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production by *Aspergillus lacticoffeatus*

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Dissertação de Mestrado
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Trabalho efetuado sob a orientação de
Professora Doutora Lígia Raquel Marona Rodrigues
Doutora Sara Isabel da Cruz Silvério

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Título da dissertação: Validation and optimization of β-galactosidase production by *Aspergillus lacticoffeatus*

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Universidade do Minho, _____/_____/_____

Assinatura:

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ABSTRACT

β-galactosidases (EC 3.2.1.23), also known as lactases, are a family of enzymes that are able to catalyze two different reactions, namely the hydrolysis of lactose and transgalactosylation. The hydrolytic activity is commonly applied in the food industries for reducing the lactose content on dairy products, but it is also important to prevent lactose crystallization problems and to increase the sweetening capacity. Transgalactosylation reactions had been used to synthesize lactose derivatives, such as galacto-oligosaccharides (GOS), lactulose and lactosucrose. These compounds are classified as prebiotics, which are functional food ingredients, are not digested on the gastrointestinal (GI) tract and are able to stimulate the growth or activity of health-promoting bacteria. The sources of β-galactosidases are extensively distributed in nature, namely in microorganisms, plants and animal organs. *Aspergillus lacticoffeatus* was chosen for this work as a potential β-galactosidase producer based on preliminary chromogenic tests performed in agar plates. Herein, additional studies carried out under submerged fermentation conditions confirmed the presence of β-galactosidase in the fermentation broth, as well as in the cell extract obtained after cell disruption by ultrasounds. Therefore, this work represents the first time that *A. lacticoffeatus* is described as a β-galactosidase producer. The enzyme production was evaluated in different fermentation media: synthetic medium composed by lactose (20 g/L), yeast extract (4 g/L), peptone (4 g/L) and salts; and alternative fermentation media with some industrial by-products such as cheese whey and corn steep liquor (CSL). The highest values of extracellular enzymatic activity (444 U/L) were obtained using the synthetic medium. The extracellular enzyme presented a molecular weight between 70–150 kDa and optimal pH and temperature in the range 3.5–4.5 and 50–55 °C, respectively. The effect of some metal ions (Na^+, K^+, Li^+, Ba^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}), detergents (Triton, SDS and Tween), additives (EDTA, PMSF and ascorbic acid) and sugars (glucose, fructose and galactose) on the enzymatic activity was also evaluated. Finally, the potential application of the enzyme for the synthesis of lactose-based prebiotics was studied and it was demonstrated that the β-galactosidase from *A. lacticoffeatus* is able to catalyze the transfer reactions involved in the formation of lactulose and GOS.

RESUMO

As β-galactosidases (EC 3.2.1.23), também conhecidas como lactases, são uma família de enzimas capazes de catalisar dois tipos diferentes de reações, nomeadamente a hidrólise da lactose e a transgalactosilação. A atividade hidrolítica é frequentemente aplicada na indústria alimentar na redução do conteúdo de lactose em produtos lácteos, mas é também importante na prevenção da cristalização da lactose e no aumento da capacidade adoçante. As reações de transgalactosilação têm sido usadas na síntese de derivados de lactose, tais como galacto-oligossacáridos (GOS), lactulose e lactosucrose. Estes compostos são classificados como prebióticos, ou seja, ingredientes de alimentos funcionais que não são digeridos no trato gastrointestinal e são capazes de estimular o crescimento ou atividade de bactérias benéficas para a saúde. As fontes de β-galactosidases estão amplamente distribuídas pela natureza, nomeadamente em microrganismos, plantas e órgãos animais. *Aspergillus lacticoffeatus* foi escolhido para este trabalho como potencial produtor de enzima tendo em conta os resultados preliminares de testes cromogénicos realizados em placas de agar. Neste trabalho, estudos adicionais conduzidos sob fermentações submersas confirmaram a presença de β-galactosidase no caldo da fermentação, assim como no extrato celular obtido após rutura celular por ultrassons. Desta forma, *A. lacticoffeatus* é aqui pela primeira vez descrito como um produtor de β-galactosidase. A produção da enzima foi avaliada em diferentes meios de fermentação: meio sintético composto por lactose (20 g/L), extrato de levedura (4 g/L), peptona (4 g/L) e sais; e meios alternativos constituídos por resíduos industriais como o soro de queijo e *corn steep liquor* (CSL). Contudo, os valores mais elevados de atividade enzimática extracelular (444 U/L) foram obtidos usando o meio sintético. A enzima produzida extracelularmente apresentou um peso molecular entre 70 e 150 kDa e o pH e temperaturas ótimos num intervalo entre 3.5–4.5 e 50–55 °C, respetivamente. O efeito de iões metálicos (Na^+, K^+, Li^+, Ba^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} e Cu^{2+}), detergentes (Triton, SDS e Tween), aditivos (EDTA, PMSF e ácido ascórbico) e açúcares (glucose, frutose e galactose) na atividade enzimática foi também avaliado neste trabalho. Finalmente, a potencial aplicação da enzima na produção de prebióticos derivados de lactose foi estudada, tendo-se demonstrado que esta β-galactosidase de *A. lacticoffeatus* é capaz de catalisar as reações de transferência envolvidas na formação de lactulose e GOS.

SCIENTIFIC OUTPUT

The work developed during this Master Thesis was presented in the international conference BIO.IBEROAMÉRICA 2016 and some results have been included in a full paper to be submitted to a peer-reviewed international journal.

