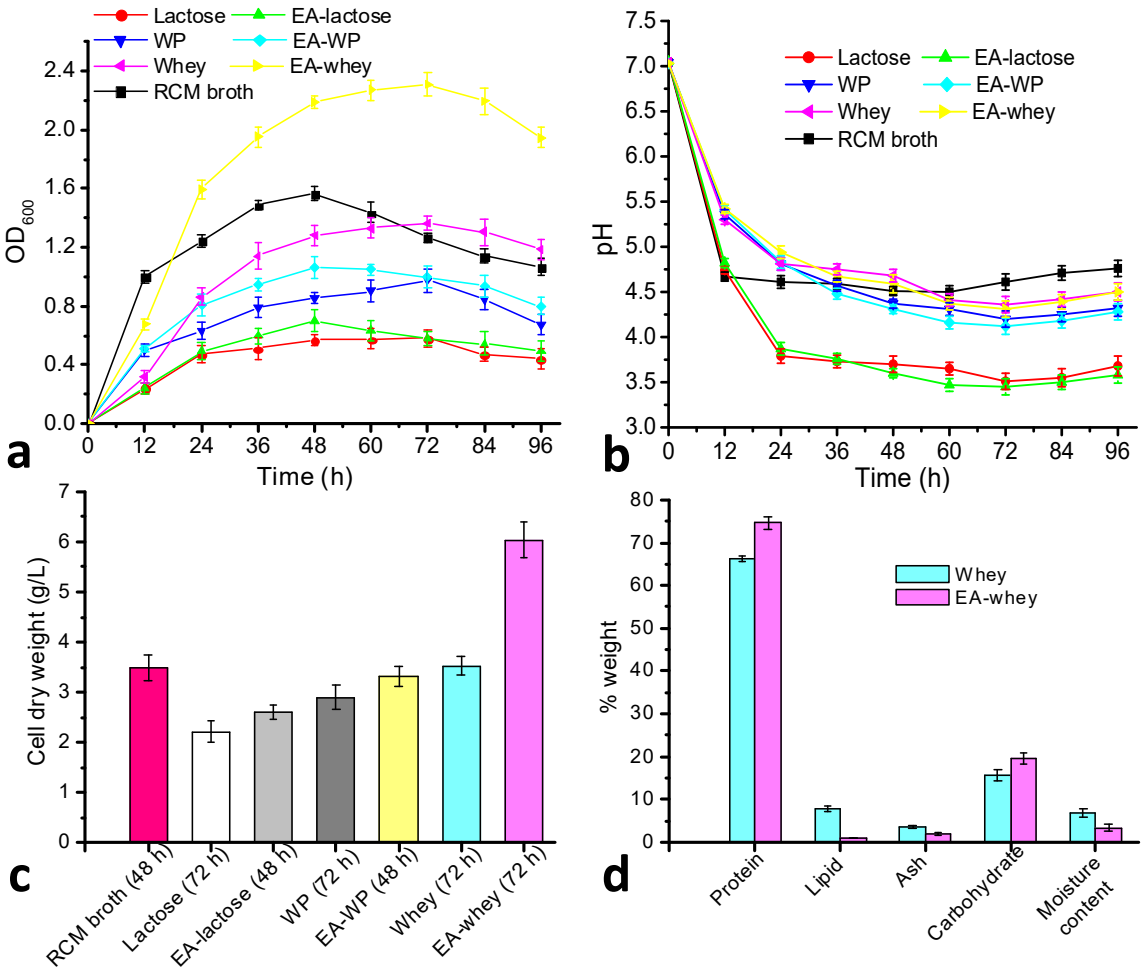

Figure 6.1: Biomass production by kefir culture using different whey-based medium at 30 °C. a) Biomass growth profile, b) pH evolution, c) maximum biomass production, and d) biomass composition.

After certain time of fermentation, the growth of microorganisms decreased which might be due to unfavorable pH of the medium (**Figure 6.1b**) or inhibitory metabolites formation or depletion of the essential nutritions. Here, it is worth noting that the different patterns of pH changes with fermentation time in the various media were likely caused due to the different mechanisms of organic acid metabolism or other metabolites formation pathway followed by the different microorganisms of the consortium (Chua et al., 2018).

Intriguingly, the cease of growth observed in the WP medium at a relatively higher pH values than that of noticed for lactose medium, suggesting that the medium pH was not only the limiting factor, but the lack of nutrients could also cause growth limitation. In fact, the deficiency in vitamins and specific minerals can lead to a growth limitation (Charalampopoulos et al., 2002a). Among the different EA-substrates used, the EA-whey attained the maximum biomass production (6.04 g/L), followed by whey (3.53 g/L), RCM broth (3.49 g/L), EA-WP (3.23 g/L), WP (2.97 g/L), EA-lactose (2.52 g/L), and lactose (2.21 g/L) (**Figure 6.1c**). Furthermore, the analysis of proximate composition of the biomass obtained after 72 h of cultivation showed that the biomass produced from the EA-whey medium was relatively rich in protein content (~76%) and carbohydrate (~20%) than the non-EA-whey (**Figure 6.1d**). Thus, the results demonstrated the potential of EA-whey to be used as a highly efficient nutrient (carbon and nitrogen) source for the bioprocesses of producing protein rich biomass, which could be utilized as a protein supplement in animal feeds or human foods.

6.3.2 Sugar consumption

The consumption of predominant sugars (lactose and lactulose) was correlated to the cell growth (**Figure 6.1a**) and decreased by following a similar fashion for all substrates (**Figure 6.2**). Lactose concentration slowly decreased during the first 12 h and then rapidly reduced during 24 h of cultivation (**Figure 6.2b**). Thereafter, it gradually decreased with fermentation time until the end of the fermentation process. During the 12 h, the consumption of lactose was sluggish probably due to the adaptation of the microorganisms in the new environment or they assimilated only the simple monosaccharides (glucose and galactose) present in the medium.

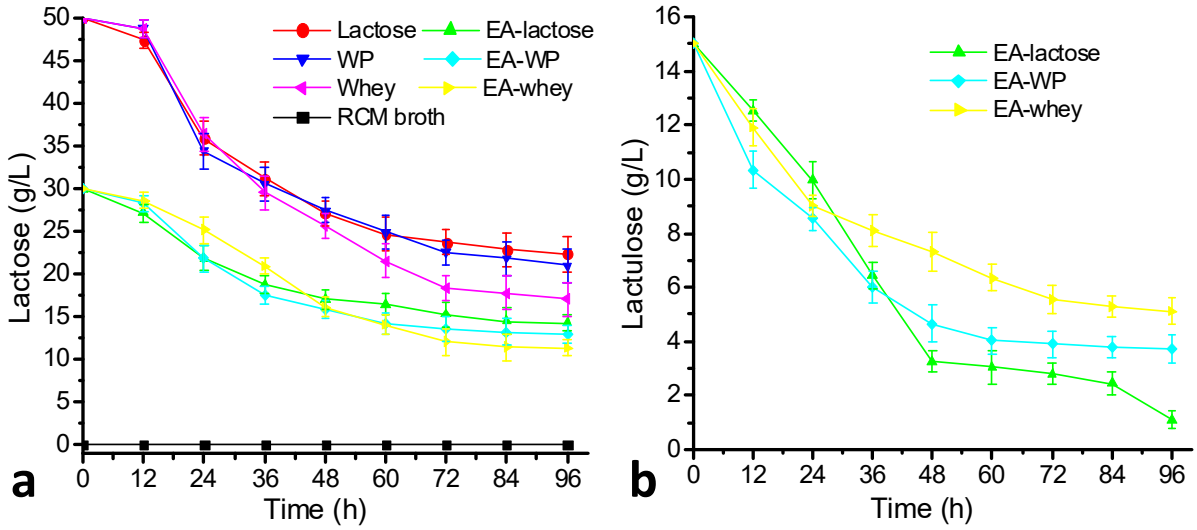

Figure 6.2: Sugar consumption a) lactose and b) lactulose during the biomass production by kefir culture in different whey-based medium.

Indeed, after electro-activation, the amount of galactose increased in the substrate and this monosaccharide is easily assimilated by microorganisms composing the kefir grain. This agrees with the microbial metabolism which is more adapted to a preferential fermentation of monosaccharides such as glucose, fructose, and galactose. Thereafter, they might have utilized the lactulose, which was sharply reduced until the end of the fermentation (**Figure 6.2b**). However, the total residual carbohydrates concentrations after 96 h fermentation suggest that the growth of microorganisms decreased which might be due to the unfavorable pH (**Figure 6.1b**) or inhibitory metabolites formation (organic acids) and/or unsuitable concentration of the nitrogen sources. The residual sugar contents in the samples (e.g., ~11 g/L of lactose and ~5 g/L of lactulose in the EA-whey) may bring about a sweeter taste in the fermented broth (**Figure 6.3**).

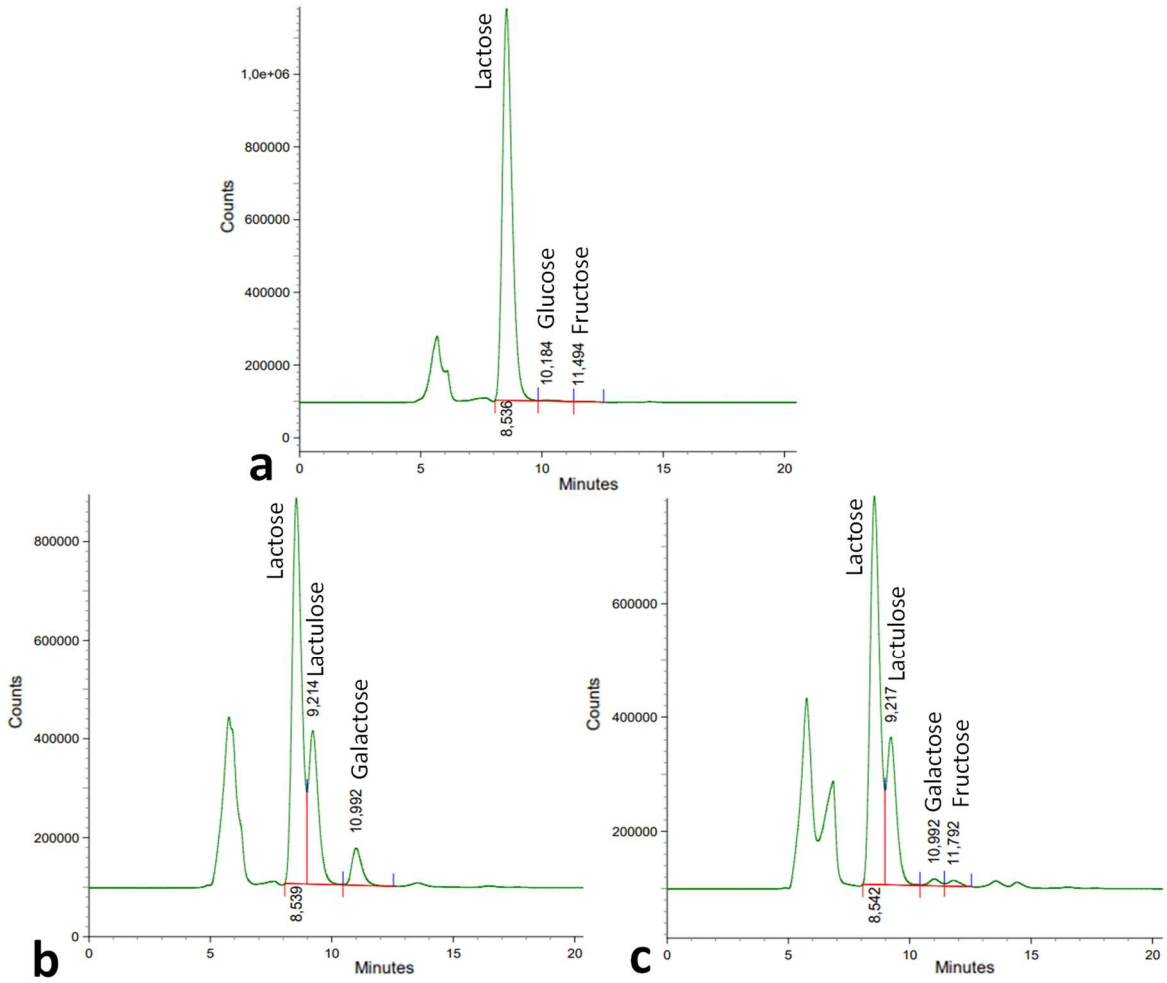

Figure 6.3: High performance liquid chromatography (HPLC) chromatograms for sugar profile a) whey, b) EA-whey, and c) EA-whey at the end of the fermentation by kefir culture.

6.3.3 Organic acids production

Production of different organic acids (i.e., lactic, acetic, citric, and propionic acids) in all the EA-substrates were more efficient than the non-EA media while the EA-whey was the most efficient medium for all organic acids measured (**Figure 6.4** and **Figure A10**). EA-whey produced significantly ($p < 0.05$) higher lactic acid and citric acid compared to all other media, however, acetic acid and propionic acid were lower than the standard RCM broth. Finally, EA-whey produced a maximum of 8.46 (84 h), 3.97 (48 h), 0.60 (48 h), 1.02 (60 h) g/L of lactic, acetic, citric, and propionic acids, respectively. Nevertheless, butyric acid was also found in several stages of fermentation (**Figure A10**), but it was not quantified in this study.

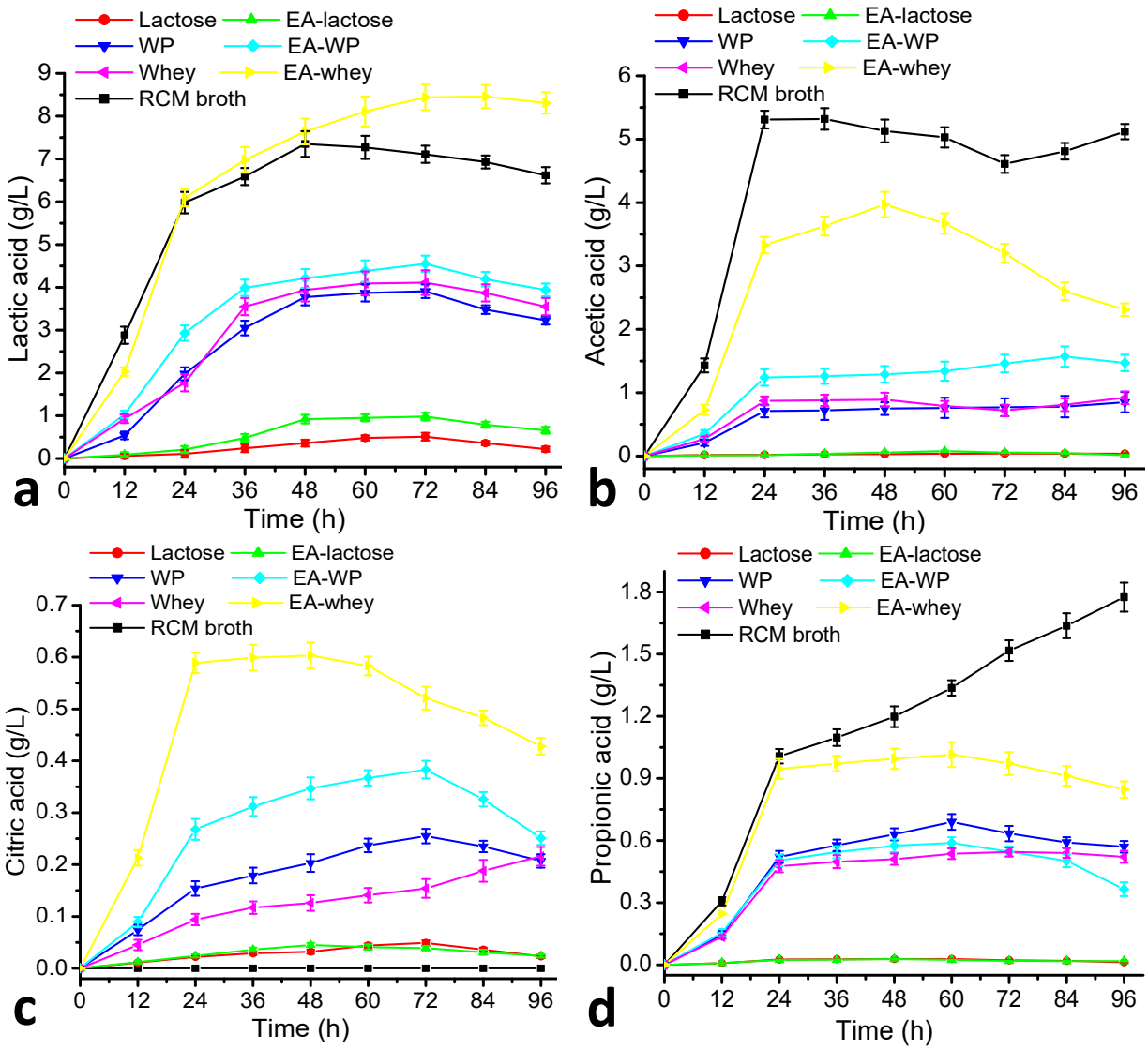

Figure 6.4: Organic acids production during the fermentation of different whey-based media at 30 °C a) lactic acid, b) acetic acid, c) citric acid, and d) propionic acid.

Lactic acid was the predominant organic acid in the present study that might be produced as a result of LAB fermentation of hexoses such as glucose, galactose or fructose, which were formed by hydrolysis of lactose and lactulose (Amirdivani and Baba, 2015). Moreover, lactic acid production is also associated with the sugar hydrolysis that are released from the glycomacropeptide of casein micelles and glycoproteins related with the fat globule membrane (Magalhães et al., 2011a). In fact, the main source of energy for bacterial growth is carbohydrates and the LAB can metabolize them through either heterofermentative or homofermentative fermentation pathway. Nevertheless, some LAB could change

homofermentative pathway to mixed acid metabolism during low growth rates and glycolytic flux (Zaunmüller et al., 2006). Basically, only lactic acid is formed from hexose metabolism in the homofermentative process, whereas volume of acetic acid, lactic acid, ethanol, and carbon dioxide are produced from hexose or even from pentose in the heterofermentative process (Liu et al., 2020). The production of different organic acids suggesting that both homo- and hetero-fermentative pathways were occurred in the present study by following different pathways (**Figure 6.5**). The results of lactic acid production were comparable to a study reported by Magalhães et al. (2011a), where they obtained 6.34 and 6.81 g/L of lactic acid using kefir grains in whey and deproteinized whey fermentation, respectively. In another study, Tu et al. (2019) demonstrated that a maximum of 10.96 g/L lactic acid could be achieved after five days of soy whey fermentation using water kefir grains.

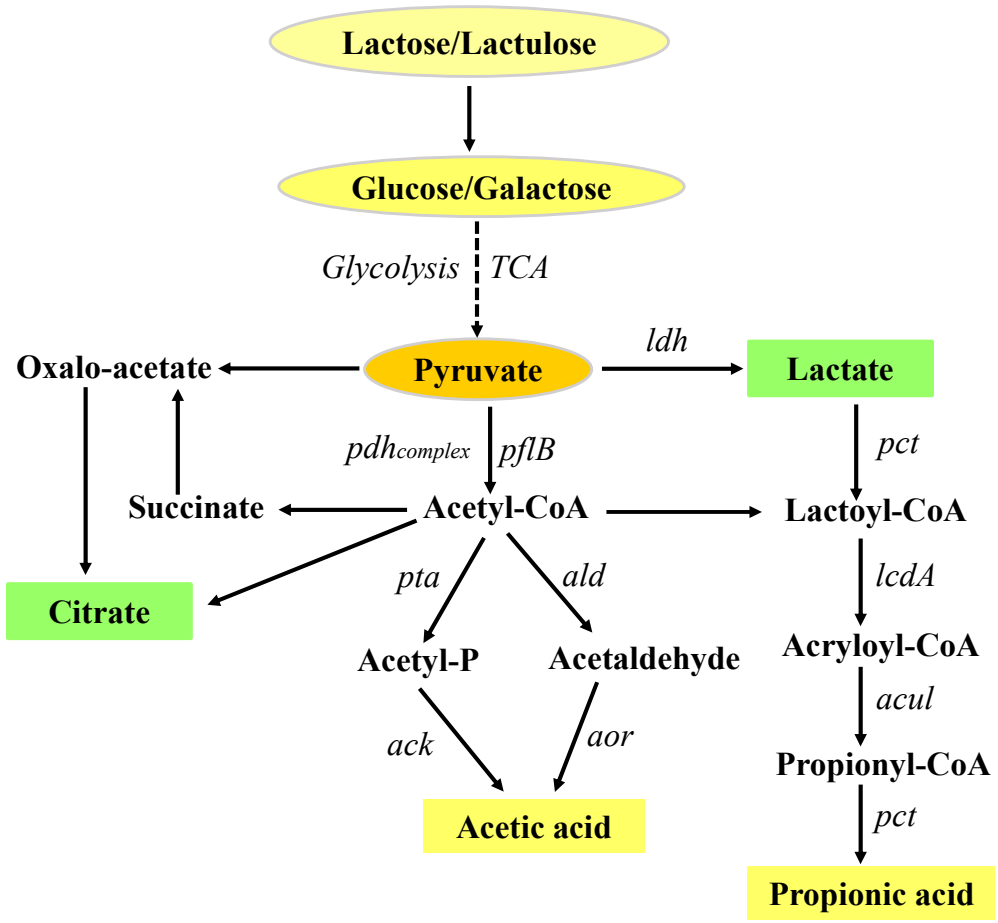

Figure 6.5: Schematic of several possible pathway for organic acids (lactic, acetic, citric, and propionic acids) production. Various enzymes coded by the genes are lactate dehydrogenase (ldh), pyruvate formate-lyase (pflB), pyruvate dehydrogenase complex (pdhcomplex), phosphotransacetylase (pta),

acetate kinase (ack), aldehyde dehydrogenase (ald), aldehyde oxidoreductase (aor), propionyl-CoA transferase (pct), lactoyl-CoA dehydratase (lcdA), acrylyl-CoA reductase (acul). TCA is tricarboxylic acid cycle.