Abstract: The enzyme β-galactosidase, also known as lactase, is widely used in the dairy industry to produce lactose-free milk. This enzyme is able to hydrolyse lactose from milk into galactose and glucose, thus enabling the consumption of milk by lactose-intolerant people. Under suitable conditions, some β-galactosidases can also catalyze transgalactosylation reactions and produce interesting compounds with recognized prebiotic effect, namely galacto-oligossacharides (GOS) or lactulose. The enzyme can be obtained from different biological sources such as microorganisms, plants and animals. Nevertheless, the most interesting β-galactosidases for technological applications are those obtained through microbial routes since higher production yields can be achieved. In this study, the fungus *Aspergillus lacticoffeatus* is described as a new and promising source of β-galactosidase. Preliminary chromogenic tests performed in agar plates suggested that this strain was able to produce the enzyme and additional studies carried out under submerged fermentation conditions confirmed the presence of β-galactosidase in the fermentation broth, as well as in the cell extract obtained after ultrasonic cell disruption. The enzyme production was evaluated in different fermentation media: synthetic medium composed by lactose (20 g/L), yeast extract (4g/L), peptone (4g/L) and salts; and fermentation media with some industrial by-products as cheese whey and/or corn steep liquor. However, the higher values of enzymatic activity (444 U/L) were obtained using the synthetic medium. The enzyme presented a molecular weight around 130 kDa and optimal pH and temperature in the range 3.5–4.5 and 50–55 ºC, respectively. The effect of some metal ions (Na^+, K^+, Li^+, Ba^{2+}, Fe^{2+}, Mg^{2+}, Zn^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}), detergents (Triton, SDS and Tween), additives (EDTA, PMSF and ascorbic acid) and sugars (glucose, fructose and galactose) on the enzymatic activity was also evaluated. Afterwards, the potential of the enzyme for the synthesis of prebiotics was studied and it was demonstrated that β-galactosidase from *A. lacticoffeatus* is able to catalyze the transfer reactions involved in the formation of lactulose and GOS.

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LIST OF ABBREVIATIONS

APS – Ammonium Persulfate

CSL - Corn Steep Liquor

DNS – 3,5-Dinitrosalicylic Acid

EDTA – Ethylenediamine Tetraacetic Acid

ELSD – Evaporative Light Scattering Detector

FFase – β-Fructofuranosidase

FOS – Fructo-oligosaccharides

GI – Gastrointestinal

GRAS – *Generally Recognized As Safe*

GOS – Galacto-oligosaccharides

HPLC – High-Performance Liquid Chromatography

HPLC-FD – High-Performance Liquid Chromatography with Fluorescence Detection

MEA – Malt Extract Agar

MUM – Mycology Collection of University of Minho

ONPG – *O*-Nitrophenyl-β-D-Galactopyranoside

OTA – Ochratoxin A

PDA – Potato Dextrose Agar Medium

RPM – Revolutions Per Minute

SD – Standard Deviation

SDS – Sodium Dodecyl Sulfate

SDS-PAGE – Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis

TCA – Trichloroacetic Acid

TEMED – Tetramethylethylenediamine

PMSF – Phenylmethanesulfonyl Fluoride

U – Enzymatic Unity

X-gal – 5-Bromo-4-chloro-3-indolyl β-D-Galactopyranoside

1. INTRODUCTION

1.1. The β-galactosidase enzyme

β-galactosidases (EC 3.2.1.23), also known as lactases, are a family of enzymes that are characterized by their ability to hydrolyze terminal non-reducing β-D-galactosyl residues from β-D-galactosides. These enzymes are able to catalyze two different reactions, namely the hydrolysis of lactose and transgalactosylation (**Figure 1**). The hydrolytic activity has been widely applied in the food industries for reducing the lactose content of dairy products, while transgalactosylation reactions had been used to synthesize galacto-oligosaccharides (GOS) and other lactose derivatives, such as lactulose and lactosucrose (Oliveira, Guimarães, & Domingues, 2011; Raol, Raol, Prajapati, & Patel, 2015; Silvério, Macedo, Teixeira, & Rodrigues, 2015, 2016).

These enzymes are also used in pharmaceutical industries, analysis and environment protection (Husain, 2010; Oliveira et al., 2011; Panesar, Panesar, Singh, Kennedy, & Kumar, 2006).
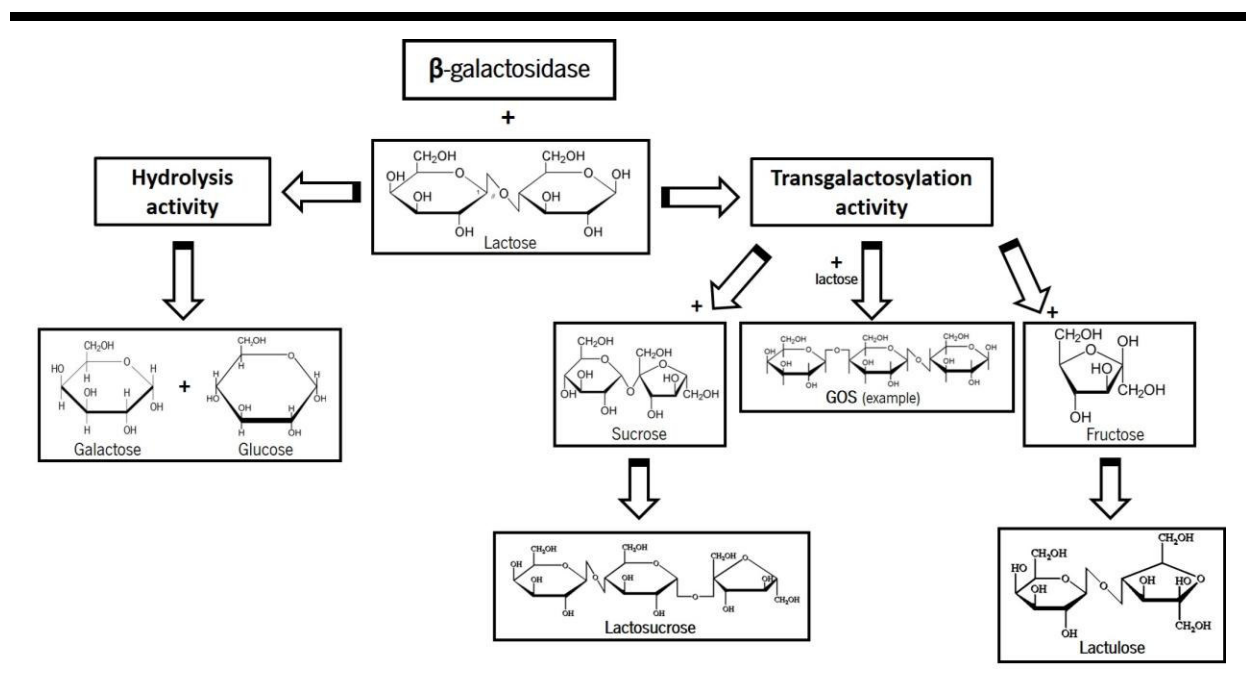
Figure 1. Enzymatic activities of β-galactosidases.