Besides lactic acid production, the acetic acid was also formed in the present study probably by the hetero-lactic bacteria present in the kefir microbiota. Generally, the formation of acetic acid in the fermented products was probably the result of lactose, citric and lactic acid metabolism of LAB or the catabolism of amino acids. Furthermore, the formation of acetic acid in the medium was in agreement with higher concentration of the acetyl esters identified during GC-MS analyses (Chua et al., 2018). Typically, several microorganisms including *Acetobacter*, *Acetomicrobium*, *Acetothermus*, *Thermoanaerobacter*, and *Clostridium* can produce acetic acid (Nayak and Pal, 2013, Pal and Nayak, 2017). These microorganisms can metabolize digestible free sugars via glycolysis pathway into pyruvate and oxidatively decarboxylate into acetyl-CoA, which further converting into acetate and secreting out (Kadere et al., 2008). Nevertheless, there might be a change of the metabolic pathway towards acetic acid production instead of ethanol in the presence of oxygen or other electron acceptors like fructose, malic or citric acids (Charalampopoulos et al., 2002a). The yield of the acetic acid was well corroborated with a study reported by Tu et al. (2019), where a maximum yield of 2.66 g/L acetic acid was produced by water kefir grains using soy whey as a feedstock. A much higher yield of acetic acid (96.9 g/L) production by *Acetobacter aceti* using cheese whey was reported by Nayak and Pal (2013); however, a continuous membrane-integrated hybrid method was used in their study. In another study, a co-culture of *Saccharomyces cerevisiae* and *Acetobactor pasteurianus* was employed to generate acetic acid using food waste as the carbon source in microaerobic fermentation, where *S. cerevisiae* facilitated to fermenting glucose into ethanol and *A. pasteurianus* to produce acetic acid (25.88 g/L) from ethanol (Li et al., 2015). In the present study, acetic acid was noticed to increase throughout the fermentation of RCM media (5.12 g/L), while it decreased from 3.97 g/L at 48 h to 2.31 g/L at the end of the process using EA-whey. This is interesting because too much acetic acid may contribute to a vinegary taste which is considered as one of the disadvantages of fermented products by bifidobacteria (Amirdivani and Baba, 2015). The results of lactic and acetic acids are of immense significance as they could provide pleasant taste of fermented beverages and prevents the

proliferation of pathogens or undesirable microorganisms because of the increased acidity of the substrate.

Citric acid produced in the samples might be due to the excretion of intermediates from the tricarboxylic acid cycle. Several LABs like *Tetragenococcus halophilus* can transform citric acid to acetic acid via pyruvate formate lyase and ethanol, whereas some other possess the capacity to transform citric acid to lactate (Gaenzle, 2015). In this study, citric acid concentration deceased rapidly at the mid-exponential growth phase in most of the cases, for example, in the EA-whey (**Figure 6.4c and 6.1a**), could be ascribed to the fact that the citric acid may possibly be utilized and metabolized by some microorganisms (Liu et al., 2020). Other than citric acid, the propionic acid was also produced in the present study. Indeed, several strains of *Propionibacterium* ssp. such as *P. acidipropionici, P. freudenreichii, P. shermanii, and P. thoenii* that naturally present in the kefir culture could produce propionic acid. These strains can use various carbon sources including lactose and glucose for production of propionic acid (Bhatia and Yang, 2017). Coral et al. (2008) reported that *P. acidipropionici* could produce 6.77 and 8.23 g/L of propionic acid from glycerol and sugarcane molasses in the batch fermentation, respectively. Nonetheless, it was reported that the propionate could inhibit the fermentation process even at a lower concentration, such as 10 g/L (Goswami and Srivastava, 2001). In the present study, only 1.02 g/L of propionic acid was produced at 60 h using EA-whey while the maximum production was 1.78 g/L at the end of fermentation for RCM broth. In a study, Liang et al. (2012) obtained a much higher yield (68.5 g/L) than the present study using Jerusalem artichoke hydrolysate. However, an immobilized *P. acidipropionici* cell and a fibrous fed bioreactor were used in their study. The production of propionic acid in this study is interesting because it is broadly utilized in the production process of cellulose-based plastics, herbicides, perfumes, and as an additive in the animal feed and a preservative in food against molds and deteriorating fungi (Coral et al., 2008). Indeed, the organic acids originating from biological process have various applications in the pharmaceuticals, cosmetics, textiles, bioenergy, and food industry (Bhatia and Yang, 2017).

6.3.4 Kefiran production

The exopolysaccharide kefiran production in the fermented broths was explored for 96 h using different lactose and whey-based mediums and the obtained results are displayed

in **Figure 6.6**. Kefiran production was increased proportionally to the cell growth and reached the maxima at 24 h for the EA-substrates while the maxima were at 48 h for all non-EA-substrates. EA-whey achieved the highest kefiran production of 2.99 g/L, followed by the whey (2.67 g/L), EA-WP (2.31 g/L), WP (1.88 g/L), RCM broth (1.42 g/L), EA-lactose (1.37 g/L), and lactose (0.91 g/L). Moreover, a simple visual observation showed viscous consistency of the fermented EA-whey and generally all the EA-substrates which is in indication of the accumulation of highly water binding macromolecules, namely exopolysaccharides.

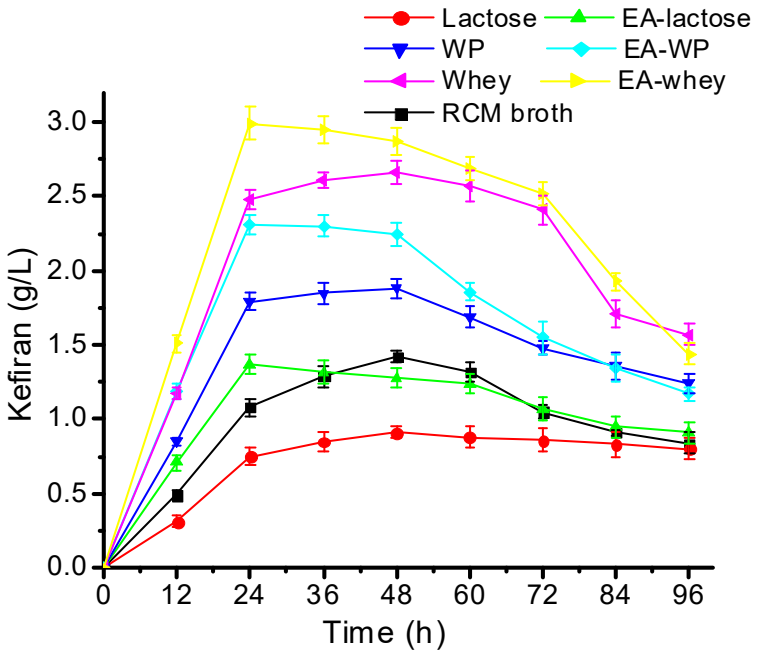

Figure 6.6: Kefiran production during the fermentation of different whey-based medium using kefir culture.

It can be seen from the **Figure 6.6** that kefiran was produced for all medium used in this study. However, EA-substrates achieved a significantly ($p < 0.05$) higher kefiran production than the non-EA-substrates because of the higher biomass growth. Indeed, the formation of kefiran in the media is associated with the cell growth. Dailin et al. (2016) observed that the production of kefiran by *Lactobacillus kefiranofaciens* was associated with cell growth, which produced kefiran during the exponential growth phase. Although kefiran is mostly generated by *L. kefiranofaciens*, some other species of *Lactobacilli* could also contribute to the kefiran production (Zajšek et al., 2013). Moreover, Cheirsilp et al. (2018b)

demonstrated that kefiran can be produced during both growth and non-growth phases. Zajšek et al. (2013) observed that the production of kefiran was the highest using lactose in the medium that was probably because of the assimilation of galactose created by hydrolysis of lactose, required for kefiran biosynthesis. The reality is that lactose is the key energy source for the bacteria, *Lactobacillus*, which correspond to 65-80% of the kefir microflora. The catabolism of lactose begins with the degradation this disaccharide into galactose and glucose. Nevertheless, both glucose and galactose participate in further syntheses of ATP, biomass, and biosynthesis of exopolysaccharides (Zajšek et al., 2013). Another possible reason for the higher production of kefiran in the fermented medium with EA-substrates was due to the results of readily available galactose for consumption by LAB to biosynthesis kefiran (Zajšek et al., 2013). Lactose medium obtained a less kefiran production was probably due to less growth and lack of nitrogen sources for kefiran biosynthesis. Taniguchi et al. (2001) demonstrated that the cells cultivated in the medium without yeast extract (used as nitrogen source) resulted in poor growth and kefiran production. It is interesting to note that even the RCM broth yielded a lower production than whey and WP although it contains enough nitrogen sources. EA-whey achieved the maximum kefiran production indicating its favorable composition (e.g., suitable C/N ratio, sufficient mineral source, etc.) for exopolysaccharide production.

After certain time, the cease in kefiran production could be ascribed to the repressive effects of high concentration of lactic acid produced in this stage. This fact was comparable to the study by Cheirsilp and Radchabut (2011). Furthermore, the inhibitory effects of lactic acid would become more severe at acidic pH than they would at neutral initial pH because the pH decreased with fermentation time (Cheirsilp et al., 2018b). Cheirsilp et al. (2018b) reported that a high amount of lactic acid was produced by *L. kefiranofaciens,* which quickly dropped the medium pH and hindered the cell growth and kefiran production. Thereafter, the decrease in kefiran upon prolonged fermentation might be ascribed to the enzymatic decomposition of the polysaccharides by a depolymerizing enzyme or a shift in the physical parameters in the culture medium. It has been reported in several studies that the LAB release cytoplasmic hydrolases after certain time of incubation, which would contribute to degrade the polysaccharides (Pham et al., 2000, Rimada and Abraham, 2001). Pham et al. (2000) observed the presence of a wide-ranging glycol-hydrolases (α-D-glucosidase, β-D-

glucosidase, α-D-galactosidase, β-D-galactosidase, β-D-glucuronidase, and some traces of α-L-rhamnosidase) in the fermentation medium, which degraded the exopolysaccharides. It can be noted here that the kefiran is a water-soluble exopolysaccharide, which is extensively used as stabilizer, thickener, emulsifier, fat substitute or gelling agent. Moreover, it possess antifungal, antibacterial, and antitumor activities, and retains positive effects on cholesterol metabolism (Saadat et al., 2019). Therefore, kefiran could widely be utilized in the food and pharmaceutical industries.

6.3.5 Production of volatile flavour substances

The production of volatile flavour substances in the different media were analyzed during the fermentation of EA-lactose, -whey and -whey permeate as well as their corresponding non-EA-substrates, and the obtained results are shown in **Figure 6.7**. The results demonstrated that the main volatile compounds in the media were aldehydes, esters, alcohols, carboxylic acids, furans, and aromatic hydrocarbons. Generally, the microorganisms present in the kefir microbiota yield a plethora of enzymes that could contribute to produce different volatile compounds through carbohydrate degradation, lipolysis, and proteolysis during fermentation (Magalhães et al., 2011a). Such enzymes are peptidases which are involved with the conversion of proteins and peptides into free amino acids (FAAs), and further degradation to volatile aroma compounds. Other enzymes are lipases and esterases, which hydrolyse triglycerides into various free fatty acids (Dragone et al., 2009b).

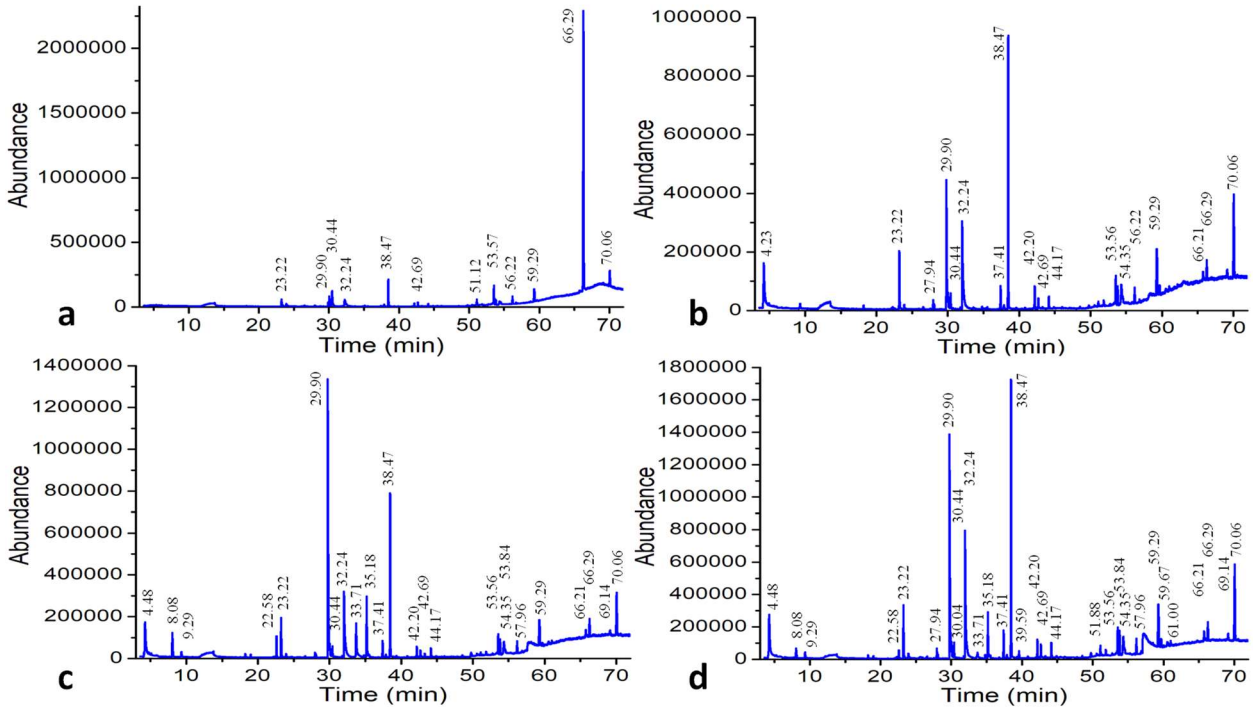

Figure 6.7: GC-MS chromatograms for volatile flavour substances a) whey, b) EA-whey, c) whey after 72 h of fermentation, and d) EA-whey after 72 h of fermentation. Ethylene oxide (4.23), acetaldehyde (4.48), ethanol (8.08), 3-methyl-1-butanol (9.29), 3-hydroxy-2-butanone (22.58), 2-methyl-1-propanol (23.22), 2-propenoic acid, 2-hydroxyethyl ester (27.94), acetic acid (29.90), 1-propanol (30.04), furfural (30.44), formic acid (32.24), 2-propanol (33.71), 2,3-butanediol (35.18), butyrolactone (37.41), 2-furanmethanol (38.47), 1-methyl-2-pyrrolidinone (39.59), 2(5H)-furanone (42.20), 1,2-cyclopentanedione (42.69), 2,5-dihydro-furan (44.17), 3-furancarboxylic acid-methyl ester (51.12), malic acid (51.88), ethanimidic acid-ethyl ester (53.57), hexanal (53.84), thiazole (54.35), 2-oxo-butanoic acid (56.22), 2-pentanol (57.96), butanal (59.29), alpha.-hydroxy-2-furanacetic acid (59.67), ethyl ethanoate (61.00), 2-fluorobenzyl alcohol (66.21), 2-5-(hydroxymethyl)-furancarboxaldehyde (66.29), formic acid, 1-methylpropyl ester (69.14), penta-ethylene glycol (70.06).

Specially, the yeasts present in the whole kefir culture such as *Kluyveromyces marxianus*, *Saccharomyces cerevisiae*, *Saccharomyces unisporus*, *Torulaspora delbrueckii*, *Candida kefir*, *Pichia fermentans*, *Yarrowia lipolytica*, perform a pivotal role to produce flavour and aroma (Simova et al., 2002). More specially, being a lactose-positive yeast, *K. marxianus* var. *lactis* present in the kefir grains' microbiota confirms the metabolism of whey lactose via alcoholic fermentation and the production of classic yeasty aroma and flavour (Simova et al., 2002, Karim et al., 2020a). Furthermore, lactose-negative yeasts could have contributed to the formation of stronger yeasty aroma with the refreshing pungent taste (Simova et al., 2002). Nevertheless, most of the volatile flavour substances produced in the

present study are comparable to those studies reported for other fermented beverages (Güzel-Seydim et al., 2000, Magalhães et al., 2011a, Ayseli and Ayseli, 2016). Therefore, the findings of the present study argue that novel beverages of acceptable organoleptic properties could be produced using whey-based fermentation by whole kefir microflora as starter culture. Indeed, the volatile flavour substances produced in the present study could potentially be used in food, flavour and fragrance, pharmaceutical, and other chemical industries, as depicted in **Table 6.2**.

Table 6.2: Predominant aroma compounds produced during the fermentation of whey-based substrates by kefir culture and their applications.

Aroma compounds	**Flavours**	**Applications**
Ethyl acetate (ethyl ethanoate)	Sweet and fruity	To bring a fruity and sweet flavor to candy, baked goods, gum, etc. To extract tobacco from cigarettes, decaffeinate tea leaves, and decaffeinate coffee. To produce adhesives, inks, photoresists, and coating formulations (Löser et al., 2013b)
Isoamyl alcohol (3-methyl-1-butanol)	Banana like flavour	Fragrances and flavours industry (Hoşoğlu, 2018a)
Isobutyl alcohol (2-methyl-1-propanol)	Sweet, musty, and wine-like smell	In the manufacturing of paints and lacquers, synthesis of organics, and resin coatings (Cseri et al., 2018)
Furfuryl alcohol (2-furanmethanol)	mild alcohol or ether-like odor	To synthesize furan resins, molds, laminating resins, food flavoring, fragrance in personal care products, and pesticide (Kumar et al., 2018)
Furfural	Grainy, biscuity, or almond-like	Furfural derivatives are used in the food industry for artificial flavors, colors, and packaging of food items (Ershov et al., 2017)

2,3-butanediol	Fruity, creamy, buttery, sweet	Cosmetics, food, transport fuels, medicines, antifreeze agents, polymers, and others (Chandel et al., 2018)
Isopropyl alcohol (2-propanol)	Sharp aromatic odor	Used in solvents, disinfectants, pharmaceuticals, antifreeze, inks, and other household products (Cseri et al., 2018)
Ethyl alcohol	Sweet smell, stinky	Flavours and aromas, cosmetics, disinfecting agents, medicine, etc. (Singh et al., 2017b)
Hexanal	Fruity flavours	Flavor ingredient in foods, fragrance in perfumes (Surburg and Panten, 2016)
Butyrolactone	Smells like acetone or mint	In food industry, this is used as a food additive to increase the aroma of food while in agriculture, for production of plant growth agent and pesticides (Ayseli and Ayseli, 2016)
Acetic acid	Pungent smell and vinegar like	Utilized for creating pickling liquor and also used in flavorings and confectionary items (Janiszewska-Turak et al., 2016)
Formic acid	Pungent and vinegar like	Used as Disinfectant, antiseptic, wine making ingredient, juice and food preservative (Anyasi et al., 2017)

6.4 Discussion

In this study, a whole kefir grain microbiota was used as a starter culture to utilize several whey derived waste substrates including lactose, whey, and WP and their corresponding EA-solutions as the potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids, kefiran which is a functional exopolysaccharide, and volatile compounds. It can be observed that all substrates used in this study showed acceptable growth of the microorganisms. This fact reflects that all these media have sufficient nutrients to support growth of microorganisms and their metabolism. It is well established that the most of LAB use glucose and galactose as the primary source of energy,

but some of them also prefer lactose. This is probably because lactose is the key sugar component of milk and fermented milk or kefir, from where the consortium derived. Besides LAB, certain yeast strains are also capable of utilizing the disaccharides (lactose and lactulose) by producing *β*-galactosidase enzyme to hydrolyze *β-(1,4)* link (Cheirsilp et al., 2018b). Lactose is transported into the cell by LacS permease and phosphoenolpyruvate dependent phosphotransferase system, and subsequently, 6-P-*β*-galactosidase hydrolyzes it to glucose by the glycolytic pathway and to galactose-6-P by the D-tagatose-6-P pathway, or glucose and galactose by the Leloir pathway (Liu et al., 2020). Generally, lactose is broken down to galactose and glucose by *β*-galactosidase while lactulose to galactose and fructose (Karim et al., 2020a). Thus, lactose and lactulose quickly decreased during their growth phase because both sugars were efficiently utilized by kefir consortium and this fact was consistent with the previous reports (Magalhães et al., 2011a, Cheirsilp et al., 2018b). Moreover, Güzel-Seydim et al. (2000) reported that the lactose could immediately be degraded to galactose and glucose by Group N streptococci, *Lactobacillus*, and by several strains of *Kluyveromyces*.