1.1.1. Sources of β-galactosidase

Sources of β-galactosidases are widely distributed in nature, namely microorganisms (bacteria, fungi, yeasts) (**Tables 1, 2** and **3**), plants (almonds, peaches, apples) and animal organs (Guerrero et al., 2015; Husain, 2010; Panesar et al., 2006). β-galactosidases from bacteria have been extensively used for the hydrolysis of lactose due to their ease of fermentation, high activity and good stability (Picard et al., 2005). In plants, these enzymes are widely distributed in the tissues and they have been shown to be involved in some biological processes, such as plant growth, fruit ripening and lactose hydrolysis (Carrillo-López, Cruz-Hernández, Cárabez-Trejo, Guevara-Lara, & Paredes-López, 2002; S. C. Li, Han, Chen, & Chen, 2001).

Table 1. Nature and molecular weights of microbial β-galactosidases (adapted from (Mahoney, 1997; Mlichová & Rosenberg, 2006)).

β-galactosidase sources	Enzyme nature	Molecular weight (kDa)
Aspergillus niger	Extracellular	124
Aspergillus oryzae	Extracellular	90
Bacillus circulans	Intracellular	240
Bacillus stearothermophilus	-	116
Bacillus subtilis	-	88
Escherichia coli	Intracellular	464
Kluyveromyces lactis	Intracellular	228
Kluyveromyces fragilis	Intracellular	201
Lactobacillus acidophilus	Intracellular	540
Streptococcus thermophiles	-	464

However, only a few number of β-galactosidases sources are Generally Recognized As Safe (GRAS status) and eligible for usage in the pharmaceutical and food industries (Numanoğlu & Sungur, 2004). For example, *Escherichia coli* produces β-galactosidase and it is the most extensively explored source but its industrial applications are hampered because it is not considered safe for food production (González Siso, 1996; Panesar et al., 2006).

At the industrial level, only microbial sources are used to produce β-galactosidases (Guerrero et al., 2015; Sanz Valero, 2009) since, compared with plant and animal sources, the microorganisms produce enzymes at higher yields which can result in a lower price of β-galactosidases (Santos, Ladero, & García-Ochoa, 1998). The industrial enzymes are mainly produced by *Kluyveromyces* spp. and *Aspergillus* spp., as they can be obtained with acceptable productivities and yields and these

microorganisms are generally considered as GRAS (Guerrero et al., 2015; Husain, 2010; Panesar et al., 2006).

Kluyveromyces spp. are lactose fermenting yeasts and are good sources of intracellular β-galactosidases (Numanoğlu & Sungur, 2004) with optimal pH at 6–7 which is suitable to produce lactose-free products (Genari, Passos, & Passos, 2003). One disadvantage of these enzymes is the need to be extracted by disruption or permeabilization of cells since they are produced intracellularly (Panesar et al., 2006).

Table 2. Biochemical properties of microbial β-galactosidases with hydrolytic activity.

Source of β-galactosidase	Temperature (ºC)	pH	Specific activity (U/mg)	Reference
Aspergillus aculeatus	60	3.5	7.2	(Guerrero et al., 2015)
Aspergillus niger	65	3.5	2.7	(Guerrero et al., 2015)
Aspergillus oryzae	65	4.5	419.3	(Guerrero et al., 2015)
Bacillus circulans	60	6	60	(Guerrero et al., 2015)
Kluyveromyces lactis	50	7	194.4	(Guerrero et al., 2015)
Kluyveromyces marxianus	30	5	59	(Bansal, Oberoi, Dhillon, & Patil, 2008)

Table 3. Biochemical properties of microbial β-galactosidases with transgalactosylation activity (adapted from (Park & Oh, 2010)).

Source of β-galactosidase	Temperature (ºC)	pH	Specific activity (U/mg)
Aspergillus aculeatus	55 – 60	5.4	24
Aspergillus oryzae	30	4.8	40
Bacillus circulans	50	5 – 6	5.1
Bifidobacterium adolescentis	50	6	526
Cryptococcus laurentii	58	4.3	12
Geobacillus stearothermophilus	65	6.5	0.5
Kluyveromyces lactis	50	7	-
Lactobacillus acidophilus	55	6.5 – 8	230
Streptococcus pneumonia	30	5.5 – 7.5	-
Sulfolobus solfataricus	75	6.5	116
Thermotoga maritima	80 - 85	6.5	70
Thermus aquaticus	80	5.5	5.7

β-galactosidases from *Aspergillus* spp. are acidic (optimal pH between 2.5–4.5) and their main application is in the hydrolysis of lactose in acidic cheese whey (Boon, Janssen, & Van 't Riet, 2000; Panesar et al., 2006; Yang & Silva, 1995). Some advantages of these fungal enzymes are the low price and high thermal stability (temperatures up to 50 °C can be used (Panesar et al., 2006; Zadow, 1984)). Furthermore, they are generally secreted by fungi, thus making their recovery easier (Guerrero et al., 2015; Panesar et al., 2006; Raol et al., 2015; Zadow, 1984). As a disadvantage, they are described as more sensitive to galactose inhibition (Boon et al., 2000). The use of enzymes from fungi, such as *Aspergillus niger* and *Aspergillus oryzae*, predominates due to their extracellular nature, high production levels and high stability (Nakayama & Amachi, 1999).

Aspergillus lacticoffeatus was chosen to be further studied in this work because it has been poorly described in the literature, indeed it has not yet been documented as a β-galactosidase producer and it presents a high similarity with *Aspergillus* spp., namely with *A. niger* (Maciel, 2013), which means that it could hold all the advantages mentioned before for the enzyme production.

1.1.2. β-galactosidase production

Stability and activity of enzymes are influenced by some environmental factors, such as temperature, pH, reaction medium, agitation, aeration, among others (Henley & Sadana, 1984; Sadana & Henley, 1986; Zale & Klibanov, 1983). For example, some studies showed that the enzyme production by *K. marxianus* was strongly influenced by agitation conditions, while aeration was less significant (Alves, Filho, De Medeiros Burkert, & Kalil, 2010). Thus, to improve the β-galactosidase production and activity, environmental factors should be optimized also considering the favorable conditions for microorganism growth.