Nevertheless, the accumulation of various organic acids such as lactic and acetic acids generated by different metabolic pathways gradually decreased the medium pH (**Figure 6.1b**) as the experiments were carried out under uncontrolled conditions. Thus, the formation of these organic acids inhibited microbial growth (**Figure 6.1a**) both in their dissociated form or undissociated form or indirectly by the protons (H^+) which were released in the media (Charalampopoulos et al., 2002a). It is interesting to note that the lactose media exhibited the lowest concentration of organic acids measured (**Figure 6.4**), however, the pH was rapidly decreased and experienced a lower pH value than other media (whey and WP). This is probably due to the production of other acids or acidic metabolites in the lactose medium (**Figure A10**). Another possible reason could be the low buffering capacity of the lactose media compared to the other whey-based media used in this study, which validating the importance of substrate composition on the overall performance of the fermentation process (Charalampopoulos et al., 2002a).

The growth pattern and overall fermentation dynamic indicate the microbial interaction based on the substrate utilization and metabolites production. Basically, kefir microflora contains various kinds of microorganisms including homofermentative LAB,

heterofermentative LAB, lactose-positive yeast, and lactose-negative yeast (Adachi, 1990). LAB are generally considered fastidious and need several additional nutrients such as amino acids and vitamins, whilst yeast can flourish in a comparatively simple medium (Ponomarova et al., 2017). Therefore, it could be speculated that yeast renders the waste substrates to be easily hydrolyzed in a micro-aerobic environment because the sugars and proteins in the media converted to alcohol and water, so that the decomposition of organic substances accelerates due to rapid completion of hydrolysis (Li et al., 2015). It is apparent that the lactose assimilating yeasts commenced the fermentation through the production of enzymes to hydrolyze the lactose into glucose and galactose and lactulose to galactose and fructose. Consequently, they were producing alcohol with glycerol, succinic acid, fatty acids, etc. as their metabolites in the media. The lactose-negative yeast was supposed to persist through the consumption of galactose and lactic acid as their carbon and energy sources (Tada et al., 2007, Cheirsilp and Radchabut, 2011). On the other hand, the glucose, fructose, and galactose were utilized by LAB as their carbon source while the ethanol in the media probably became nutrient source for the AAB. Consequently, the enzymes secreted by AAB could oxidize ethanol to produce acetic acid, glycerol to create fructose and diketone, and glucose to malic acid, gluconic acid, and other organic acids, respectively (Li et al., 2015). Nevertheless, the lactic acid produced by LAB might again be utilized by non-lactose assimilating yeast (Cheirsilp et al., 2018b, Tu et al., 2019). Furthermore, the yeast could eliminate hydrogen peroxide (H_2O_2) that is known as an inhibitor for LAB growth (Cheirsilp et al., 2003). Therefore, it can be urged that the intriguing interactions of microorganisms based on synergetic relationship enabled the LAB, yeast, and AAB to mutually co-exit and function collectively in the fermentation media. However, the regulatory decisions which provoke a microorganism to produce beneficial metabolites that establish the basis of interspecies metabolite exchange is yet to be studied in detail. In a study, Ponomarova et al. (2017) stipulated some insights of regulatory processes motivating yeast to secrete metabolites. They demonstrated that the different metabolites, especially, amino acids secreted by yeast would have a pivotal role in the synergistic interaction of yeast-LAB and the production of amino acids by yeast was dependent on total nitrogen sources in the medium.

The EA-substrates achieved a higher growth and biomass production compared to the non-EA-substrates ascribed to the favorable composition of different sugars such as lactose

(62%), lactulose (31%), and galactose (~5%) (Kareb et al., 2018a). Furthermore, the enhanced growth in the EA-substrates could partially be explained by their improved functionalities with prebiotic lactulose which was generated through *in situ* electro-isomerization of lactose (Karim and Aider, 2020d, Karim and Aïder, 2020). Notably, lactulose is a renowned prebiotic, which is recognized as a bifidogenic factor contributing to stimulate the growth of different probiotics (Kareb et al., 2018a) including yeasts such as *Saccharomyces cerevisiae* and *Kluyveromyces marxianus*. Beside prebiotic lactulose, EA-media contain lactose, glucose, and galactose (Karim and Aider, 2020a), which may increase carbohydrate availability and thus, boosting the fermentation media for optimum growth of probiotic microorganisms (Kareb et al., 2018a). It can be postulated that the microbial growth was promoted by easy assimilation of readily available monosaccharides i.e., galactose, followed by the disaccharides: lactulose and lactose (Kareb et al., 2018a). Furthermore, EA-substances transformed to a metastable state during the EA process, which was characterized by a high alkalinity and a negative oxidation-reduction potential (-500 to -800 mV) (Karim and Aider, 2020a, d). Indeed, the higher reductive potential of EA-substrates (antioxidative medium) than the non-EA-substrates (Djouab and Aïder, 2019c, Karim and Aider, 2020a, d), could have promoted microbial growth by lowering/balancing the redox potential of the fermentation medium and creating reducing non-stressing growth conditions (Kareb et al., 2018a). In addition, the EA-substrates required a shorter time compared to non-EA-substrates to obtain the maximum growth attributed to the excessive internal potential energy of the highly active EA-solutions (Karim and Aider, 2020a), which could reduce the activation energy required for the metabolism of microorganisms in the electro-activated growth media and escalated the microbial growth. Consequently, a higher biomass growth and metabolite production was obtained using EA-substances. Nevertheless, formation of free radicals including hydroxyl radicals and superoxide anion radicals, is an unavoidable consequence during respiration of aerobic microorganisms. The free radicals and reactive oxygen species (ROS) are very unpredictable and react promptly with other groups or molecules in living cells, thus may influence cellular functions by damaging lipids, proteins, and DNA (Saini et al., 2017b). EA-media could have a high radical scavenging activity due to their improved antioxidant property.

Notably, EA-whey was the most efficient medium among the different EA-substrates used, which could be accredited to the favorable composition (e.g., C/N ratio) of the medium. This is because the EA-whey contains enough free amino acids, vitamins, small peptides, and minerals, which could be interesting to stimulate the growth of microorganisms (Kareb et al., 2018a). It has been previously reported that the proteins, peptides, and acid casein hydrolysates in the whey are essential to promote microbial growth (Gomes et al., 1998b). Furthermore, the whey proteins were partially hydrolyzed to small peptides during the EA process (Kareb et al., 2017a), which makes them readily accessible for direct uptake using minimal energy to release FAAs than the intact proteins in the non-EA-whey. Thus, EA-whey provides an easy availability of the requisite bioactive peptides or FAAs as efficient nitrogen source (Kareb et al., 2018a). Likewise, the EA-WP and EA-lactose achieved a lower growth than the EA-whey, which might be attributed to their insufficient FAAs and small peptides content, that are crucial as nitrogen sources.

In addition, EA-whey possess increased buffering capacity because of having whey proteins and this could increase the efficiency of a fermentation process. Basically, whey, especially EA-whey, contains various buffering compounds such as whey proteins and their corresponding degradation products, small peptides and other inorganic or organic compounds, which may increase their buffering capacity (Salaün et al., 2005b). This increased buffering capacity would result in sluggish pH decrease (slow acidification rate) and enhance the efficiency of fermentation process in terms of improved fermentation time and products formation (Charalampopoulos et al., 2002a). Indeed, the evolution of pH in the medium substantially influence the fermentation time and the levels of various metabolite formation in the final product. Nevertheless, the hydrolysis of whey proteins and concurrent glycation with reducing sugars would occur under EA process, leading to the formation of increased Maillard reaction products, which may greatly enhance the antioxidant capacity and bifidogenic effect of EA-whey than other media (Kareb et al., 2017a, Nooshkam et al., 2020). In a recent study, Kareb et al. (2018a) claimed that the EA-whey capable of preventing the accumulation of toxic H_2O_2 produced by some LAB under aerobic condition. Thus, it could act as an antioxidant paying attention to the cell membrane lipid oxidation by ROS and limit their undesirable effect during the growth of microorganisms. Henceforth, it can be disputed that not only the symbiotic interactions among the microorganisms of kefir

consortium but also a substrate-inoculum synergistic relationship exits in the fermentation process using EA-whey based substrates and a whole kefir microbiota as inoculum. Therefore, the present study argues that the EA-substrates, especially, EA-whey could efficiently be utilized in the bioprocess to produce fermented products and valuable metabolites.

ANNEXE D

Standard calibration curves for sugar consumption (Figure A8); Standard calibration curves for organic acids production (Figure A9); HPLCchromatograms for organic acids production in different media (Figure A10).

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Chapitre 7: Bioconversion of electro-activated lactose, whey and whey permeate to produce single cell protein, ethanol, aroma volatiles, organic acids and fat by *Kluyveromyces marxianus*

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RÉSUMÉ

Le but de cette étude était de produire de la biomasse et des biomolécules possédant des propriétés aromatiques par la fermentation de lactosérum électro-activé (EA-Whey), de perméat de lactosérum électro-activé (EA-WP) et de lactose électro-activé (EA-lactose) en utilisant *Kluyveromyces marxianus* ATCC 64884. Le processus de fermentation a été réalisé pendant 96 h à 30 °C sous une agitation à 150 rpm. Les résultats ont montré que tous les substrats ont permis d'obtenir une accumulation satisfaisante de la biomasse et que la fermentation dans du EA-Whey a atteint une biomasse maximale (4,23 g/L) à 96 h de fermentation suivie de celle réalisée dans du bouillon YM (4,85 g/L). La biomasse produite avait une teneur élevée en protéines et en lipides (24,43-57,83 et 15,44-25,64 %, respectivement) en fonction des substrats utilisés et des conditions de fermentation. Plusieurs acides organiques majeurs dont les acides lactique, acétique, citrique, propionique ont été produits pendant la fermentation sur tous les milieux, avec des différences significatives entre les substrats électro-activés et non électro-activés. *K. marxianus* a produit divers composés aromatiques volatils aux propriétés organoleptiques appréciées. Le bouillon YM a entraîné la plus faible production d'éthanol (8,42 g/L à 48 h) tandis que la plus forte production d'éthanol a été produite dans le lactosérum non électro-activé (28,13 g/L à 48 h), suivi du lactose (27,85 g/ L à 48 h), du EA-lactose (26,77 g/L à 36 h), du perméat de lactosérum (WP) (25,99 à 72 h), du EA-WP (24,66 g/L à 36 h) et du EA-Whey (22,06 g/L à 48 h). De plus, un maximum de 393,85 à 988,22 mg/L de 2-phényléthanol a été atteint, selon les substrats utilisés. Ces résultats suggèrent que l'électro-activation peut être utilisée pour améliorer l'utilisation du lactosérum et de produire des dérivés à partir de bioprocédés en utilisant *K. marxianus* comme souche de fermentation.

Mots-clés: Substrats à base de lactosérum; Électro-activation; *Kluyveromyces marxianus*; Production de biomasse; Acides organiques; éthanol; 2-phényléthanol; Substances aromatiques volatiles; gras.

ABSTRACT

The aim of this study was to produce biomass and valuable biomolecules with aroma and flavor properties through fermentation of electro-activated whey (EA-Whey), electro-activated whey permeate (EA-WP), and electro-activated lactose (EA-lactose) using *Kluyveromyces marxianus* ATCC 64884. The fermentation process was performed for 96 h at 30 °C and 150 rpm. The results showed that all substrates provided a satisfactory biomass growth and EA-Whey achieved a maximum biomass (4.23 g/L) at 96 h of fermentation followed by YM broth (4.85 g/L). The produced biomass had high protein and lipid content (24.43-57.83, and 15.44-25.64%) depending on the used substrates and fermentation conditions. Several major organic acids including lactic, acetic, citric, propionic acids were produced during the fermentation on all media, with significant differences between electro-activated and non-electro-activated substrates. *K. marxianus* produced various volatile aroma compounds with valued organoleptic properties. The YM-broth resulted in the lowest ethanol production (8.42 g/L at 48 h) while the highest ethanol was produced in the non-electro-activated whey (28.13 g/L at 48 h), followed by lactose (27.85 g/L at 48 h), EA-lactose (26.77 g/L at 36 h), WP (25.99 at 72 h), EA-WP (24.66 g/L at 36 h), EA-Whey (22.06 g/L at 48 h). Moreover, a maximum of 393.85 to 988.22 mg/L of 2-phenylethanol was achieved, depending on the substrates used. These findings suggest that electro-activation can be used to improve the use of whey and its derivatives in *K. marxianus* driven bioprocesses to produce valuable metabolites for different applications, including in foods.

Keywords: Whey-based substrates; Electro-activation; *Kluyveromyces marxianus*; Biomass production; Organic acids; Ethanol; 2-phenylethanol; Volatile flavour substances; fat.

7.1 Introduction

Whey and whey permeate (WP) are the main agro-industrial by-products in the cheese or casein production process. Whey is the liquid residue remaining after milk has been curdled and strained and WP is a co-product which is obtained after recovering whey proteins from liquid whey by a combination of ultrafiltration with diafiltration to remove lactose and minerals. The global production of whey is estimated to 190×10^6 tons/year, and Canada produces 4% of the total whey (Kaur et al., 2020, Ramos et al., 2021). In 2019 for example, Canada was reported as one of the ten major cheese manufacturer countries, recording 5.2×10^5 tons of cheese (Valdez Castillo et al., 2021). Typically, a volume of ≈9 kg of whey is generated in the production process of 1 kg of cheese (Park and Haenlein, 2013). Thus, it can be estimated that the production of whey was approximately 4.7×10^6 tons in Canada in 2019 (Valdez Castillo et al., 2021). The major constituents of whey/WP is lactose (and its derivatives galactose and glucose), minerals, whey proteins such as α-lactalbumin and β-lactoglobulin, as well as other small molecules such as peptides and free amino acids (Yadav et al., 2015c). Discarding raw cheese whey or/and WP creates huge disposal problems because they have high chemical oxygen demand (COD) and biological (biochemical) oxygen demand (BOD) with a value of 60,000-80,000 and 30,000-50,000 mg/L O_2, respectively, which is mainly due to their high lactose content (Karim and Aïder, 2020). Therefore, this remains a huge challenge for the dairy industry and researchers to develop economical and sustainable approaches of valorization of these industrial co-products and that is why industries are multiplying different processes to produce various products with good commercial value utilizing these low cost co-products from dairy industries (Yadav et al., 2015c).

Recently, biological routes are gaining attention for valuable metabolites production by utilizing the low-cost by-products as carbon and nitrogen sources (Yadav et al., 2015c). The microbial conversion of lactose and whey/WP to valuable fermented metabolites such as organic acids, enzymes, volatile flavour substances, fatty acids, and single-cell proteins could be a sustainable approach for whey management and valorization (Karim et al., 2020b). Also, this way of valorization presents some advantages such as a possibility of using whey and whey permeate as a basic growth media and conduction of the fermentation processes at

temperatures around 25-30 °C, making the process highly energy saving. However, it is also important to mention that raw whey/WP is a tricky-type of waste by-product to handle, which can be quickly acidified because of its high organic matter content with inadequate bicarbonate alkalinity (Seo et al., 2014a). Another main problem is low total solids content and high lactose to glucose ratio, that makes fermented beverages from these raw materials watery with unpleasant taste (Tesfaye et al., 2019). Furthermore, many microorganisms including probiotic bacteria showed poor growth in the milk-based substrates because of their limited lactose assimilation ability, weak proteolytic activity, and inadequate oxidation-reduction potential of the substrates (Kareb and Aïder, 2018). Hence, there is still an immense need to explore the efficient substrates or suitable supplements to incorporate with substrates for promoting the growth of microorganisms when whey and whey permeate are used as growth media.

Several recent studies (Kareb and Aïder, 2018, Karim and Aïder, 2020) demonstrated that the electro-activation of lactose and whey/WP is highly efficient to convert lactose into lactulose and other simple sugars such as galactose, glucose, and fructose at a desired alkaline condition, which in turn may promote microbial growth by ease assimilation of readily available monosaccharides and prebiotic lactulose. Indeed, lactulose is a well-documented disaccharide with proven prebiotic potential. Therefore, it is hypothesized that the challenges of raw whey and its derivatives utilization could be defeated by using electro-activated (EA) substrates to produce value-added metabolites via fermentation of whey-based media. Recently, *Kluyveromyces marxianus*, a nonconventional yeast species, has piqued immense research interest, because of their ability to utilize a broad range of sugars including lactose and lactulose, thermotolerance, secretion of lytic enzymes, the highest growth rate than other eukaryotes, and the production of ethanol by fermentation (Karim et al., 2020b). This yeast also showed an ability to enhance organoleptic properties through the formation of numerous volatile compounds (e.g., ethyl acetate, acetaldehyde, isobutyl alcohol, etc.), to produce valuable enzymes and metabolites such as β-galactosidase, functional exopolysaccharides and free amino acids (Karim et al., 2020b). In addition, the yeast produces carbon dioxide and alcohol during the fermentation, which could increase mouthfeel and taste of the fermented products. Moreover, *K. marxianus* has obtained Qualified Presumption of Safely (QPS) and Generally Regarded As Safe (GRAS) status in European Union and the United

States, respectively, which makes it particularly suitable to produce pharmaceuticals and food-grade proteins (Karim et al., 2020b).

The main objective of this study is to explore the feasibility of a bioprocess utilizing electro-activated whey-based substrates including electro-activated lactose (EA-lactose), electro-activated whey (EA-whey), and electro-activated whey permeate (EA-WP) as the potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids and volatile compounds using *K. marxianus*.

7.2 Materials and methods

7.2.1 Yeast strain and maintenance

The yeast strain used in this study, *Kluyveromyces marxianus* (ATCC® 64884™) was purchased from American Type Culture Collection (ATCC). Yeast cells were maintained in M-BCG Yeast and Mold Broth (YM broth) medium containing glucose (50 g/L), yeast extract (9 g/L), biopeptone (10 g/L), magnesium sulphate (2.1 g/L), potassium dihydrogen phosphate (2 g/L), diastase (0.05 g/L), thiamine hydrochloride (0.05 g/L), bromocresol green (0.026 g/L), with the addition of 40% glycerol (v/v) at -80 °C. Briefly, a volume of ≈1 mL water was withdrawn with a sterile pipette from a single test tube of sterile distilled water (≈10 mL) and applied directly to the pellet (lyophilized cells) to form a suspension. Then the suspension was aseptically transferred back into the test tube of sterile distilled water and kept it at 25 °C undisturbed for overnight for rehydration. Thereafter, the cells were grown in 10 mL YM broth for 24 h until the exponential growth phase. Finally, 0.5 mL yeast culture was added to 0.5 mL of 40% sterile glycerol (v/v) solution to a final concentration of 20% (v/v) in a 1.5 mL cryovials. The vials with 1 mL aliquots of this culture were inverted thoroughly to mix cells and glycerol, then were stored frozen at -80 °C. The stock culture was revitalized and precultured in YM broth or agar medium before any experiment.

7.2.2 Inoculum preparation

To revive the stored cells, the cultures were activated from glycerol stock in the M-BCG Yeast and Mold Agar (YM agar) plates comprising of glucose (50 g/L), yeast extract (9 g/L), biopeptone (10 g/L), magnesium sulphate (2.1 g/L), potassium dihydrogen phosphate (2 g/L), diastase (0.05 g/L), thiamine hydrochloride (0.05 g/L), bromocresol green (0.026 g/L), agar (15 g/L). Briefly, a frozen stock cryovial was collected and scraped off splinters of solid ice with a sterile inoculation loop/toothpick and streaking onto the used YM agar to

grow at 30 °C. The inoculum for fermentation was prepared by transferring a loopful of yeast cells from freshly grown culture (the colonies that appeared after 48 h) into 250 mL baffled Erlenmeyer flask containing 50 mL of YM growth medium. Thereafter, the flask was incubated in a rotary shaker incubator operating at 150 rpm at 30 °C for 20 h, so that the concentration of cells in the medium corresponded to 0.8 OD (optical density, $OD_{600} = 0.8$). Finally, the tested cultivation media (electro-activated whey EA-Whey, electro-activated whey permeate EA-WP, electro-activated lactose EA-Lactose, and their corresponding none electro-activated media) as described below were inoculated with 5% (v/v) of this revitalized *K. marxianus* preculture.

7.2.3 Culture media and growth conditions

K. marxianus was inoculated to seven growth media to study the efficacy of different substrates for producing biomass and metabolites. The composition of the selected media is summarized in **Table 1**. The media are comprising mainly of a number of carbon sources including lactose, lactulose, and galactose. The 6% WP and 7% whey (Agropur Co-operative, Longueil, Canada) were chosen considering the equivalent concentration of 5% lactose (Sigma-Aldrich, St. Louis, MO, USA), that are usually obtained from cheese manufacturing industry as co-products. Thus, they could be utilized directly after their production from industry without further diluting or concentrating step. The initial pH of all media was adjusted to 6.0 ± 0.1 using acidic solution produced in the anodic compartment during the electro-activation process (Karim and Aïder, 2020) or 0.1 N NaOH. All media were sterilized by passing through a micro-cellulose filter of 0.22 µm pore size (Sigma-Aldrich, St. Louis, MO, USA). It can be noted that the whey-based substrates were used in this study without addition of any nutrient supplements.