Regarding the source of β-galactosidase, it is well-known that this enzyme can be produced intracellularly or secreted to the extracellular medium (**Table 1**). When the enzyme is intracellular it becomes necessary to proceed to the cell rupture in order to obtain the enzyme. There are several methods to disrupt the cells, each exhibiting advantages and disadvantages. Some studies showed that the excretion method using ultrasounds is the most effective to recover β-galactosidases from *K. lactis*, *E. coli* and *Lactobacillus* strains (Becerra, Rodríguez-Belmonte, Cerdán, & González Siso, 2001; Özbek & Ülgen, 2000; D. Wang & Sakakibara, 1997).

The fermentation operation mode also influences the production and recovery of the enzyme. Batch operation is the traditional procedure since the enzyme is continuously synthesized and it is simple and easy to control. For *Kluyveromyces* spp., the maximum yield is obtained at the end of the exponential growth phase and the beginning of the stationary phase (Pinheiro, Belo, & Mota, 2003; Ranzi, Porro, Compagno, & Martegani, 1987; Rios, Belleville, Paolucci, & Sanchez, 2004). However, some studies reported that fed-batch fermentations using lower levels of substrate concentration led to higher biomass concentrations and consequently higher enzyme production and activity. To achieve a high productivity (g enzyme/g cells. h), the industrial fermentations must be planned to reach high cell density and this can be accomplished through the use of fed-batch operation mode (Panuwatsuk & Da Silva, 2003; Prytz et al., 2003).

Nowadays, the "waste valorization" concept has drawn considerable interest, mainly due to the stricter environmental legislation. Furthermore, the use of these residues is also an advantage, since they could be employed as inexpensive substrates in enzyme production.

Cheese whey is the main by-product of the dairy industry and it contains a large amount of lactose (Table 4). Around 9 L of whey is produced during the production of 1 kg of cheese, which leads to an estimated accumulation around 180 to 190 million ton of whey each year (Guimarães, Teixeira, & Domingues, 2010; Mollea, Marmo, & Bosco, 2013). In Portugal, around 1 million liter of cheese whey are discarded to the environment per day (Domingues, Lima, & Teixeira, 2004). This residue is a very polluting product and it can cause an excess of oxygen consumption, impermeabilization, eutrophication and toxicity in the receiving environment (Marwaha & Kennedy, 1988; Mawson, 1994; Rech & Ayub, 2007; Tejayadi & Cheryan, 1995). Additionally, the dumping of cheese whey constitutes a significant loss of food and energy, since it can be used, for example, as culture medium for microorganisms able to metabolize lactose and produce added-value compounds, such as prebiotics, biogas and bioethanol (González Siso, 1996; Guimarães et al., 2010; Nath et al., 2016; Rech & Ayub, 2007; Shete & Shinkar, 2013).

Table 4. Typical composition of cheese whey (Fuquay & Jelen, 2011; Panesar et al., 2007).

Components	Acid whey (g/L)	Sweet whey (g/L)
Total solids	63–70	63–70
Lactose	44–46	46–52
Proteins	6–8	6–10
Calcium	1.2–1.6	0.4–0.6
Phosphate	2–4.5	1–3
Lactate	6.4	2
Chloride	1.1	1.1

Another example of industrial residue is the corn steep liquor (CSL), which is the main by-product of the corn wet-milling industry and it is largely used as an inexpensive substrate, replacing yeast extract and peptone, due to its high amounts of nitrogen, vitamins and amino acids (Parekh, Formanek, & Blaschek, 1999; Pereira, Guimarães, Teixeira, & Domingues, 2010). CSL has been successfully used in the production of antibiotics (De Azeredo, De Lima, Coelho, & Freire, 2006), biosurfactants (Gudiña, Fernandes, Rodrigues, Teixeira, & Rodrigues, 2015), cellulase (Grigorevski De Lima, Pires Do Nascimento, Da Silva Bon, & Coelho, 2005), protease (De Azeredo et al., 2006) and glucose isomerase (W. P. Chen, Anderson, & Han, 1979), by some microorganisms and also as a ruminant feedstuff (Azizi-Shotorkhoft, Sharifi, Mirmohammadi, Baluch-Gharaei, & Rezaei, 2016; Nascimento, Junior, Pereira, Bon, & Coelho, 2009).

1.1.3. β-galactosidase activities

The main application of the hydrolytic activity of β-galactosidases is in the hydrolysis of lactose in milk or derived products in order to produce lactose-free dairy products (Benavente et al., 2015; Husain, 2010; Oliveira et al., 2011; Raol et al., 2015). Actually, around 70% of the World population is lactose intolerant (**Figure 2**), resulting from absent or reduced β-galactosidase activity (Adam, Rubio-Texeira, & Polaina, 2004; Benavente et al., 2015; Husain, 2010). Nowadays, there is already a huge market of lactose-free dairy products which are obtained through the use of β-galactosidases (Oliveira et al., 2011). This hydrolytic activity is also important because it prevents lactose crystallization problems and increases the sweetening capacity which is advantageous in the production of several food products such as ice creams, condensed milk, among others (Rodríguez, Leiro, Cerdán, González Siso, & Fernández, 2008; Zadow, 1984).
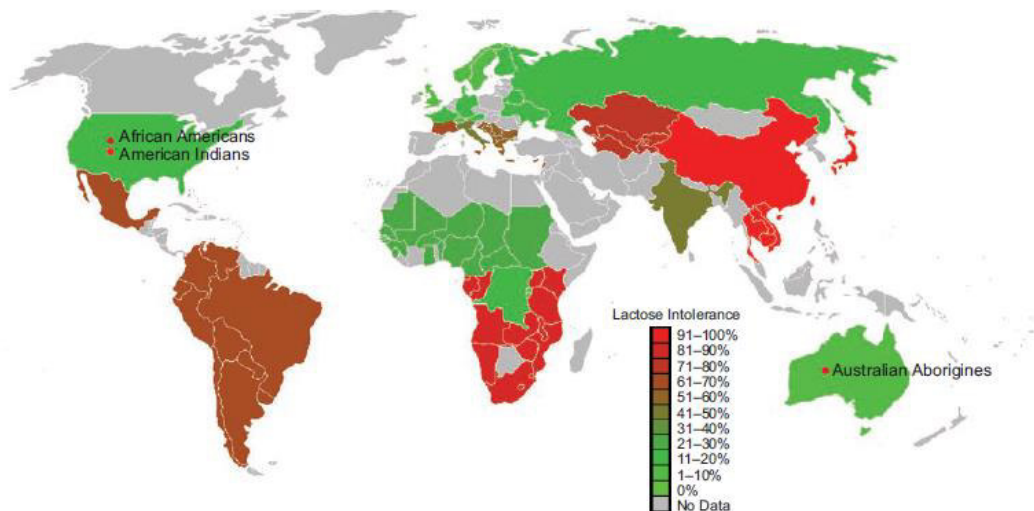
Figure 2. Distribution of lactose intolerance in the World (taken from (Husain, 2010)).

The transgalactosylation activity of β-galactosidases has been extensively explored and is well documented. This reaction occurs in two consecutive steps: the formation of galactosyl-enzyme complex followed by the transfer of the galactose moiety to a galactose acceptor (Albayrak & Yang, 2002; Oliveira et al., 2011; Prenosil, Stuker, & Bourne, 1987; Vera, Guerrero, & Illanes, 2011). β-galactosidases are non-specific for the galactose acceptor, thus water, carbohydrates (like lactose, fructose or sucrose) (Gänzle, Haase, & Jelen, 2008; Kim, Park, & Oh, 2006; W. Li et al., 2009) and also alcohols (Irazoqui et al., 2009; Klewicki, n.d.; Stevenson, Stanley, & Furneaux, 1993) could act as such, leading to the formation of diverse transgalactosylated compounds, such as GOS, lactulose and lactosucrose (**Figure 1**).

When lactose is present in the reaction medium, the hydrolytic activity competes with the transgalactosylation activity. The transgalactosylation reaction generally prevails at the beginning, when the lactose concentration is high, while the hydrolytic activity becomes predominant as the reaction proceeds (Prenosil et al., 1987; Vera et al., 2011). Nevertheless, some recent studies show that balance between both activities is extremely dependent on the enzyme source (Guerrero et al., 2015; Rabiu, Jay, Gibson, & Rastall, 2001). For example, for β-galactosidase from *A. oryzae* galactose is a stronger inhibitor of the transgalactosylation activity rather than the hydrolysis reaction (Vera et al., 2011).

1.2. Functional food and Prebiotics

In the last years, the concept of food has been changing and food is no longer intended just to provide nutritional support for the body, but also to improve mental and physical well-being and prevent nutrition-related diseases (Menrad, 2003). As many studies have demonstrated, there is a tight equilibrium between the health and the intestinal microflora of the host. Therefore, some food products that beneficially influence the intestinal microbiota had been developed and they are called functional food (Sangwan, Tomar, Singh, Singh, & Ali, 2011).

Functional food is defined as 'natural or processed foods that contain known biologically-active compounds which provides a clinically proven and documented health benefit, and thus, an important source in the prevention, management and treatment of chronic diseases of the modern age (Di Bartolomeo, Startek, & Van Den Ende, 2013)'. Besides, a food can only be considered as a functional food if it performs health and benefits in doses which can be consumed in a normal diet in a natural form (Di Bartolomeo et al., 2013). A 'normal' food can be made functional by increasing the concentration, adding or enhance the bioavailability of a specific component. Many compounds of animal and plants origin can be used to complement functional foods, but nowadays, the most commonly used are probiotics and prebiotics (Sangwan et al., 2011).

The main difference between probiotics and prebiotics is that the first are viable components while prebiotics are unviable food components. However, both are considered safe and effective agents that regulate the gut's micro-environment. Probiotics are normally used to replenish bacteria levels in the gut and, although they are commonly added to fermented dairy products, they do not have a long shelf life due to their active form.

On the other hand, prebiotics are non-digestible functional food ingredients or substances that are not digested on the GI tract and are able to modify the already established intestinal microflora, stimulating the growth or activity of health-promoting bacteria (Gibson & Roberfroid, 1995; Intanon et al., 2014; Oliveira et al., 2011; Roberfroid et al., 2010). Prebiotics have been successfully incorporated in a wide variety of food products like breads, baked goods, nutritional bars, meet products, salad dressings, sweeteners and yoghurts (Sangwan et al., 2011). Contrasting with the probiotic strategy, which provides living organisms, the prebiotic application is considered simpler and more effective to modulate the microflora (Di Bartolomeo et al., 2013). Lactulose, lactosucrose and GOS are some examples of prebiotics derived from lactose.

1.2.1. Lactulose

Lactulose (4-O-β-D-galactopyranosyl-D-fructose) is a ketose disaccharide with a molar mass of 342.30 g/mol and a melting point of 169 ℃. It is a white and odorless powder with a sweet taste, and it is about 1.5 times sweeter than lactose (Ait-Aissa & Aider, 2014; Panesar & Kumari, 2011). The global demand for this sugar has grown considerably in the last decade and, nowadays, it has mainly been used as a medical drug, being registered as such in over 100 countries, against constipation and hepatic encephalopathy (Panesar & Kumari, 2011; Christian Schumann, 2002). Moreover, lactulose is well-recognized as a prebiotic and it has many applications in food industry. For instance, it is added to infant milk formulas, used as bifidus factor or functional ingredient to replicate the prebiotic effect of human milk for a long time (Ait-Aissa & Aider, 2014; Olano & Corzo, 2009).

As lactulose is not found naturally it has to be synthetized through three possible production methods (Silvério et al., 2016) (**Figure 3**):

1 - Chemical synthesis - that is based in the alkaline isomerization of lactose via the Lobry de Bruyn-Alberda van Ekenstein rearrangement, through the transformation of the glucose moiety in lactose to a fructose, yielding lactulose (**Figure 3 – A**). Many catalysts have been employed, such as calcium hydroxide, tertiary amines, sodium aluminate, borates, sodium hydroxide, magnesium oxide and also zeolites and sepiolites (Villamiel, Corzo, Foda, Montes, & Olano, 2002; Zokaee, Kaghazchi, Zare, & Soleimani, 2002). However, this chemical synthesis has several disadvantages, like the high level of lactose degradation, low yields and a vast amount of by-products that are difficult to separate (Aider & Halleux, 2007; H. Wang, Yang, Hua, Zhao, & Zhang, 2013; Zokaee et al., 2002).