Table 7.1: The definition and composition of seven cultivation media used for cell growth and metabolites production.

No.	Medium Name	Characteristics	Composition
1	YM broth	Yeast-Mold medium	Yeast extract, peptone, soluble starch, glucose, cysteine hydrochloride, sodium chloride, sodium acetate, agar: 13, 10, 1, 5, 0.50, 5, 3, 0.50 g/L, respectively.
2	Lactose	5% lactose solution	Lactose (50 g/L)
3	EA-Lactose	5% lactose solution after electroactivation with 300 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~4%, and traces of glucose and fructose (Karim and Aider, 2020a)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~1 g/L)
4	WP	6% whey permeate solution (as equivalent to 5% lactose solution)	Lactose (50 g/L) and traces of glucose and galactose
5	EA-WP	6% whey permeate solution after electroactivation with 600 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~6%, and traces of glucose and fructose (Karim and Aïder, 2020)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~1.52 g/L)
6	Whey	7% whey solution (as equivalent to 5% lactose solution)	Lactose (50 g/L) and traces of glucose and galactose
7	EA-Whey	7% whey solution after electroactivation with 900 mA current intensity for 60 min; lactose: ~62%, lactulose: ~31%, galactose: ~7%, and traces of glucose and fructose (Karim and Aider, 2020d)	Lactose (~30 g/L), lactulose (~15 g/L), galactose (~2.55 g/L)

EA-Lactose: Electro-activated lactose. EA-WP: Electro-activated whey permeate. EA-Whey: Electro-activated whey.

7.2.4 Growth profile and cell concentration determination

The growth profile of *K. marxianus* cells in the different tested media were studied for 96 h of cultivation. In brief, 100 mL of each cultivation (growth) media was prepared in the sterilized baffled Erlenmeyer flasks (250 mL) and was injected with 5% (v/v) of inoculum. The flasks were then covered with cotton rolled and aluminum foil. The inoculated flasks were nurtured in an incubator shaker (Innova®44, NJ, USA) at 30 ℃ and 150 rpm for 96 h. The medium pH was observed during the cultivation by a pH meter (model: Oakton pH 700) equipped with an Oakton pH probe (Oakton Instruments, Vernon Hills, IL, USA). Samples were collected at 12 h intervals during the cell enrichment to evaluate the growth pattern based on optical density at 600 nm (OD_{600}) using a Biotek power wave spectrophotometer and Gen5 2.09 software (Biotek Instruments, model-XS2, Winooski, VT, USA).

7.2.5 Biomass collection and cell dry weight

The dry cell weight of biomass was determined in triplicate using a gravimetric method. Briefly, the fermented broths were taken in a centrifuge tube (50 mL falcon tubes) at different time of cell growth and centrifuged at 10,000 × g for 15 min at 4 ℃ (Beckman Coulter Avanti® J-E centrifuge, Indianapolis, IN, USA). The supernatant was then transferred to tube for further analysis and the precipitated cells were then centrifuged again with 0.9 M sodium chloride solution followed by deionized water. Thereafter, the supernatant was discarded immediately, and the cells were lipolyzed using a laboratory scale freeze dryer (Lyph-lock®4.5, Labconco corp., Kansas City, MO, USA). Finally, the dry cell weight was measured using a high precision analytical balance.

7.2.6 Protein and lipid production analysis

The freeze-dried biomass was used to analyze protein content following the combustion method using an Elementar rapid MICRO N Cube Nitrogen analyzer (Elementar Analysen systeme GmbH, Langenselbold, Germany). A conversion factor of 6.38 was used to calculate protein content (Mariotti et al., 2008a). Lipid content was determined using Folch's extraction (Folch et al., 1957) method with minor modifications (Islam et al., 2018, Karim et al., 2018b). Briefly, 100 mg of dry yeast cells were extracted with a solvent of 7.5 mL composed of a mixture chloroform/methanol (ratio 2/1, v/v) under ultrasonication (Bransonic, model: 5510R, 135 W, 42 KHZ) for 15 min (Ren et al., 2017). Thereafter, the mixture was centrifuged at 4000 rpm and 4 ℃ for 10 min. The solvent phase samples were

transferred into a pre-weighted watch glass using a Pasteur pipette. Then again, the remaining cell pellets were re-extracted with fresh solvent followed by water repeating the procedure as described above. The lower solvent phase was pipetted out for lipid analysis. The solvent and water were removed using a Buchi R-100 Rotavapor (Buchi, New Castle, DE, USA) system to obtain dry lipids. Finally, the lipid content in the extract was estimated gravimetrically after evaporation of the extracting solvent.

7.2.7 Evaluation of organic acids production

The organic acids were analyzed using a high-performance liquid chromatography (HPLC) system (Waters, Milford, MA, USA) equipped with a refractive index detector (Hitachi, L-7490 model, Tokyo, Japan) and an ICSep-ion300 (7.8 × 300 mm) column (Transgenomics, San Jose, CA, USA). The column temperature was set to 40 ℃. An isocratic mobile phase of 10 mM H_2SO_4 solution was used at a flow rate of 0.4 mL/min. A volume of 50 µL of sample was injected and analyzed for 50 min. The composition of various organic acids (lactic, citric, butyric, acetic, and propionic, etc.) was analyzed using ChemStation software and recognized by comparing their retention time to their corresponding standards. Finally, they were quantified using the standard calibration curves (**Figure A11**).

7.2.8 Volatile flavour compounds analysis

The samples were collected from the corresponding fermented broths at different time intervals and were analyzed directly without any pre-treatment according to Magalhães et al. (2011a) with slight modification to detect the volatile compounds in the fermented media using gas chromatography (GC). The experiment was performed by using a G1530A Agilent GC system (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an Agilent 5973N quadrupole mass spectrometer (MS) and was connected to a Split⁄Split-less automatic MPS injector (GERSTEL GmbH & Co. KG, Mülheim an der Ruhr, Germany). A DB-WAX capillary column with dimensions 60 m × 0.250 mm and 0.25 µm film thickness (Agilent, USA) was used. The carrier gas was helium maintaining a flow rate of 1 mL/min. The temperature of oven was programmed as the following: the temperature was set to 50 ℃ for 5 min and then increased to 220 ℃ at 3 ℃/min (held for 15 min). The quadrupole, source, and transfer line temperatures were retained at 150, 230, and 250 ℃, respectively. The injector inlet temperature was maintained at 250 ℃. A volume of 1.0 µL of samples were injected in the split mode (split ratio, 1:10). The mass spectra of electron ionization were

recorded at 70 eV in a full scan mode with the electron energy ranging from 35 to 325 amu. Finally, the compounds were identified in respect to mass spectra matching using a standard NIST05 library and retention index of authentic reference standard by Enhanced ChemStation software (version: MSD ChemStation E.02.02.1431). Ethanol, 2-phenylethanol, isoamyl alcohol were quantified using the standard calibration curves.

7.2.9 Statistical analysis

The experiments were performed for three times and the variation within the replicates were evaluated by calculating the standard deviation of the means. The mean values ± standard deviations were considered for the analysis. The differences at $p < 0.05$ were termed as significant. The differences between the different substrates were evaluated by analysis of variance (ANOVA) of the obtained data using the SAS software V9.3 (SAS Institute Inc., Cary, NC, USA).

7.3 Results and discussion

7.3.1 Cell growth and biomass production

The growth and biomass production by *K. marxianus* strain was studied using several whey-based media and the obtained results are presented in **Figure 7.1**. As it can be observed, **Figure 7.1a** showed that the cell growth kinetics followed similar trends for all substrates, where the YM broth achieved the highest growth followed by the electro-activated whey (EA-Whey) medium. No significant difference ($p > 0.05$) was observed in growth kinetics in whey permeate (WP) and electro-activated whey permeate (EA-WP) or lactose and electro-activated lactose (EA-lactose). However, a significant difference ($p < 0.05$) was noticed between the growth of *K. marxianus* in whey and EA-Whey media. As it can be seen from **Figure 7.1b,** the pH significantly ($p < 0.05$) decreased within 12 h of fermentation for all the used media. However, the pH in the YM medium was observed to increase with the fermentation time. The initial decrease in pH (increased acidity) in the whey-based media could be attributed to the adaptation of *K. marxianus* to the media with the production of organic acids including lactic and acetic acids or other acidic products (Alves et al., 2020). Another possible reason could be the production of CO_2 by yeast which contributed to increase the growth medium acidity, due to the presence by forming carbonic acid in the whey-based media (Alves et al., 2020). It is interesting to noting here that the various patterns

of pH adjustments were observed with fermentation time in the different media. This is probably due to the different mechanisms of metabolites formation pathway that were followed by *K. marxianus* in different media (Chua et al., 2018).

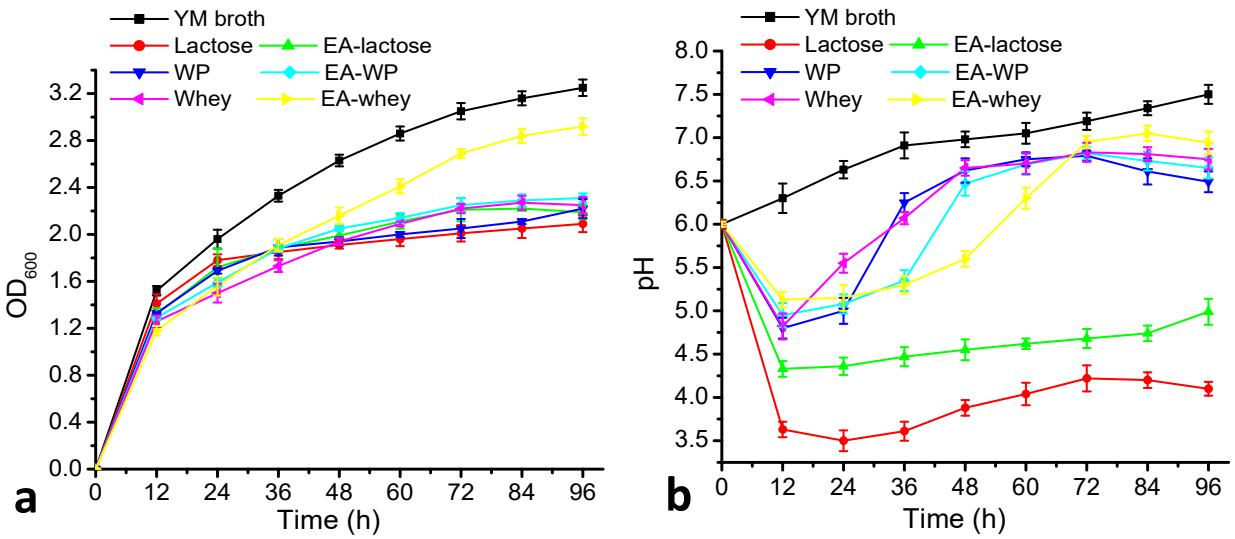

Figure 7.1: Growth of Kluyveromyces marxianus in different whey-based substrates at 30 °C for 96 h of fermentation. a) Biomass growth profile and b) pH evolution. EA-lactose: Electro-activated lactose. WP: Whey permeate. EA-WP: Electro-activated whey permeate. EA-Whey: Electro-activated whey.

Initially, the growth media composed of lactose was characterized by higher growth compared to the WP or whey ascribed to the fact that *K. marxianus* easily adapted to the simple substrates than the complex substrates. However, the decreased growth after certain time might be due to unfavorable pH of the lactose media (**Figure 7.1b**) or to some inhibitory effect of the formed metabolites or increased competition for essential nutrients. In fact, the deficiency in vitamins and specific minerals can lead to a growth limitation of *K. marxianus* (Charalampopoulos et al., 2002b). In contrast, the whey media achieved a higher growth than the lactose or WP which might be due the favorable composition of carbon and nitrogen sources such as whey proteins, peptides, and some residual casein hydrolases, which are vital to promote microbial growth (Gomes et al., 1998a). Furthermore, whey proteins also exhibit antioxidative potential via chelation of transition metal ions by lactoferrin or free radical scavenging activity by sulfhydryl containing amino acids (Khan et al., 2019). Fermentation of whey proteins probably enhanced the release of reductants such as cysteine present in

peptide chains of whey protein, thereby neutralizing the free radicals present in the medium (Alves et al., 2020).

The EA-substrates attained a better performance than the non-EA-substrates attributed to the favorable composition of various sugars such as lactose, lactulose, and galactose. Moreover, the superior growth in the EA-substrates might be partially justified by their improved functionalities by the presence of a prebiotic lactulose that was produced during the *in situ* electro-isomerization of lactose into lactulose following the electro-activation process of lactose, whey or whey permeate (Karim and Aider, 2020d, Karim and Aïder, 2020). Besides the favorable carbon sources and prebiotic effect of lactulose, other growth promoting factors of the EA-substrates such as high antioxidant activity might be associated to the elevated growth of *K. marxianus*, as observed in the present study (Kareb et al., 2018b, Djouab and Aïder, 2019b). The EA-Whey medium demonstrated better performance than EA-WP and EA-lactose, which could be attributed to the sufficient content of free amino acids (FAAs) and small peptides in the EA-Whey media. Indeed, the whey proteins were partially hydrolyzed to small peptides and free amino acids during the EA process (Kareb et al., 2017b), which makes them readily accessible for direct uptake by *K. marxianus* using minimal energy. Thus, EA-Whey provides an easy availability of the requisite bioactive peptides or free amino acids as efficient nitrogen source (Kareb et al., 2018b).

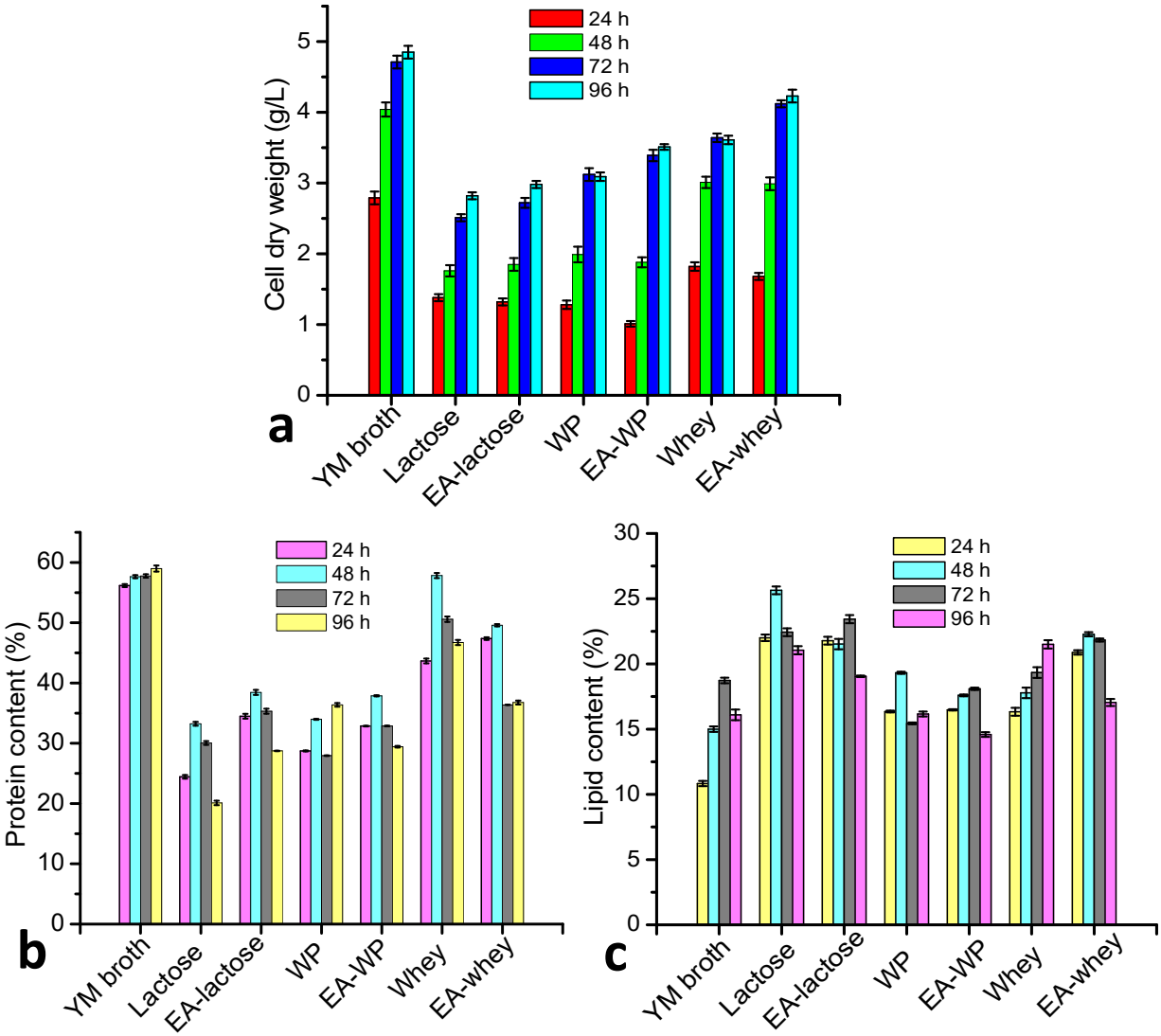

Figure 7.2: Biomass production by Kluyveromyces marxianus using different whey-based media. a) Cell dry weight, b) protein content, and c) lipid content in biomass. EA-lactose: Electro-activated lactose. WP: Whey permeate. EA-WP: Electro-activated whey permeate. EA-Whey: Electro-activated whey.

The production of biomass and its protein and fat contents were studied and is shown in **Figure 7.2**. It can be seen from **Figure 7.2a** that biomass considerably increased with increasing fermentation time and EA-Whey achieved a maximum biomass (4.23 g/L) at 96 h of fermentation, followed by the YM broth (4.85 g/L). Furthermore, a considerable increase of protein content in the biomass was noticed with increasing growth rate and it was reached a plateau after 48 h of fermentation (**Figure 7.2b**). The fermented biomass could be considered for protein enriched livestock feeds as they comprised high protein content

ranging from 24.43 to 57.83%, depending on the substrates used. The maximum protein content (57.83%) was obtained in the biomass of whey medium at 48 h, which was almost same as for the YM broth (58.99%) at 96 h. The protein content of *K. marxianus* cells during the batch fermentation in this study is in good agreement with previously reported values (35.9-71.9%) for the same yeast species (Fonseca et al., 2007).

Nevertheless, the *K. marxianus* biomass produced in all whey-based substrates was also rich in lipid content (**Figure 7.2c**). The highest lipid content of 25.64% was obtained in the biomass of the lactose-based fermentation medium while it was 22.27% in the biomass produced in the EA-Whey medium. This is probably because the lactose media would have reached carbon and nitrogen depletion stage prior to the other whey-based media used, and subsequently, the starvation of nutrients triggers lipid accumulation (Karim et al., 2021b). It was observed that the lipid content increased with increasing biomass accumulation, however, a reduction in the lipid content, in some cases, was also noted at the end of fermentation and this could be ascribed to the degradation of stored lipid inside the *K. marxianus* cells (Karim et al., 2021a). This is highly recognized that oleaginous microbes typically start to store lipids under the carbon/nitrogen limiting conditions, and consequently, the stored lipids begin to degrade after certain time (Karim et al., 2019b). The lipid content of *K. marxianus* obtained in the range of 15.44-25.64% is well corroborated with the study previously reported by Aggelopoulos et al. (2014), where they achieved a maximum fat content of 25.5% using a mixed food waste as substrate. Herein, it can be argued that the results in this study indicate the potential of EA-whey-based substrates to be used as highly efficient nutrient (carbon and nitrogen) sources for developing bioprocesses to produce protein/lipid enriched yeast biomass, which could be utilized as a supplement in animal feeds or human foods or for the extraction of fat for other uses.

7.3.2 Production of organic acids

The production of various organic acids such as acetic, butyric, citric, lactic, and propionic acids in the fermented broths were evaluated using HPLC and the obtained results are presented in **Figure 7.3** and **Figure A12)**. The EA-substrates were less pronounced to produce organic acids compared to the none-electro-activated substrates. The highest content of acetic (1173.84 mg/L) and propionic (358.23 mg/L) acids were produced in the whey medium after 72 and 96 h of fermentation, respectively. However, the highest amount of

lactic acid (1480.12 mg/L) was obtained in the WP after 12 h while citric acid (1908.43 mg/L) reached a maximum after 24 h in the whey medium. It is worth noting that the lactic and citric acids were reached a plateau within 12-24 h for all substrates used, then gradually decreased to lean towards zero at the end of the fermentation. Nevertheless, butyric acid was also observed in some cases, however, it was not quantified in this study. The production of organic acids in this study are comparable with those previously reported for different dairy substrates using the strains of *Kluyveromyces* (Leandro et al., 2019).

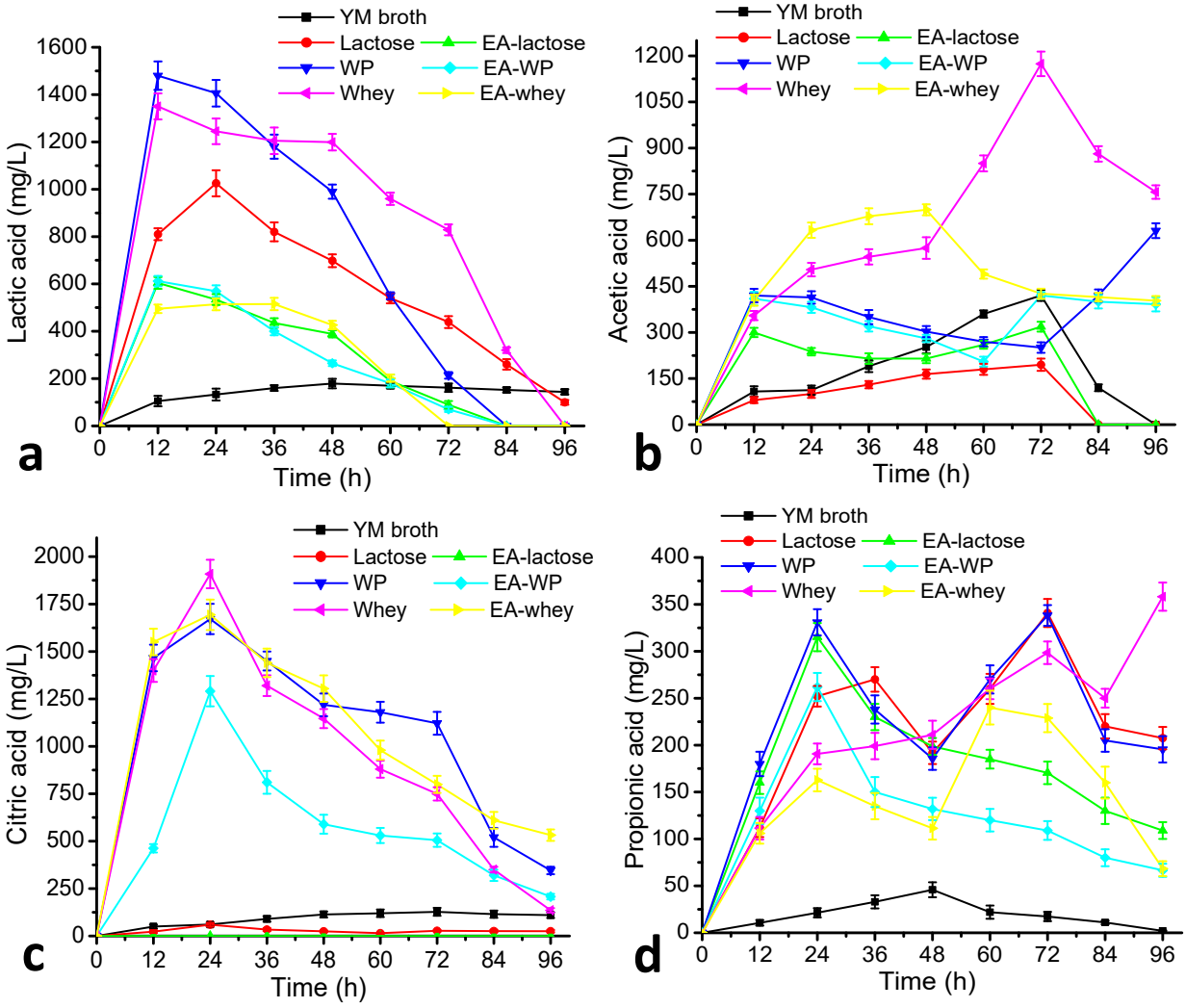

Figure 7.3: Organic acids production during the fermentation of different whey-based media at 30 °C a) lactic acid, b) acetic acid, c) citric acid, and d) propionic acid. EA-lactose: Electro-activated lactose. WP: Whey permeate. EA-WP: Electro-activated whey permeate. EA-Whey: Electro-activated whey.

It can be postulated that β-galactosidase enzyme was produced by *K. marxianus* in the tested media, which hydrolyzed lactose into glucose and galactose, and then transformed into lactic acid through a homo-lactic fermentation pathway. However, ethanol or acetate can be produced along with lactate by hetero-lactic fermentation pathway, which may decrease lactate yield. Nevertheless, lactate can also be converted into acetic, propanoic, or/and butyric acids via lactate consuming pathway, also decreasing the lactate yield (Asunis et al., 2020). Thus, it can be argued that *K. marxianus* produced organic acids such as lactic and citric acids utilizing the sugars present in the media, then they were again transformed into acetic, propanoic, and/or butyric acids, and other fermented compounds.

7.3.3 Production volatile flavouring compounds and bioethanol

The fermentation process is often related to the release of variety of metabolites which are accountable to improve the organoleptic properties of the fermented dairy products. To determine the key metabolites produced by *K. marxianus* in the whey-based substrates in the present study, volatile aroma-related compounds were analyzed during the fermentation and the result is shown in **Figure 7.4**. The findings showed that the most important volatile substances in the fermented media were aldehydes, esters, alcohols, carboxylic acids, and aromatic hydrocarbons. Specially, several well-recognized aroma compounds including acetaldehyde, ethyl acetate, ethyl alcohol or ethanol, isobutyl alcohol (2-methyl-1-propanol), isoamyl alcohol (3-methyl-1-butanol), isopropyl alcohol (2-pentanol), 2,3-butanediol, vinyl ether, 2-phenylethanol (2-PE) or phenylethyl alcohol, 2,3-butanediol, butanoic acid, diglycerol were predominantly observed in the tested media.

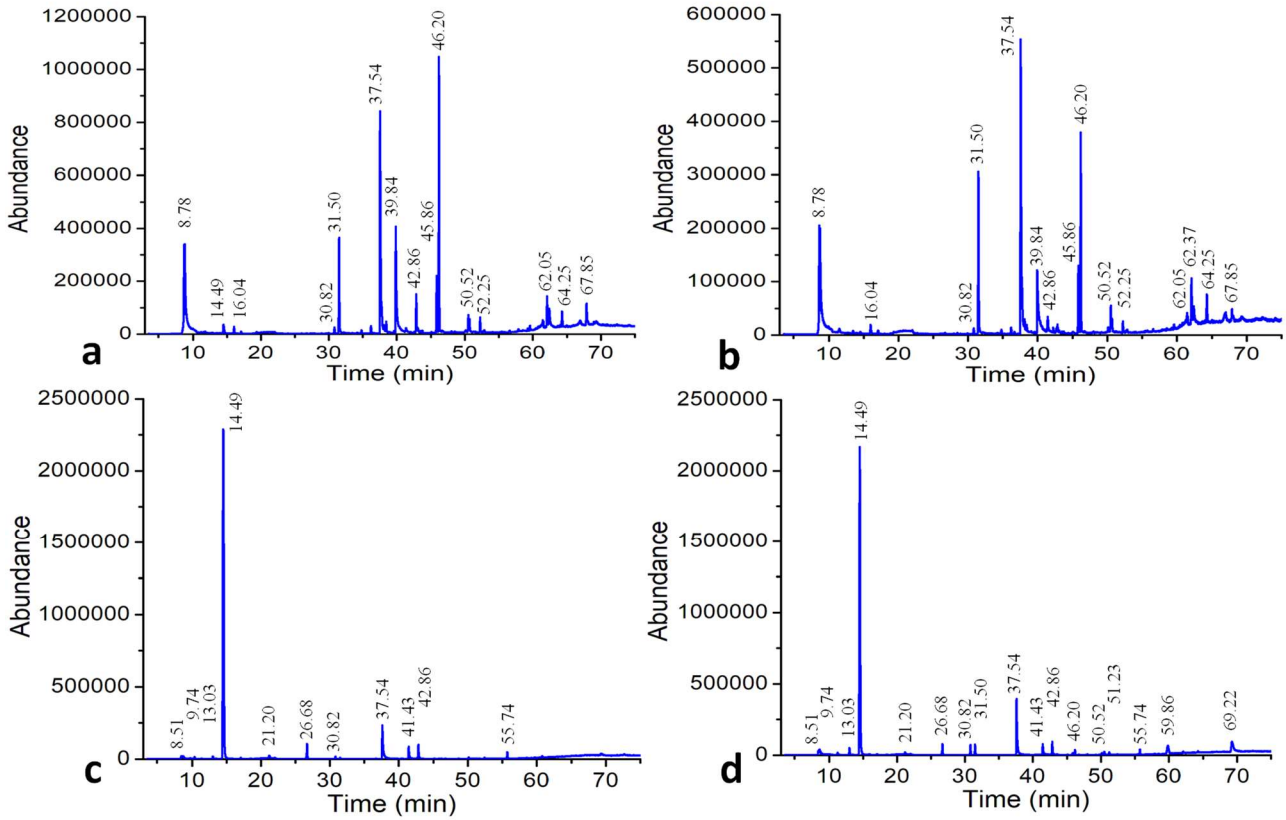

Figure 7.4: GC-MS chromatograms for volatile aroma compounds. a) whey before fermentation, b) EA-Whey before fermentation, c) whey after 72 h of fermentation, and d) EA-Whey after 72 h of fermentation. Ethylene oxide (8.51), carbon dioxide (8.78), acetaldehyde (9.74), ethyl acetate (13.03), ethyl alcohol (14.49), 3-methyl-2-butanone (16.04), 2-methyl-1-propanol or isobutyl alcohol (21.20), 3-methyl-1-butanol or isoamyl alcohol (26.68), 3-hydroxy-2-butanone (30.82), 1-hydroxy-2-propanone (31.50), acetic acid (37.54), formic acid (39.84), 2-pentanol or isopropyl alcohol (41.43), 2,3-butanediol (42.86), butyrolactone (45.86), 2-furanmethanol (46.20), 2(5H)-furanone (50.52), vinyl ether (51.23), 2,5-dihydro-furan (52.25), 2-phenylethanol or phenylethyl alcohol (55.74), trimethyl-urea (59.86), 2,2'-oxybis-ethanamide (61.47), 3-methyl-butanal (62.05), thiazole (62.37), 2-oxo-butanoic acid (64.25), 4H-pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl- (67.85), diglycerol (69.22).

The evolution of ethanol, 2-phenylethanol (2-PE), and isoamyl alcohol in the different fermented media were studied and is shown in **Figure 7.5**. The YM-broth obtained the lowest ethanol production of 8.42 g/L at 48 h while the highest ethanol produced (28.13 g/L at 48 h) in the none-electro-activated whey followed by lactose (27.85 g/L at 48 h), EA-lactose (26.77 g/L at 36 h), WP (25.99 at 72 h), EA-WP (24.66 g/L at 36 h), and EA-Whey (22.06 g/L at 48 h). Production of sufficient ethanol in all whey-based media without any additional nutrients like N and P sources, indicated that they contained enough nutrients for bioethanol production by *K. marxianus* (Koushki et al., 2012). The yields in the whey-based

media were significantly higher ($P < 0.05$) than ethanol produced in YM broth. It is worth mention that the electro-activated substrates (EA-substrates) achieved a lower yield than the none-electro-activated substrates though they reached a plateau at a reduced fermentation time. The higher yield could be correlated to the higher initial lactose concentration in the none-electro-activated substrates (~50 g/L) than the EA-substrates (~30 g/L). This can be also a possible reason behind the lowest yield in the YM-broth. Beniwal et al. (2017) explored the lactose utilization by *K. marxianus* in mixed sugars (glucose, galactose, and lactose), and they observed that the yeast preference was glucose, lactose, and galactose in descending order. Both galactose and lactose were simultaneously metabolized when they are present together in the fermenting broth of *K. marxianus*, however, the rate of lactose utilization is preferentially higher over galactose. Moreover, the galactose and fructose were utilized at a similar rate, and this could be due to the separate transporter for fructose and galactose (Beniwal et al., 2017). Likewise, Tesfaw et al. (2021) concluded that the higher ethanol might be associated with higher lactose and longer incubation time (80 h) using crude whey and *K. marxianus* ETP87. However, the fermentation time in this study was dependent on the substrates and different media archived optimum yield at different time. After certain time of fermentation, ethanol yield was decreased which could be attributed to the production of some inhibitory metabolites. As such, trimethyl-urea was found in the EA-Whey media (**Figure 7.4**), which might be responsible for lower yield in EA-Whey than whey. Beniwal et al. (2017) obtained the lowest yield of ethanol while whey was fortified with urea.

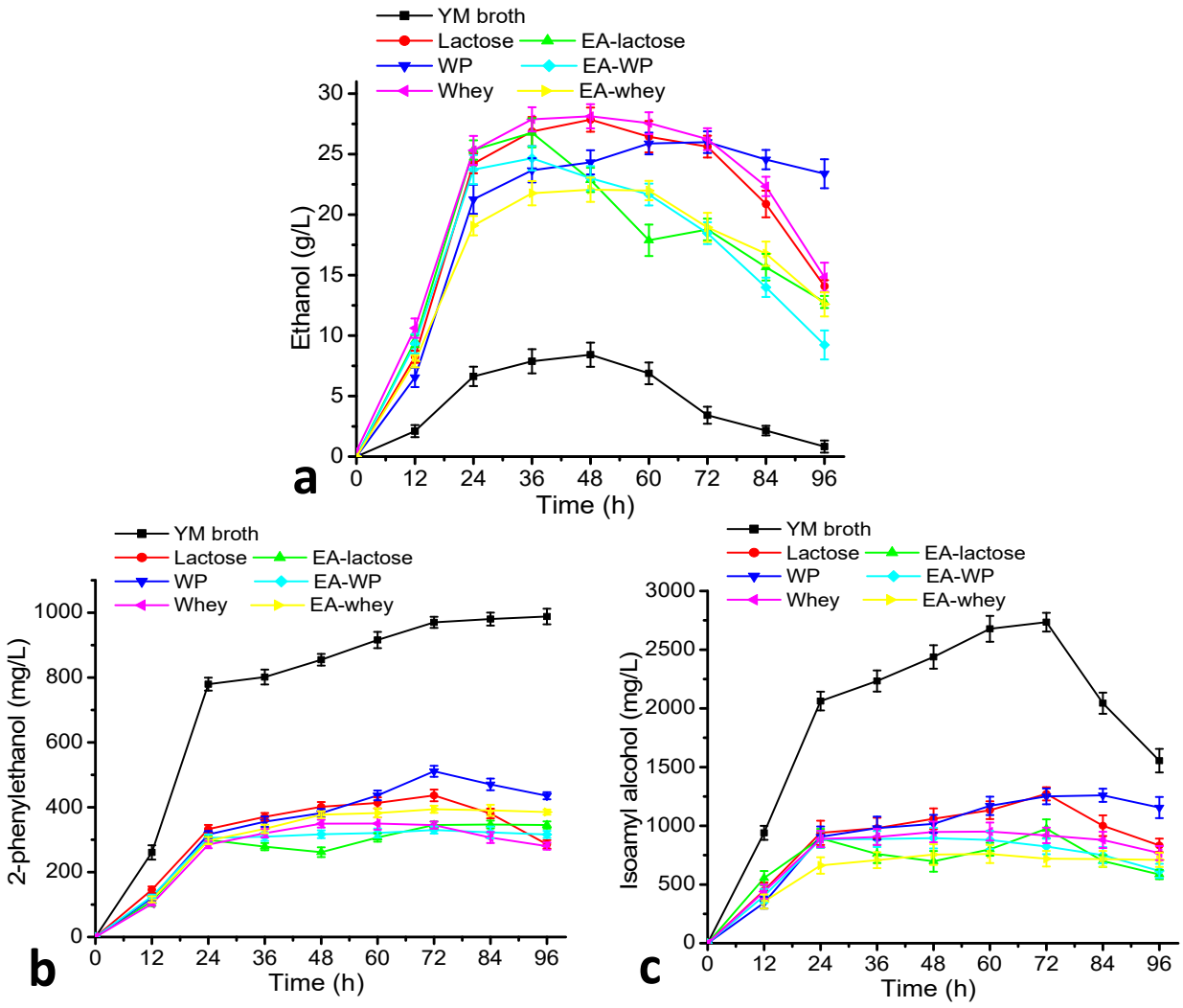

Figure 7.5: Production of a) ethanol, b) 2-phenylethanol, and c) isoamyl alcohol during the fermentation of several media at 30 °C. EA-lactose: Electro-activated lactose. WP: Whey permeate. EA-WP: Electro-activated whey permeate. EA-Whey: Electro-activated whey.

The performance of bioethanol production in the present study was in good agreement with the results previously reported for different cheese whey-based substrates using several *K. marxianus* strains and is presented in **Table 7.2**. Gabardo et al. (2014) obtained a higher ethanol yield of 42.80% with a productivity of 6.0 g/L/h from whey permeate, however, a higher concentration of lactose (150 g/L) and a continuous fluidized-bed bioreactor were used in their study. Moreover, they used an alginate-immobilized *K. marxianus* CCT 4086 strain as fermenting agent. In fact, the continuous fermentation and cell immobilization technique could increase ethanol production with a reducing fermentation time (Christensen

et al., 2011, Dragone et al., 2011). In another study, Güneşer et al. (2016b) obtained a much higher ethanol production yield of 46.46 g/L using *K. marxianus* LOCK0024 strain at 25 ℃ and 220 rpm after 5 days. However, Na-alginate entrapped the *K. marxianus* cells was used because of using a traditional liquid-droplet-forming method from a foamed alginate solution. It can be seen from **Table 7.2** that the ethanol yield is strongly dependent on the different operating factors including substrate concentration, temperature, and pH. Diniz et al. (2014) demonstrated that biomass concentrations between 2.4 and 3.3 (at OD_{600}), pH between 4.7 and 5.7, temperatures between 33.3 and 38.5 ℃, and lactose concentrations between 50 and 108 g/L are optimal for ethanol production from cheese whey using *K. marxianus*.

Table 7.2: Performance of bioethanol production through fermentation of different cheese whey-based substrates using several *Kluyveromyces marxianus* strains.

Substrate	Substrate concentration (lactose, g/L)	Strain	Reactor	Operating conditions	Ethanol production, g/L	Reference
Cheese whey	46.8	*K. marxianus* DSMZ 7239	Continuous fluidized-bed bioreactor (alginate-immobilized cells)	Dilution rate: 0.2/h, pH: 4.26-4.76, 32 °C	17.60 (~4.5 g/L/h)	(Christensen et al., 2011)
Cheese whey permeate	150	*K. marxianus* CCT 4086	Continuous fluidized-bed bioreactor (alginate-immobilized cells)	Dilution rate: 0.3/h, pH: 7, 30 °C	42.80 (6.0 g/L/h)	(Gabardo et al., 2014)
Cheese whey	48		Fed-batch reactor	Uncontrolled condition, 30 °C	8.00	(Ariyanti et al., 2014)
Cheese whey	43.6	*K. marxianus* var. *marxianus* CBS 712	Batch reactor	Uncontrolled condition, 30 °C	17.00	(Zoppellari and Bardi, 2013)
Cheese whey	150	*K. marxianus* NCIM 3217	Batch reactor	pH: 4.5, 35 °C	43.70	(Das et al., 2016)
Cheese whey	50-70	*K. marxianus* URM 7404	Batch reactor	pH: 4-6, 32-36 °C	15.56-20.60	(Murari et al., 2019)

Substrate	Substrate concentration (lactose, g/L)	Strain	Reactor	Operating conditions	Ethanol production, g/L	Reference
Whey permeate	50	*K. marxianus* ATCC® 64884™	Batch reactor	Uncontrolled conditions with an initial pH: 6.0, 30 °C, 150 rpm	25.99	**Present study**
Electro-activated whey permeate	30	*K. marxianus* ATCC® 64884™	Batch reactor	Uncontrolled conditions with an initial pH: 6.0, 30 °C, 150 rpm	24.66	**Present study**
Whey	50	*K. marxianus* ATCC® 64884™	Batch reactor	Uncontrolled conditions with an initial pH: 6.0, 30 °C, 150 rpm	28.13	**Present study**
Electro-activated whey	30	*K. marxianus* ATCC® 64884™	Batch reactor	Uncontrolled conditions with an initial pH: 6.0, 30 °C, 150 rpm	22.06	**Present study**

Isoamyl alcohol is another predominant alcoholic aroma compound produced in all substrates used in the present study. Naturally, isoamyl alcohol possess banana like flavour and is widely used in the fragrances and flavours industry (Hoşoğlu, 2018b). **Figure 7.5c** shows that YM broth achieved the highest yield of 2734.26 mg/L (72 h), followed by the lactose (1272.22 mg/L at 72 h), whey permeate (WP, 1250.46 at 72 h), electro-activated lactose (EA-lactose, 974.51 at 72 h), whey (951.01 at 60 h), electro-activated whey permeate (EA-WP, 895.36 at 48 h), and electro-activated whey (EA-Whey, 760.16 at 60 h). The results are comparable with a study reported by Dragone et al. (2009a), where they characterized 41 volatile compounds in an alcoholic beverage (35.4% v/v ethanol) that was produced by distillation of the fermented broth obtained by continuous whey fermentation with *K. marxianus*. They observed that the higher alcohols were the most abundant group of volatile compounds, including isoamyl (887 mg/L), isobutyl (542 mg/L), 1-propanol (266 mg/L), and isopentyl (176 mg/L) alcohols being found in highest quantities. In the present study, *K. marxianus* also produced 2-PE in all the media used (**Figure 7.5b**). The 2-PE reached a plateau at around 72 h for all whey-based media, while they achieved a maximum of 436.75, 345.29, 510.97, 329.95, 345.06, and 393.85 mg/L of 2-PE in the lactose, EA-lactose, WP, EA-WP, whey, and EA-whey, respectively. However, the highest amount of 2-PE was produced in the YM broth (988.22 mg/L) at the end of the fermentation process.