2 - Enzymatic synthesis – that is commonly carried out with β-galactosidases and glycosidases by transgalactosylation reactions. The synthesis of lactulose from lactose and fructose by β-galactosidase (**Figure 3 – B**) with transgalactosylation activity could overcome the disadvantages of the chemical method, being beneficial in respect to purification and classification as a natural product (Ait-Aissa & Aider, 2014; Guerrero, Vera, Plou, & Illanes, 2011; H. Wang et al., 2013). A problem associated with this strategy is the eventual lactulose degradation by β-galactosidases, which compromises the yields. However, some strategies have been developed to overcome this limitation, mainly related to protein engineering (Silvério et al., 2016).

3 - Electro-activation – which is based in the electrocatalytic method. The electro-activation occurs in a reactor that is composed of two electrodes submerged into an electrolyte solution and it comprises three compartments: anodic, central and cathodic. The central and anodic compartments can be filled with an electrolyte and the cathodic, where occurs the electro-isomerization, is filled with lactose solution (Aider & Gimenez-Vidal, 2012; Aït Aissa & Aïder, 2013b). The isomerization requires protons, which can be ensured with an alkaline solution (**Figure 3 – C**).
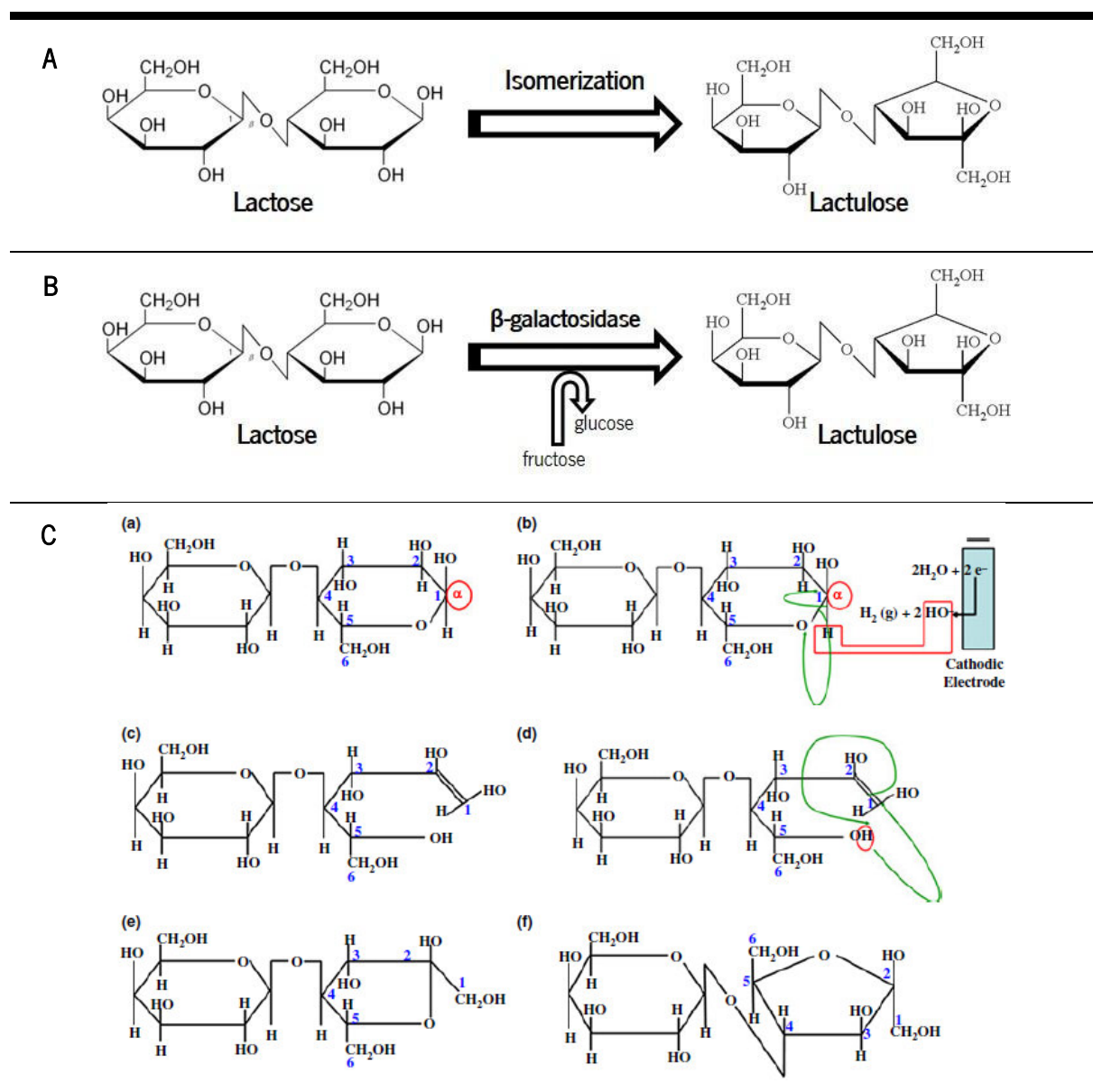
Figure 3. Schematic representation of lactulose production. A – Chemical method. B – Enzymatic method. C – Electro-activation method (taken from (Aït Aissa & Aïder, 2013a): (a) – Lactose molecule; (b), (c), (d) – Formation of the enediol intermediate; (e), (f) - Lactulose molecule.

As previously mentioned, lactulose has its main applications in the pharmaceutical and food industries, and its mechanism of action is related to the bacteria that are present in the lower part of the gut. In the food industry, lactulose is used to produce health-promoting food, once it stimulates the proliferation of good colonic bacteria, such as *Bifidobacteria* and *Lactobacilli*, due to its prebiotic effect (Ait-Aissa & Aider, 2014). One example of this kind of food is the yogurt, since some studies have been reported that lactulose improves its functional properties by improving the stability and survival of probiotic strains (Ozer, Akin, & Ozer, 2005; Tabatabaie & Mortazavi, 2008). Due to its prebiotic effect, lactulose also inhibits the growth of pathogens, once its fermentation by colon bacteria results in the formation of short-chain fatty acids, which leads to a reduction of the intestinal pH and inhibition of the development of harmful bacteria like *E. coli*, *Clostridia* and Bacteroides (Ait-Aissa & Aider, 2014). Additionally, lactulose can also be used as a sugar substitute in bakery products, infant milk powders, yogurts and desserts and as a sweetener for diabetics (Panesar & Kumari, 2011).

In the medico-pharmaceutical field, lactulose is widely used in the treatment of constipation, since it is an osmotic laxative and increases the action of the intestine, and hepatic encephalopathy (Ait-Aissa & Aider, 2014; Panesar & Kumari, 2011). However, several studies made over the years suggest that lactulose has many other potential applications, which are summarized in **Table 5**. Additionally, this prebiotic is also applied to diagnostic colonic disorders through the breath hydrogen test, which consists of the oral administration of lactulose and further determination of the exhaled hydrogen during the next hours. The hydrogen is the result of the bacterial metabolism of lactulose in the intestine, and the amount and time of its production provides information about the colonic transit and microbial colonization (Simrén & Stotzer, 2006). This test is also used for the diagnosis of bacterial overgrowth in the small intestine (Nucera et al., 2005).

Due to its versatile applications, the interest and production of lactulose have increased significantly in the recent years. Nowadays, lactulose is commercially available in either dried form (powder, crystals, granulated) or as syrup (50–72%, w/v). The two major manufacturers are Solvay Pharmaceuticals that is focused on pharmaceutical applications, and Morinaga Milk Industry, which production is geared to food and feed markets (McSweeney & Fox, 2009).

Table 5. Potential lactulose applications in the medico-pharmaceutical field.

Application	Mechanism of action	Reference
Salmonella carriers	- Reduces the colonic pH, which makes *Salmonella* difficult to survive - Clears the fecal *Salmonella* and reduces the incidence of urinary and respiratory infections	(Christian Schumann, 2002) (Liao, Cui, Jin, & Floren, 1994)
Inflammatory bowel disease	- Reduces in production and absorption of gut endotoxins - Regulates the bowel habit - Reduces of oxidative stress and ameliorates of the symptoms	(X. Chen, Zuo, Hai, & Sun, 2011; Panesar & Kumari, 2011)
Anti-endotoxins	- Reduces endotoxemia - Prevents endotoxin-dependent operation complications, such as renal dysfunction	(Koutelidakis et al., 2003)
Reducing blood ammonia levels	- Increases the incorporation of ammonia - Inhibits the synthesis and activity of enzymes that generate ammonia	(Ait-Aissa & Aider, 2014)
Laxative	- Stimulates peristaltic movements by increasing acidification and gas formation - Reduces the colon transit time and increases the sense of urgency by increasing the volume of intestinal contents	(Ait-Aissa & Aider, 2014)
Colon carcinogenesis	- Prone to reduce the risk of colon cancer, once colonic flora influences colon carcinogenesis	(Wollowski, Rechkemmer, & Pool-Zobel, 2001)
Tumor prevention and immunology	- Enhances anti-tumor and immunology effects of *Bifidobacteria* - Prevent complications after surgery of obstructive jaundice	(Schumann, 1997) (Greve, Gouma, von Leeuwen, & Buurman, 1990)
Mineral absorption	- Promotes the absorption and retention of Ca, Mg, Zn, Cu and Fe	(Ait-Aissa & Aider, 2014)
Inhibition of secondary bile acid formation	- Decreases the concentration and excretion of the secondary bile acids	(van Berge Henegouwen, van der Werf, & Ruben, 1987)

1.2.2. Lactosucrose

Lactosucrose (O-β-D-galactopyranosyl-(1,4)-O-α-D-glucopyranosyl-(1,2)-β-D-fructofuranoside) is a trisaccharide composed of glucose, galactose and sucrose, with a molecular weight of 504.44 and a melting point around 181 °C. It is a white powder with bland taste, highly hygroscopic and has a high moisture-retaining capacity (McSweeney & Fox, 2009; Silvério et al., 2015). There are some evidences that this trisaccharide is poorly hydrolyzed by the human digestive enzymes on the GI tract, thus reaching the colon almost intact, where it is selectively fermented by intestinal *Bifidobacterium* (Fujita, Ito, & Kishino, 2009; Oku, Tanabe, Ogawa, Sadamori, & Nakamura, 2011). However, lactosucrose is not yet

considered a prebiotic ingredient and additional studies are needed to prove that it satisfies all the criteria established for prebiotic compounds (Silvério et al., 2015).

This carbohydrate is obtained through the use of enzymes that are able to catalyze both hydrolysis and transfer reactions, like β-fructofuranosidases and β-galactosidases, using lactose and sucrose as substrates. When the reaction is catalyzed by β-fructofuranosidases or levansucrases, the transfer of the fructosyl residue from sucrose (donor) to lactose (acceptor) occurs. On the other hand, when β-galactosidase is the catalyst, the transfer of the galactosyl residue from lactose to sucrose occurs. However, in both enzymatic routes, other compounds can act as acceptors, such as water, sucrose, and even the formed lactosucrose, leading to the formation of undesired by-products, hence compromising the yield and productivity (Silvério et al., 2015). Another problem associated with these strategies is the product degradation by the enzyme, and some approaches have been developed in order to minimize this limitation. Examples include thermal deactivation of the enzyme, the use of immobilized enzymes, the process integration combining synthesis and product separation and protein engineering that can improve the performance of the enzymes (Kawase, Pilgrim, Araki, & Hashimoto, 2001; Mikuni et al., 2000; Pilgrim, Kawase, Matsuda, & Miura, 2006; Silvério et al., 2015).

The benefits associated to the ingestion of lactosucrose have already been reported, and some studies clearly showed that it has a beneficial effect in the intestinal microflora maintenance and intestinal protection, since it promotes *Bifidobacterium* multiplication, reduces intestinal pH, inhibits the proliferation of pathogenic bacteria and enhances mineral bioavailability (Mu, Chen, Wang, Zhang, & Jiang, 2013; Roberfroid et al., 2010).