It is important to mention that 2-PE is related to flowery and honey-like smell that positively effects aroma of beverage and had been previously reported for *K. lactis* (Kadyan et al., 2021) and *K. marxianus* (Karim et al., 2020b). Martínez et al. (2018a) achieved a maximum 10.21 mg/g of 2-PE using solid-state fermentation of sugarcane bagasse supplemented with L-phenylalanine (L-phe) and *K. marxianus* as inoculum. In another study, *K. marxianus* CCT7735 was the most outstanding strain among 267 strains to produce the maximum 2-PE (3.44 g/L) titer under optimized conditions (De Lima et al., 2018). However, the growth inhibition of *K. marxianus* was also reported for a concentration of 2-PE around 1.4 g/L (Fabre et al., 1998). Moreover, Garavaglia et al. (2007) observed an optimum yield of 2-PE of 0.59 g/L using *K. marxianus* CBS6556 on grape must at a pH of 7.0, L-phe concentration of 3.0 g/L, temperature of 37 ℃, and oxygen mass transfer of 2.0/h.

Characterized by delicate fragrance of rose petals, 2-PE is widely used as commercial alcohol after ethyl alcohol in the food industry for fruit formulas, ice cream, jams, candy, soft

drinks, gelatins, puddings, rubber/chewing gums. In the pharmaceutical industry, it is used asr antiseptic and local anesthetic. It is also used in the perfumes and cosmetics industry (Conde-Báez et al., 2017). Moreover, fragrant substances in fruit, such 2-PE, α-pinene are frequently bound in glycoside forms and are naturally discovered during ripening by endogenous enzymes associated with β-glucosidases. Hence, the production of such compounds through fermentation of natural substances comprising certain aroma precursors could be linked to the microbial β-glucosidase activity (Aggelopoulos et al., 2014).

7.4 Conclusion

This work reveals that the electro-activated lactose (EA-lactose), electro-activated whey (EA-Whey), and electro-activated whey permeate (EA-WP) could be used as potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids and volatile aroma compounds using *K. marxianus*. The fermented biomass could be used for protein and fat enriched materials as they comprised high protein and lipid content of 24.43-57.83% and 15.44-25.64%, respectively. The production of orgonic acids including lactic, acetic, citric, propionic, and butyric acids are certainly promising as they could increase the organoleptic properties and shelf life of the fermented beverages. Furthermore, the study shows the presence of various volatile compounds in the fermented broth produced by *K. marxianus*. Most of these compounds are like those reported for other alcoholic beverages though the concentration values are different. The YM-broth resulted in the lowest ethanol production (8.42 g/L at 48 h) while the highest ethanol was produced in the non-electro-activated whey (28.13 g/L at 48 h), followed by lactose (27.85 g/L at 48 h), EA-lactose (26.77 g/L at 36 h), WP (25.99 at 72 h), EA-WP (24.66 g/L at 36 h), EA-Whey (22.06 g/L at 48 h). Apart from bioethanol production, the higher alcohols mainly isoamyl alcohol, 2-phenylethanol, isobutyl alcohol were the most predominant compounds, contributing for the greatest proportion of the total aroma. The volatile compounds which can be harmful to the health and responsible for bad odor such as methanol were not found. Finally, the potential of electro-activated whey/whey permeate based substrates as growth media for biotechnological transformation can be an emerging opportunity to improve the valorization of whey by concurrently enhancing the production of value-added derivatives and reducing the effluent disposal costs.

Annexe E

Standard calibration curves for organic acids production (Figure A11); HPLC chromatograms for organic acids production in different media (Figure A12).

ACKNOWLEDGEMENTS

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General conclusion and perspectives

Conclusion

In this thesis, an original approach for the complete valorization of whey and whey permeate (WP), which are considered as highly polluting by-products from the dairy industry, into high added value ingredients is presented without any upstream and/or downstream fractionation. To achieve this goal, the applicability of a green technology, based on electro-activation (EA) in solution was carefully selected and compared with a conventional chemical process to isomerize part of the lactose contained in the whey and whey permeate (WP) into lactulose. Furthermore, the electro-activated substrates including the electro-activated lactose (EA-lactose), electro-activated whey (EA-Whey), and electro-activated whey permeate (EA-WP) produced by EA technology could efficiently be used as carbon sources to produce protein rich biomass and valuable metabolites for food and feed industry using Kefir culture and *Kluyveromyces marxianus*. Important conclusions based on the objectives are presented below:

Lactulose production through electro-isomerization of lactose using electro-activation (EA) technology is affected by several operational and experimental conditions, and the process needs to be optimized. In the first objective (Chapter 3), the EA technique was applied to isomerize lactose into lactulose in an EA reactor modulated by anion and cation exchange membranes. The effect of lactose concentrations (5, 10, 15, and 20%), applied electric fields (300, 600, and 900 mA), and processing time (0-60 min) on lactose electro-isomerization rate (lactulose formation) and coproduct (glucose, galactose, and fructose) formation were investigated. The effect of different physicochemical parameters such as pH, alkalinity, temperature, ion migration, and oxidation-reduction potential (ORP) on the conversion of lactose into lactulose was correlated with the lactulose formation to understand the involved process mechanism of action. The results revealed that the conversion of lactose into lactulose was lactose-concentration-, electric-current, and EA-time-dependent. The obtained results demonstrated that in contrast to the chemical method, the EA process was found to give a higher yield of lactulose in a reduced reaction time for all conditions. The highest lactulose yield was obtained during the electro-isomerization process of lactose and was ~38% at 40 min using a 900-mA current intensity in a 10% lactose solution with a solution pH of 11.27 and the alkalinity of 21.07 mmol/L. The highest lactulose yield was

obtained during the conventional chemical isomerization process was ~27% at 60 min in a 10% lactose solution, while the pH was 11.33 for the alkalinity of 43.20 mmol/L (equivalent to 600 mA in EA). The correlated lactulose yield with the process parameters suggested that the lactulose can be produced at ambient temperature without additional heating if the required alkaline condition is achieved, although a higher temperature was positively correlated to the lactulose formation but leading to a higher by-product formation. A highly alkaline condition was required for the formation of lactulose; however, the lactulose produced in the medium did not reduce during the EA process while the alkalinity has been declined. Furthermore, EA triggers the feed solution to transfer into a metastable state characterized by unusual values of the chemical and physical parameters such as the ORP, pH, and alkalinity. Thus, it can be concluded based on the compared approaches (EA vs chemical) that EA significantly reduced the activation energy required for the isomerization reaction of lactose into lactulose, and consequently, a higher yield of lactulose was achieved within a shorter duration at ambient temperature compared to that of the chemical isomerization using KOH as catalyst.

The isomerization reaction was found to be highly dependent on the type and concentration of feed solution. In the second objective (Chapter 4), the isomerization efficiency of lactose into lactulose *in situ* of whey by using EA technology was compared with the chemical isomerization method using KOH as catalysis under equivalent solution alkalinity. Electro-isomerization of lactose into lactulose was performed by using whey solutions of 7, 14, and 21% (w/v) dry matter under current intensities of 300, 600, and 900 mA, respectively, during 60 min. A maximum lactulose yield of ~32% was achieved under a 900-mA current intensity at 60 min of EA for the 7% whey solution. The results suggest that the formation of lactulose was dependent on the whey concentration, current intensity, and EA time. Furthermore, no lactulose was produced in the chemical isomerization although equivalent alkalinity was created as in the EA. Thus, it is obvious that other process mechanisms of action were involved with the EA technique to achieve the required alkaline conditions.

Objective three (Chapter 5) was designed to produce lactulose *in situ* of WP using EA technology and compare the productivity of EA with conventional chemical isomerization at potassium hydroxide (KOH)-equivalent solution alkalinity in the feed medium. Electro-

isomerization was conducted under different current intensities of 300, 600, and 900 mA for 60 min using 6, 12, and 18% (w/v) WP solutions. The results of the objective three suggest that, in contrast to the conventional chemical isomerization process, the EA technology was more efficient in producing a greater amount of lactulose within a short time whatever the reaction conditions. The highest lactulose yield of ~37% was achieved in the EA technique at 50 min under a 900-mA current intensity using a 6% WP solution with a medium pH and alkalinity of 11.34 and 40.00 mmol/L, respectively. Whereas a maximum of 25% lactulose was obtained in the conventional chemical isomerization for the 6% WP solution as the feed, while the medium pH was 11.60 for an alkalinity of 40.00 mmol/L (equivalent solution alkalinity to 900 mA current intensity as in the EA process). Therefore, the EA technique offers higher potential than the conventional chemical method to produce the prebiotic lactulose through the valorization of the WP, using it as a lactose source. Furthermore, the EA process was carried out under complete auto-catalytic conditions, implying that the alkalinizing catalysts and external heating are not required in the EA technology.

The findings of objective four (Chapter 6) demonstrated that the EA-lactose, EA-whey, and EA-WP could efficiently be used as carbon sources to produce valuable metabolites including organic acids (i.e., lactic, acetic, citric, and propionic acids), the exopolysaccharide kefiran, and volatile flavour compounds using the whole kefir grains as a starter culture. The results showed that the EA substrates achieved a higher biomass growth in a reduced fermentation time than their non-EA mediums. The highest cell growth (6.04 g/L) was obtained for EA-whey after 72 h which was even 1.7-fold higher than a standard nutrition broth, the reinforced clostridial medium (RCM). Furthermore, EA-whey produced a maximum of 8.46, 3.97, 0.60, and 1.02 g/L of lactic, acetic, citric, and propionic acid, respectively. Moreover, EA-whey achieved the highest kefiran production of 2.99 g/L, followed by the whey (2.67 g/L), EA-WP (2.31 g/L), WP (1.88 g/L), RCM broth (1.42 g/L), EA-lactose (1.37 g/L), and lactose (0.91 g/L). The results also demonstrated that various aromatic volatile compounds were produced during the fermentation of EA-whey, which may increase the organoleptic characteristic/sensory quality of the fermented products. Henceforth, it can be disputed that not only the symbiotic interactions among the microorganisms of kefir consortium but also a substrate-inoculum synergistic relationship exits in the fermentation process using EA-whey based substrates and a whole kefir

microbiota as inoculum. Thus, the results of this objective provide strong evidence that the EA-substrates, especially EA-whey, could be potential feedstock to develop an efficient bioprocess for producing fermented products and valuable metabolites using kefir grains as a suitable starter.

The results of objective five (Chapter 7) revealed that the EA-lactose, EA-whey, and EA-WP could be used as potential carbon sources to produce protein-rich biomass and valuable metabolites such as organic acids and volatile aroma compounds using *K. marxianus*. The results showed that all substrates provided a satisfactory biomass growth and EA-whey achieved a maximum biomass (4.23 g/L) at 96 h of fermentation followed by YM broth (4.85 g/L). The fermented biomass could be used for protein and fat enriched materials as they comprised high protein and lipid content of 24.43-57.83% and 15.44-25.64%, respectively. Several major organic acids including lactic, acetic, citric, propionic acids were produced during the fermentation on all media, with significant differences between electro-activated and non-electro-activated substrates. The production of orgonic acids including lactic, acetic, citric, propionic, and butyric acids are certainly promising as they could increase the organoleptic properties and shelf life of the fermented beverages. Furthermore, the study shows the presence of various volatile compounds in the fermented broths produced by *K. marxianus*. Most of these compounds are like those reported for other alcoholic beverages though the concentration values are different. The YM-broth resulted in the lowest ethanol production (8.42 g/L at 48 h) while the highest ethanol was produced in the non-electro-activated whey (28.13 g/L at 48 h), followed by lactose (27.85 g/L at 48 h), EA-lactose (26.77 g/L at 36 h), WP (25.99 at 72 h), EA-WP (24.66 g/L at 36 h), EA-Whey (22.06 g/L at 48 h). Apart from bioethanol production, the higher alcohols mainly isoamyl alcohol, 2-phenylethanol, isobutyl alcohol were the most predominant compounds, contributing for the greatest proportion of the total aroma. A maximum of 393.85 to 988.22 mg/L of 2-phenylethanol was achieved, depending on the substrates used. The volatile compounds which can be harmful to the health and responsible for bad odor such as methanol were not found.

To sum up, the EA technology offers higher potential than the conventional chemical method to produce the prebiotic lactulose through the isomerization of lactose or valorization whey/WP, using them as a lactose source. Furthermore, the EA process could be an

environmentally friendly and sustainable method for producing prebiotic lactulose through the isomerization of whey/WP because it can be performed under complete autocatalytic conditions, meaning that alkalinizing chemicals and external heating are not required. Nevertheless, the potential of electro-activated whey/WP-based substrates as growth media for biotechnological transformation can be an emerging opportunity to improve the valorization of whey by concurrently enhancing the production of value-added derivatives and reducing the effluent disposal costs. Therefore, the results of this work suggest that the EA technology can be an emergent sustainable technology for achieving dual objectives of prebiotic lactulose production and concurrent valorization of whey and its derivatives in Kefir culture and *K. marxianus* driven bioprocesses to produce valuable metabolites for different applications including in food and feed industry. This knowledge is not only helpful to reduce the production cost of dairy industries, but also provide an eco-friendly alternative for the disposal of whey/WP as a part of integrated approach for their complete valorization to achieve the zero-waste goal.

Perspectives

In view of the results obtained in this thesis, some perspectives can be considered, namely:

- In contrast to the chemical method, the electro-activation (EA) process was found to offer a higher potential for an economic and environmentally friendly approach to produce lactulose by the isomerization of lactose *in situ* of whey and whey permeate (WP). However, further research is still required to understand the thermodynamics and mechanism of action that is involved with EA technique to obtain the required solution alkalinity, particularly, involving its action on the reaction activation energy behind this process. Such state-of-the-art process mechanism is crucial to exploit this technology in large-scale.
- The EA technique was observed to be more selective compared to the chemical method. However, despite some meaningful achievements of the studies on EA parameters and their correlations, a standard optimized condition should be established, which is more economical in terms of the configuration and geometry of the reactor considering the concentration and type feed solutions. Nevertheless, the neutralization of a highly reactive and alkaline solution using the EA technology without any external chemicals is yet to be investigated.

- The possibility of increasing the yield of lactulose production by using the EA technology combined with other green chemistry approaches such as ion exchange resins for continuous medium regeneration could be considered in the future studies.
- In this study, the liquid fermentation process was considered. It is possible that *K. marxianus* cells could be inhibited due to a high volume of final product (such as 2-PE, bioethanol) concentration in the medium. Solid-state fermentation using electro-activated materials can be of particular interest in this regard.
- The response of *K. marxianus* to several stresses such as osmotic, oxidative, ethanol and starvation stress during fermentation of EA-substrates and the protective mechanism to control the level of reactive oxygen species (ROS), e.g., antioxidative enzymes such as superoxide dismutase, catalase (CAT), and glutathione reductase (GR) and intracellular non-enzyme molecules such as glutathione (GSH) can be studied.
- The metabolic pathway of *K. marxianus* and its mechanism showed variation and functional diversities in different EA-substrates, hence there are still many opportunities for future study. The pilot scale study as well as extensive lab-scale evolution of metabolic engineering would be employed to understand the mechanism of metabolism to justify the applicability in industrial level.

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Annexe A: Supporting figures and tables (Chapitre 3)

Global Electrical Resistance

Global system resistance, *R (Ω)* of the EA-reactor was determined from the values of applied current intensity *I (A)*, and the corresponding voltage *U (V)* according to the Ohm's law as stated in Equation:

$$R = \frac{U}{I}$$

Figure A1:

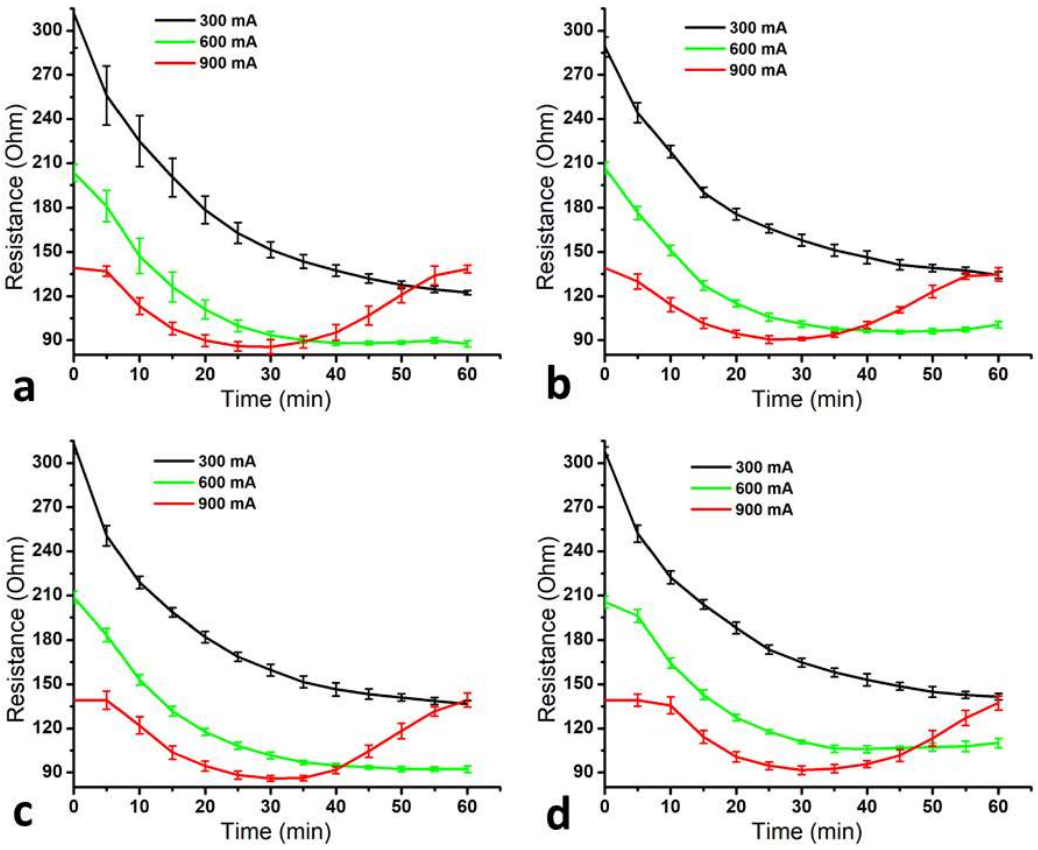

Figure A1: Global electric resistance of EA-reactor as a function of time at different current intensities (300, 600, and 900 mA) for different a) 5%, b) 10%, c) 15%, d) 20% lactose solutions.

Figure A2:

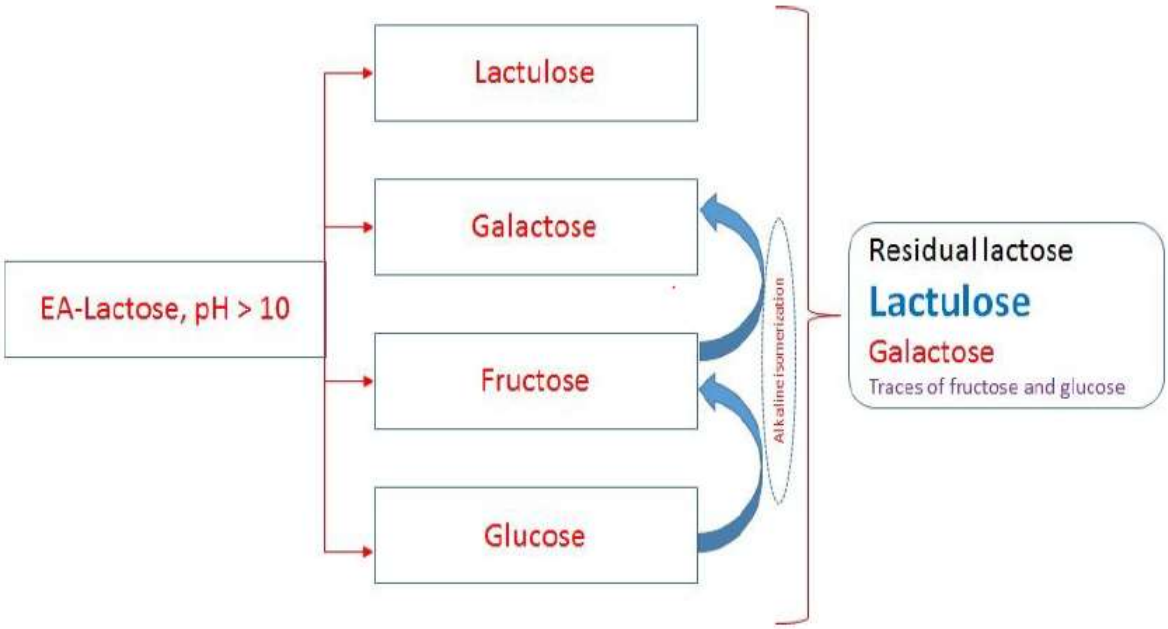

Figure A2 : The possible mechanism pathway of the electro-isomerisation of lactose into lactulose and subsequent galactose formation as a reaction by-product by using the electro-activation process. Adopted from Djouab and Aïder (2019b).

Table A1: Formation of glucose and fructose for different lactose solutions (5, 10, 15, and 20%) at different current intensities (300, 600, 900 mA) of EA.

Time	**5% lactose**						**10% lactose**					
(min)	**300 (mA)**		**600 (mA)**		**900 (mA)**		**300 (mA)**		**600 (mA)**		**900 (mA)**	
	Glucose (%)	Fructose (%)	Glucose (%)	Fructose (%)	Glucose (%)	Fructose (%)	Glucose (%)	Fructose (%)	Glucose (%)	Fructose (%)	Glucose (%)	Fructose (%)
0	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	-	-	-	-	-	-	-	-	-	-
10	-	-	-	-	-	0.11	-	-	-	-	-	0.11
15	-	-	2.12	0.12	2.36	0.18	-	-	-	-	2.15	0.15
20	-	-	2.46	0.18	2.76	0.43	-	-	2.07	0.13	2.90	0.32
25	-	-	2.43	0.36	3.12	0.65	-	-	2.23	0.18	3.10	0.52
30	-	-	2.77	0.40	3.25	0.83	-	-	2.62	0.16	3.31	0.66
35	-	-	3.18	0.48	3.84	1.09	-	-	2.93	0.35	3.68	0.87
40	-	0.12	3.04	0.54	4.05	1.25	-	-	2.70	0.38	3.90	1.06
45	2.11	0.17	3.31	0.66	4.30	1.57	-	-	3.07	0.46	4.09	1.23
50	2.93	0.27	3.08	0.79	4.55	2.03	-	-	2.88	0.49	4.52	1.51
55	2.52	0.16	3.28	0.90	4.66	2.37	-	-	3.17	0.60	4.56	1.66
60	2.49	0.28	3.20	0.97	4.91	2.70	2.38	0.14	3.16	0.69	4.70	1.84

*No glucose and fructose were found for 15% and 20% lactose solutions

Table A2: Formation of galactose and glucose in different lactose solutions (5% and 10%) during chemical isomerization for equivalent solution alkalinity of EA.

Time (min)	5% lactose						10% lactose					
	300 (mA)		600 (mA)		900 (mA)		300 (mA)		600 (mA)		900 (mA)	
	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)
0	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	0.40	9.28	0.55	7.48	-	8.03	0.35	9.13	0.30	10.24
10	0.11	-	0.42	10.01	0.74	7.29	-	8.25	0.29	9.40	0.23	11.24
15	0.38	8.49	0.80	8.85	1.66	6.92	0.27	8.49	0.57	8.17	0.65	-
20	0.38	8.51	0.92	8.63	1.59	6.49	0.30	8.51	0.74	7.99	0.76	-
25	0.37	8.76	1.41	8.67	1.35	6.84	0.38	8.39	0.90	7.96	0.82	-
30	0.38	8.39	1.45	8.77	2.49	6.80	0.37	8.76	0.98	8.08	0.84	-
35	0.37	8.76	1.95	8.70			0.52	8.39	1.14	9.02		
40	0.38	8.39	2.31	8.39			0.59	8.15	0.97	9.14		
45	0.52	8.39	2.61	8.54			0.75	8.19	1.26	-		
50	0.59	8.15	2.56	8.08			0.72	8.23	1.24	-		
55	0.76	8.18	2.96	8.14			0.92	8.34	1.31	-		
60	0.73	8.22	2.98	8.00			0.89	8.38	1.30	-		

Table A3: Formation of galactose and glucose in different lactose solutions (15% and 20%) during chemical isomerization for equivalent solution alkalinity of EA.

Time	**15% lactose**						**20% lactose**					
(min)	**300 (mA)**		**600 (mA)**		**900 (mA)**		**300 (mA)**		**600 (mA)**		**900 (mA)**	
	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)	Galactose (%)	Glucose (%)
0	-	-	-	-	-	-	-	-	-	-	-	-
5	-	-	0.20	-	0.58	-	-	-	-	-	0.25	-
10	0.15	-	0.39	-	0.70	-	-	-	0.21	-	0.25	-
15	0.28	-	0.43	-	0.95	-	-	-	0.29	-	0.31	-
20	0.26	-	0.59	-	0.76	-	0.18	-	0.31	-	0.35	-
25	0.31	-	1.10	-	0.96	-	0.16	-	0.29	-	0.38	-
30	0.32	-	0.60	-	0.94	-	0.27	-	0.34	-	0.37	-
35	0.36	-	0.95	-			0.35	-	0.49	-		
40	0.33	-	0.72	-			0.3	-	0.39	-		
45	0.39	-	1.09	-			0.27	-	0.45	-		
50	0.93	-	0.70	-			0.34	-	0.47	-		
55	0.96	-	0.92	-			0.34	-	0.47	-		
60	1.67	-	1.96	-			0.37	-	0.58	-		

Table A4: Residual lactose (%) in different electro-activated lactose solutions (5%, 10%, 15%, and 20%).

Time	5% lactose			10% lactose			15% lactose			20% lactose		
(min	300	600	900	300	600	900	300	600	900	300	600	900
	(mA)	(mA)	(mA)	(mA)	(mA)	(mA)	(mA)	(mA	(mA)	(mA)	(mA)	(mA)
0	100	100	100	100	100	100	100	100	100	100	100.00	100
5	99.80	78.87	77.03	100	83.55	76.48	100	100	99.79	100	100.00	99.84
10	99.73	72.59	69.29	100	83.47	71.43	99.81	99.67	99.68	99.83	99.82	99.75
15	89.77	67.70	64.54	83.24	74.46	66.52	99.74	99.64	99.48	99.77	99.79	99.60
20	86.28	64.63	59.08	82.76	70.75	61.74	99.69	99.43	99.20	99.74	99.68	99.34
25	85.33	61.83	55.65	82.44	67.94	58.33	99.63	99.27	76.19	99.69	99.63	98.89
30	73.10	59.93	52.51	80.81	66.30	56.22	99.46	99.23	71.48	99.70	99.50	78.46
35	66.58	58.01	49.85	76.04	62.64	53.17	99.34	86.24	66.94	99.68	99.44	74.48
40	66.86	56.43	47.43	74.88	61.85	50.59	99.26	84.19	63.26	99.67	99.32	72.01
45	64.90	54.24	44.49	73.83	60.21	49.85	99.24	80.74	60.33	99.61	99.23	67.71
50	63.43	52.87	42.10	73.05	57.90	47.97	99.07	77.65	58.06	99.65	80.86	65.18
55	64.14	51.79	39.14	71.98	56.90	47.23	98.99	76.39	51.89	99.65	78.35	64.27
60	61.89	50.90	35.51	67.41	56.12	46.28	86.24	72.68	54.5	99.60	76.86	61.82

Table A5: Residual lactose (%) in different chemically isomerized lactose solutions (5%, 10%, 15%, and 20%).

Time	5% lactose			10% lactose			15% lactose			20% lactose		
(min)	300 (mA)	600 (mA)	900 (mA)	300 (mA)	600 (mA)	900 (mA)	300 (mA)	600 (mA	900 (mA)	300 (mA)	600 (mA)	900 (mA)
0	100	100	100	100.00	100.00	100	100	100	100	100	100	100
5	100	83.77	85.01	91.97	90.52	89.46	100	99.8	79.06	100	100	99.75
10	99.89	82.39	82.69	91.75	90.31	88.53	99.85	99.61	78.24	100	99.79	99.75
15	91.13	78.40	74.91	91.24	82.81	78.50	99.72	99.57	74.01	100	99.71	99.69
20	91.11	78.34	75.51	91.19	80.31	75.77	99.74	99.41	74.20	99.82	99.69	99.65
25	85.10	73.00	71.78	91.23	78.74	74.34	99.69	98.9	73.78	99.84	99.71	99.62
30	83.95	72.63	70.33	90.87	77.52	73.15	99.68	99.4	73.84	99.73	99.66	99.63
35	83.10	69.18		82.34	75.65		99.64	99.05		99.65	99.51	
40	82.95	68.81		81.26	73.30		99.67	99.28		99.70	99.61	
45	81.34	67.90		80.77	74.21		99.61	98.91		99.73	99.55	
50	81.26	67.75		80.26	73.98		99.07	99.3		99.66	99.53	
55	78.97	66.12		79.01	72.23		99.04	99.08		99.66	99.53	
60	78.56	66.29		77.32	71.57		98.33	98.04		99.63	99.42	

Annexe B: Supporting figures and tables (Chapitre 4)

Figure A3:

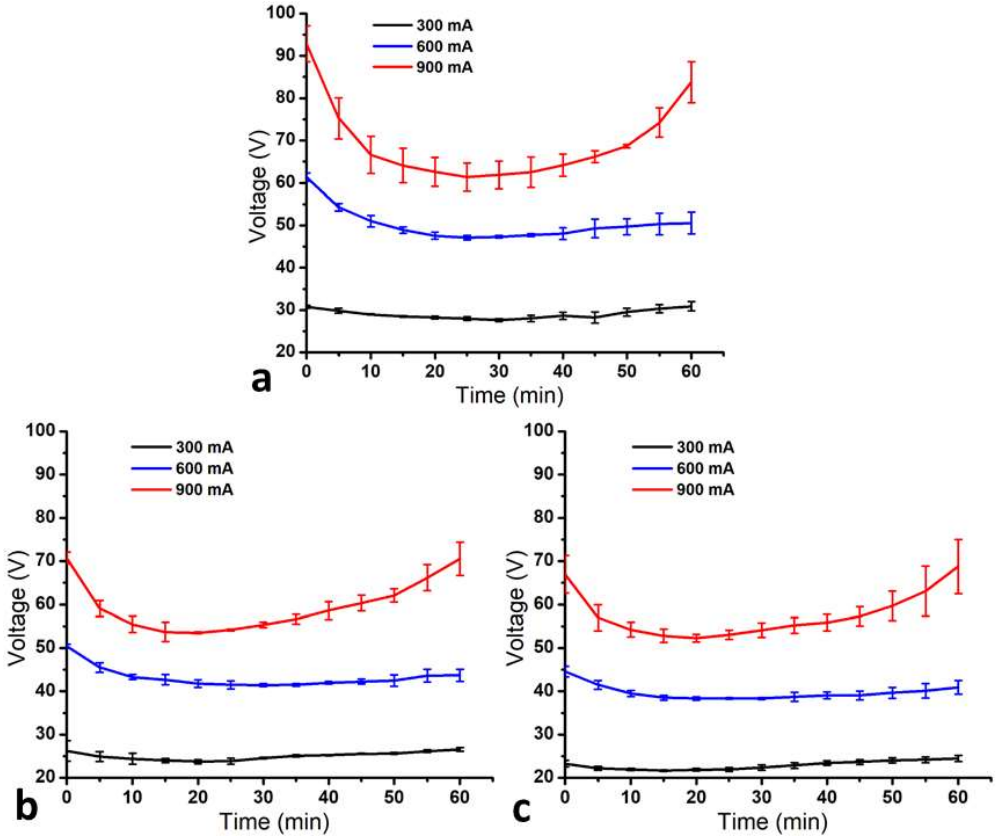

Figure A3: Variation of voltage during the EA process at different current intensities (300, 600, 900 mA) for a) 7%, b) 14%, c) 21% whey solution.

Figure A4:

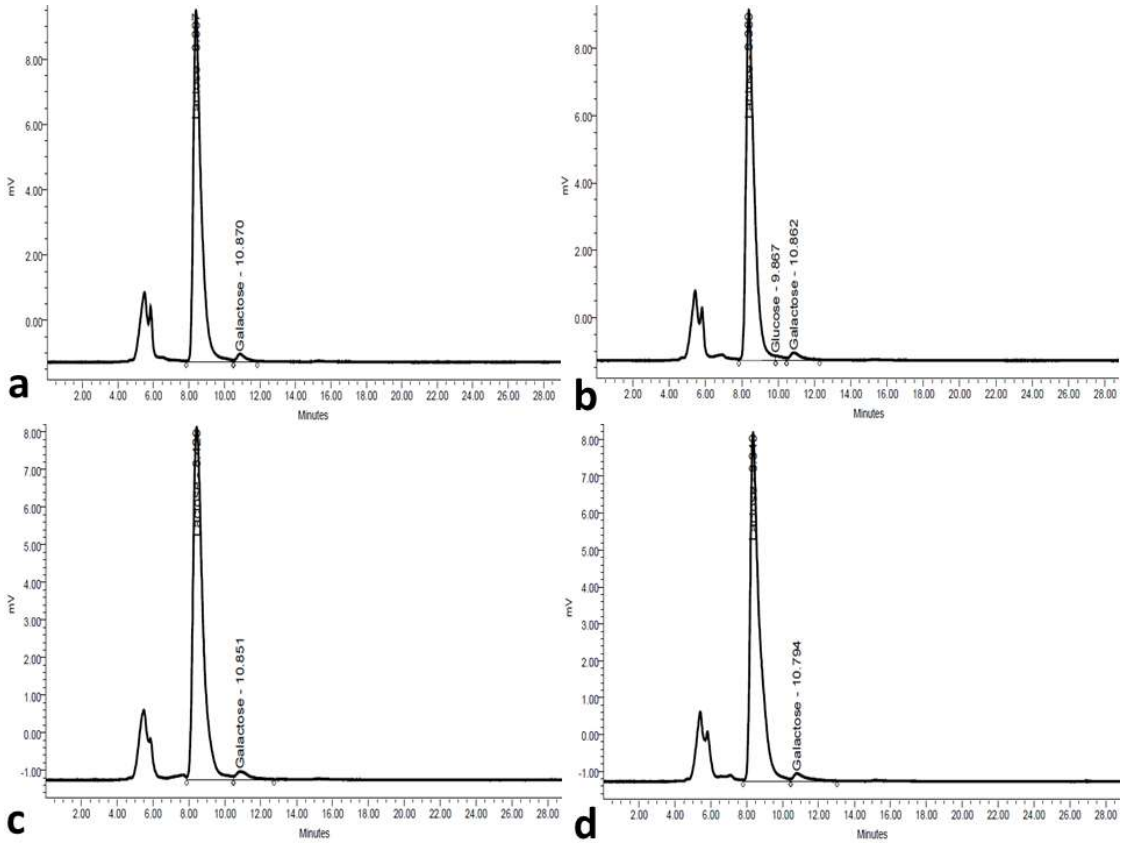

Figure A4: HPLC chromatograms for 14 and 21% whey solutions; a) initial feed solution (lactose: 97.24%, galactose: 2.76%, b) at 60 min under 600 mA for 14% whey (lactose: 95.77%, galactose: 3.12%, glucose:1.11%), c) at 60 min under 900 mA for 14% whey (lactose: 96.62%, galactose: 3.38%), d) at 60 min under 900 mA for 21% whey (lactose: 96.47%, galactose: 3.53%).

Figure A5:

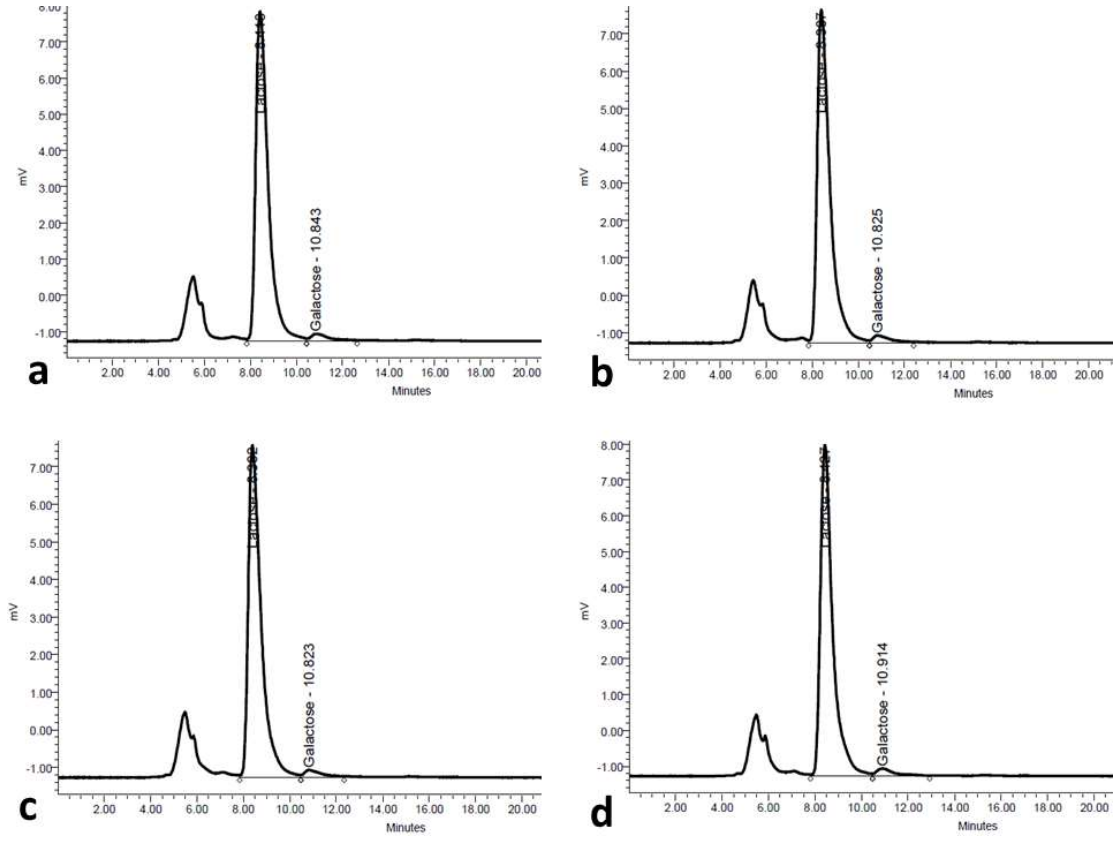

Figure A5: HPLC chromatograms for chemical isomerization in 7% whey solution; a) at 0 min equivalent to 600 mA, b) at 50 min equivalent to 600 mA, c) at 0 min equivalent to 900 mA, d) at 40 min equivalent to 900 mA.

Table A6: Formation of sugars in chemical isomerization for 7% whey solutions using equivalent solution alkalinity as in the EA of different current intensities (300, 600, 900 mA).

Time (min)	7% Whey								
	300 (mA)			600 (mA)			900 (mA)		
	Lactose	Galactose	Glucose	Lactose	Galactose	Glucose	Lactose	Galactose	Glucose
0	96.71	3.29	-	96.74	3.26	-	96.92	3.08	-
5	95.71	3.20	1.09	96.71	3.29	-	96.88	3.12	-
10	95.71	3.16	1.13	96.79	3.21	-	96.93	3.07	-
15	95.62	3.17	1.21	96.65	3.35	-	96.55	3.45	-
20	96.88	3.12	-	96.68	3.32	-	96.79	3.21	-
25	96.95	3.05	-	96.63	3.37	-	96.53	3.47	-
30	96.70	3.30	-	96.60	3.40	-	96.66	3.34	-
35	96.79	3.21	-	96.72	3.28	-	96.51	3.49	-
40				96.85	3.15	-	96.64	3.36	-
45				96.96	3.04	-			
50				96.87	3.13	-			

*No lactulose and fructose were detected

Annexe C: Supporting figures (Chapitre 5)

Figure A6:

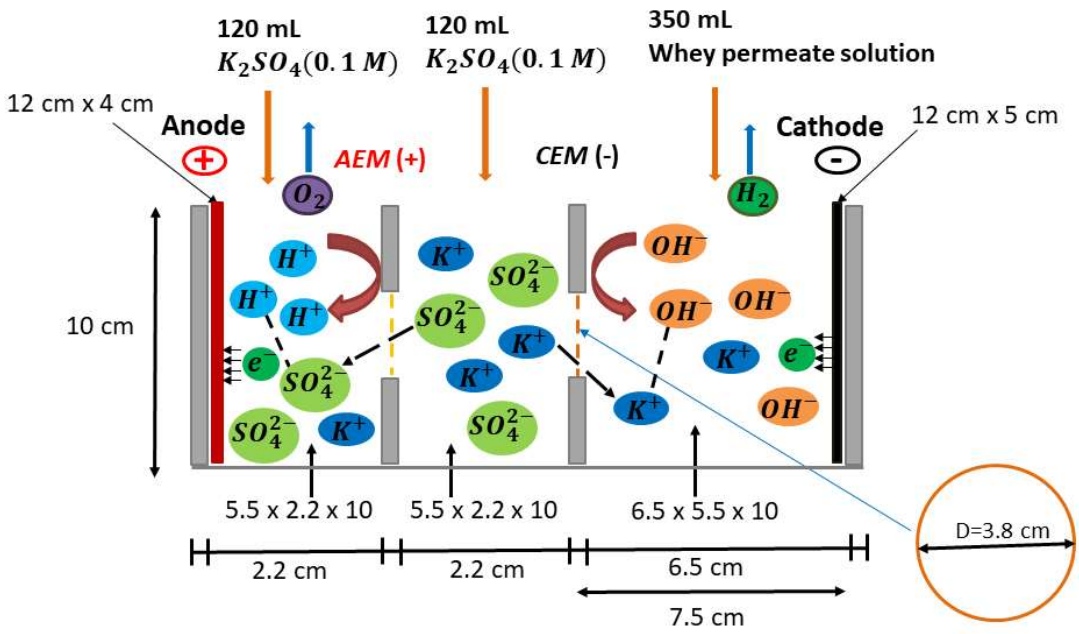

Figure A6: Schematic of the EA reactor with geometrical parameters used for lactose isomerization into lactulose *in situ* of WP.

Figure A7:

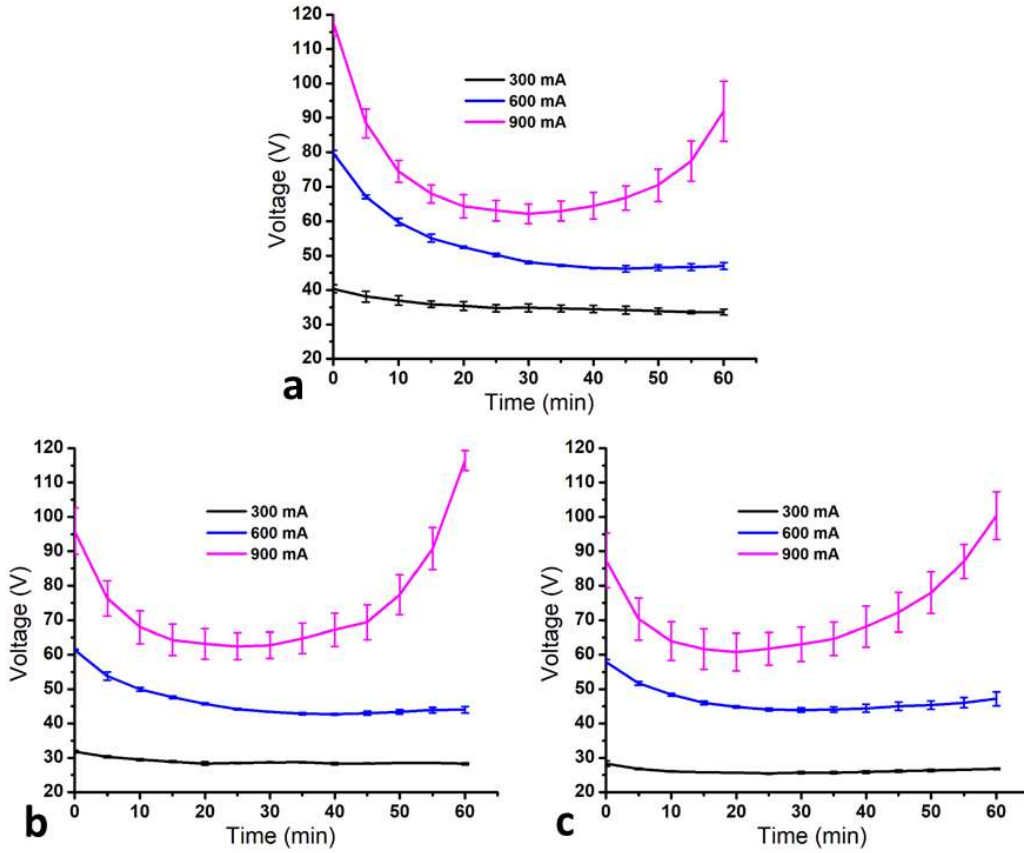

Figure A7: Evolution of voltage during the EA under different current intensities (300, 600, and 900 mA) for a) 6, b) 12, and c) 18% WP solutions.

Annexe D: Supporting figures (Chapitre 6)

Figure A8:

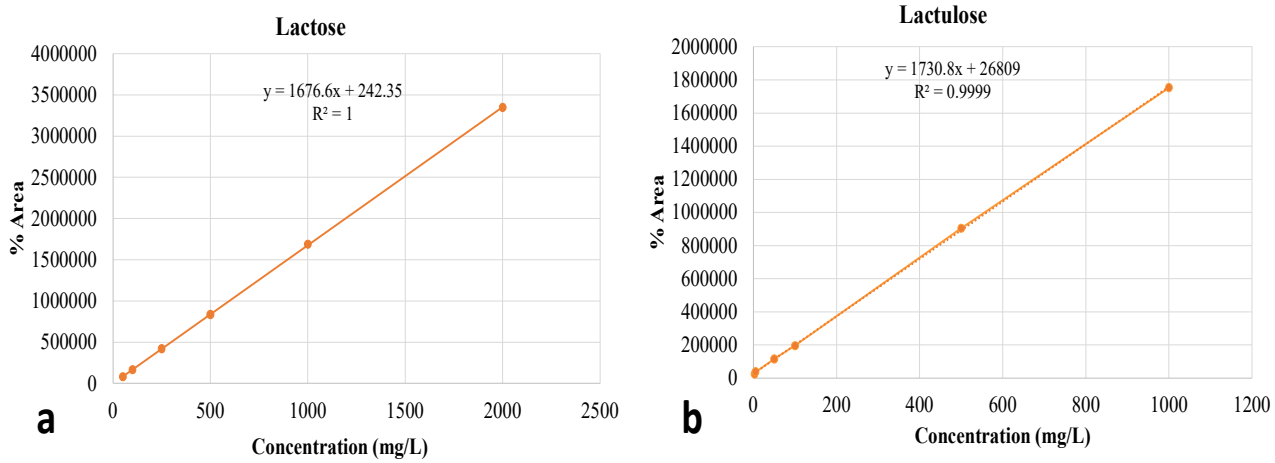

Figure A8: Standard calibration curves for sugar consumption, a) lactose and b) lactulose.

Figure A9:

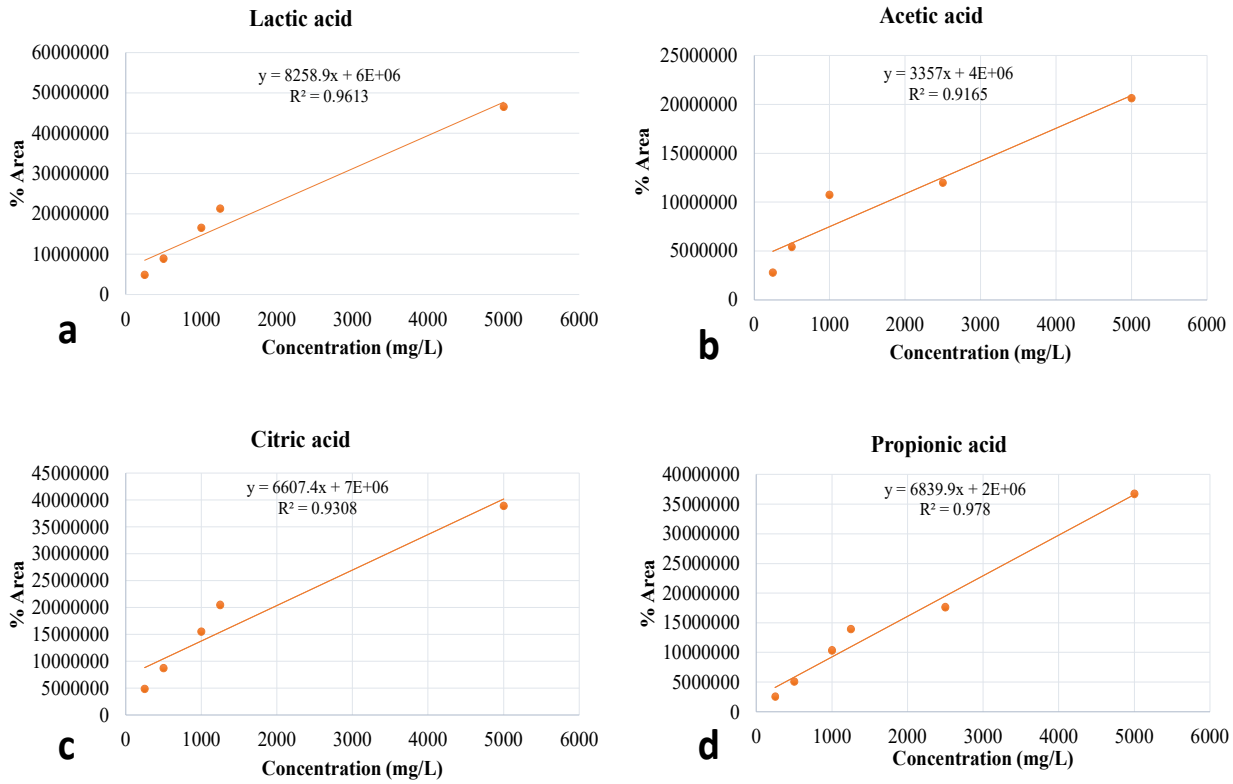

Figure A9: Standard calibration curves for organic acids production, a) lactic, b) acetic acid, c) citric acid, and d) propionic acid.

Figure A10:

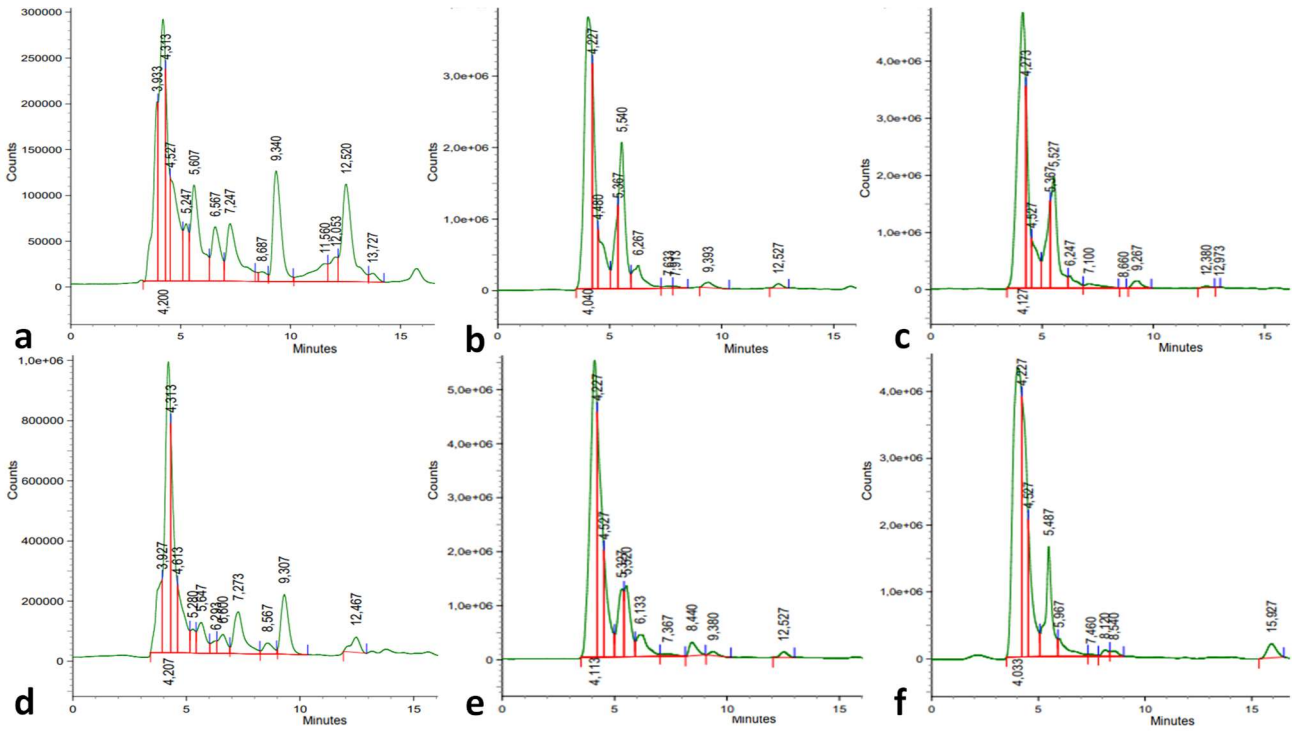

Figure A10: High performance liquid chromatography (HPLC) chromatograms for organic acids a) lactose, b) WP, c) whey, d) EA-lactose, e) EA-WP, and f) EA-whey (diluted) during 72 h of fermentation by kefir culture. Lactic acid (~4.1), acetic acid (~4.3), citric acid (~4.5), butyric acid (~5.3), and propionic acid (~5.5).

Annexe E: Supporting figures (Chapitre 7)

Figure A11:

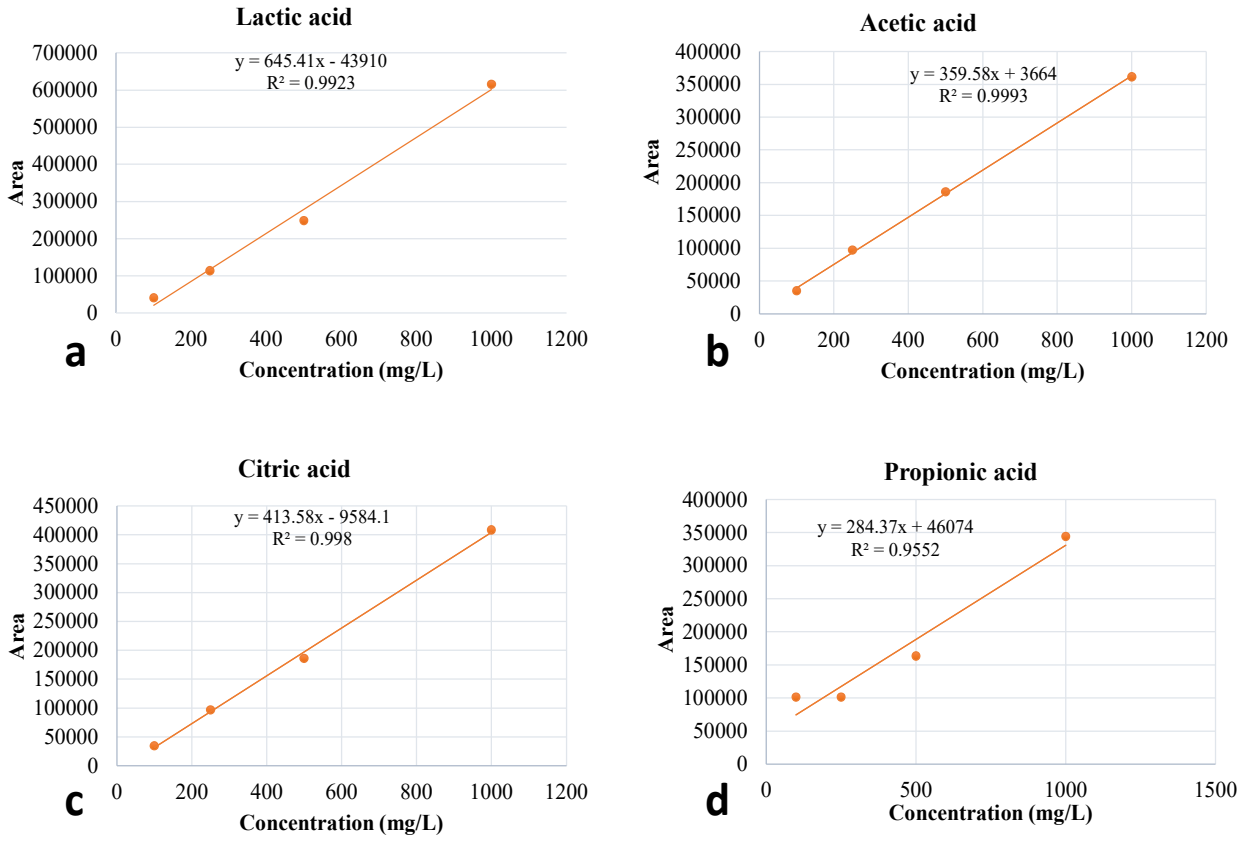

Figure A11: Standard calibration curves for organic acids production, a) lactic, b) acetic acid, c) citric acid, and d) propionic acid.

Figure A12:

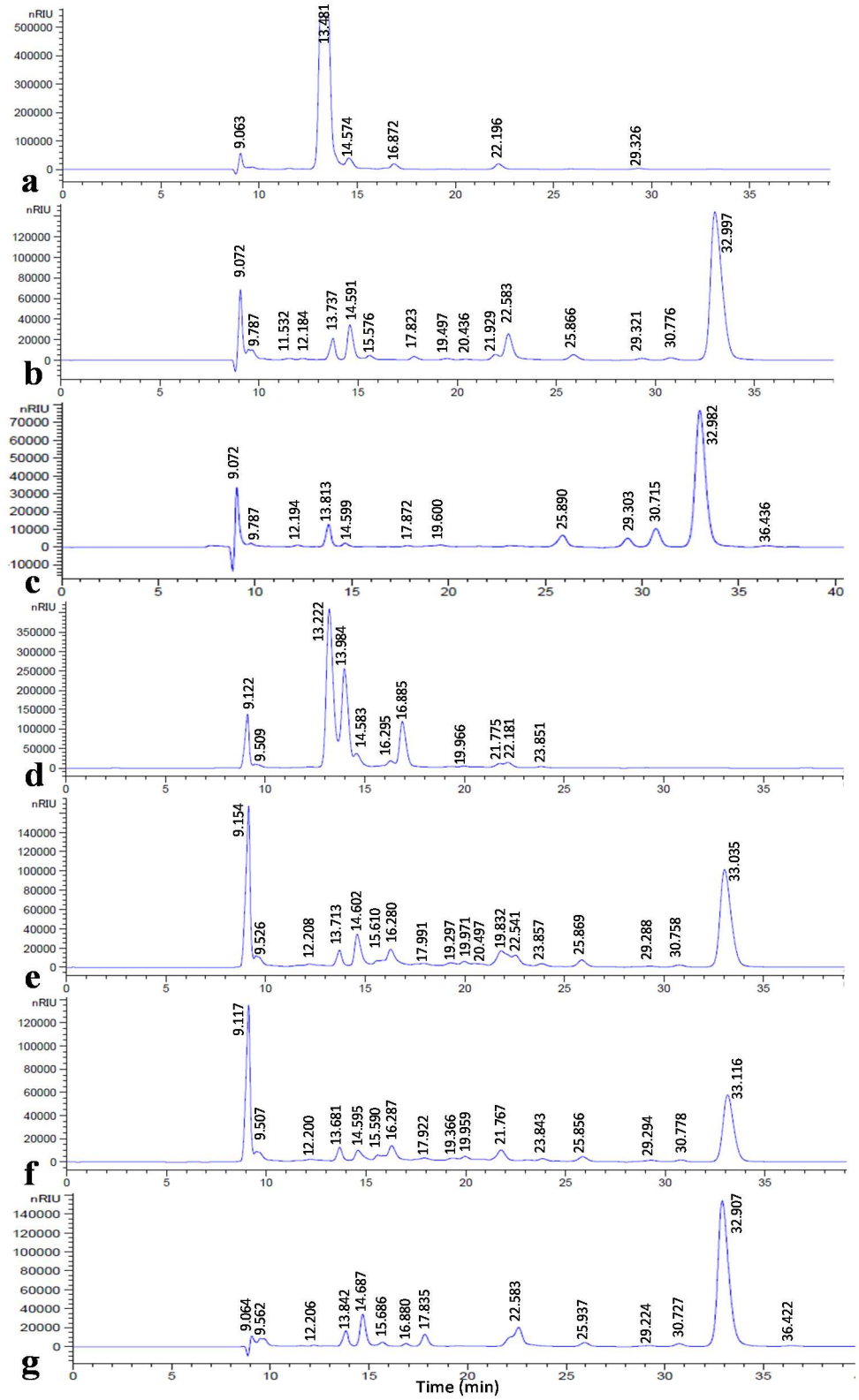
a
nRIU
9.063
13.481
14.574
16.872
22.196
29.326
b
nRIU
9.072
9.787
11.532
12.184
13.737
14.591
15.576
17.823
19.497
20.436
21.929
22.583
25.866
29.321
30.776
32.997
c
nRIU
9.072
9.787
12.194
13.813
14.599
17.872
19.600
25.890
29.303
30.715
32.982
36.436
d
nRIU
9.122
9.509
13.222
13.984
14.583
16.295
16.885
19.966
21.775
22.181
23.851
e
nRIU
9.154
9.526
12.208
13.713
14.602
15.610
16.280
17.991
19.297
19.971
20.497
19.832
22.541
23.857
25.869
29.288
30.758
33.035
f
nRIU
9.117
9.507
12.200
13.681
14.595
15.590
16.287
17.922
19.366
19.959
21.767
23.843
25.856
29.294
30.778
33.116
g
nRIU
9.064
9.562
12.206
13.842
14.687
15.686
16.880
17.835
22.583
25.937
29.224
30.727
32.907
36.422
Time (min)

Figure A12: HPLC chromatograms for organic acids production in different media a) whey (0 h), b) whey (24 h), c) whey (96 h), d) EA-whey (0 h), e) EA-whey (24 h), f) EA-whey (96 h), and g) whey permeate (24 h). Standard organic acids and their corresponding retention time: citric acid (14.562), maleic acid (16.582), melonic acid (18.545), lactic acid (22.583), formic acid (23.810), acetic acid (25.887), propionic acid (30.706), iso-butyric acid (34.286), and butyric acid (36.463).