Lactosucrose has many potential applications in the food industry and it is widely used in Japan in diverse types of food and drinks to improve the GI health and well-being. As a low-digestive sweetener, lactosucrose can be included for example in bakery products, yogurts, ice creams, cookies, infant formula, instant soups and mineral water. In addition, as it has a potential effect on the inhibition of body fat accumulations, preventing the obesity and, since it is low in calories it is also suitable as an ingredient for low-calorie food. This carbohydrate is also added to pet and fish food (Mu et al., 2013; Silvério et al., 2015; Teramoto et al., 2006). In pharmaceutical and cosmetic industries, lactosucrose has been used as a microflora regulator (Garseen, van Tol, Jben, & Verlaan, 2009), as an active ingredient to prevent some skin diseases (Nobuaki, 1998) and also as an excipient (Bassarab et al., 2005).

At the industrial level, the production of lactosucrose is dominated by two Japanese companies, Ensuiko Sugar Refining Co. and Hayashibara Shoji Inc. Lactosucrose is commercially available as a powder (> 45%) or syrup (72–75%, w/v) (McSweeney & Fox, 2009).

1.2.3. Other Oligosaccharides

Besides lactulose and lactosucrose, other non-digestible oligosaccharides have been reported as important constituents of functional food. Some examples of these oligosaccharides are the galacto-oligosaccharides (GOS) and fructo-oligosaccharides (FOS) which are properly recognized as prebiotics and are well known for their contribution to the GI tract health (Kovács et al., 2013; Sangwan et al., 2011).

GOS are defined as oligosaccharides with 3 to 5 sugar units bound through glycosidic bonds and presenting glucose or galactose at the reducing end (Gosling, Stevens, Barber, Kentish, & Gras, 2010). GOS are an ideal choice for formulating food and beverages for digestive and immune health, and they are also particularly attractive for its similarity to the human milk oligosaccharides (Sangwan et al., 2011; Venema, 2012). Additionally, due to their properties, GOS are also used in many other applications (**Table 6**). These oligosaccharides are synthesized by the transgalactosylation of lactose catalyzed by β-galactosidase and they are mostly commercially available in preparations containing up to 50- 60%, with differences in the molecular size and the type of linkage (Rastall, 2010; Sangwan et al., 2011).

Table 6. Properties and applications of GOS (McSweeney & Fox, 2009).

Properties	Applications
Low sweetness	Low glycemic index, low calorie foods
Prevention of hygroscopicity	Candies, sweets, chocolates
Prevention of coloration	Fruits, jams
Reinforcement agent or thickener	Sauces, creams, jelly
Glazing agent	Rice crackers, hard lollies
Regulation of freezing point	Ice cream, frozen foods
Humectant	Cakes, pastries
Powdering material	Coffee whitener, soups

Other interesting oligosaccharides possible to obtain by enzymatic synthesis are the FOS. These oligosaccharides are generally composed by one sucrose molecule linked to 1 to 3 units of fructose and

are commonly called 1-kestose, nystose and 1-β-fructofuranosylnystose (Ganaie, Lateef, & Gupta, 2014; Guío, Rodríguez, Alméciga-Diaz, & Sánchez, 2009). FOS exhibited functional properties such as prevention of colonic carcinogenesis, low caloric values, modulation of colonic microflora, decrease of lipids and cholesterol and improvement of the bioavailability of minerals, the GI physiology and immune functions (Dominguez et al., 2012; Guío et al., 2009). These oligosaccharides are currently produced by the transfructosylation activity of fructosyltransferase and β-fructofuranosidase, derived from plants and microorganisms (Dominguez et al., 2012).

1.3. Project Aims

The specific aims of this work consist of:

- Validation of *A. lacticoffeatus* as a β-galactosidase producer
- Evaluation of different fermentation media (synthetic media, CSL and cheese whey) and operational conditions towards the optimization of β-galactosidase production
- Enzyme characterization (size, optimal temperature and pH and the effect of several compounds)
- Potential application of β-galactosidase from *A. lacticoffeatus* in the synthesis of prebiotics (lactulose and lactosucrose)

2. MATERIALS AND METHODS

2.1. Microorganism and growth conditions

Aspergillus lacticoffeatus (MUM 06.150) was obtained from MUM (Mycology collection of University of Minho). The microorganism was grown on PDA (Potato dextrose agar) medium Petri plates at 25 °C for 7-10 days.

2.2. Chromogenic test

The chromogenic test was performed in Petri plates containing MEA (malt extract agar) medium where the glucose was replaced by lactose: malt extract (2% w/v), lactose (2% w/v), peptone (0.1% w/v) and agar (2% w/v). The MEA medium was supplemented with 0.5% (v/v) of a solution of 20 mg/mL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside). The microorganism was inoculated and the plates were incubated protected from light at 25 °C for 7 days. The appearance of a blue color in the plates suggest that β-galactosidase was produced.

2.3. Fermentation conditions

Spore suspensions for inoculums were prepared in sterile saline solution (0.85% NaCl) containing 0.01% Tween 80. Conidia density was adjusted to 10^6 conidia/mL using a Neubauer counting chamber. The fermentations were performed at different temperatures (28 °C and 37 °C, the most reported in literature to grow several *Aspergillus* spp.), using 180 revolutions per minute (rpm) and different periods of time (6-19 days). Several fermentation media were studied. Synthetic medium (Medium A) was constituted by lactose (2% w/v), peptone (0.4% w/v), yeast extract (0.4% w/v), KH_2PO_4 (0.2% w/v), $Na_2HPO_4.12H_2O$ (0.8% w/v) and $MgSO_4.7H_2O$ (0.025% w/v). Cheese whey, provided by Queizuar, S.L. (A Coruña, Spain), and Corn steep liquor (CSL), provided by COPAM: Companhia Portuguesa de Amidos, S.A. (S. João da Talha, Portugal), were also evaluated as alternative fermentation media.

CSL was used to replace peptone and yeast extract and 2 different media were prepared: Medium B – CSL (0.8% w/v), lactose (2% w/v) and salts (KH_2PO_4, $Na_2HPO_4.12H_2O$ and $MgSO_4.7H_2O$) in the same concentrations of the synthetic medium; Medium C – CSL (2% w/v), lactose (2% w/v) and salts in the same concentrations of the synthetic medium.

Cheese whey was used as lactose source and 4 different media (with similar lactose concentration (2% w/v)) were prepared: Medium D – Cheese whey; Medium E – Cheese whey supplemented with salts

in the same concentrations of the synthetic medium; Medium F – cheese whey supplemented with extracts (peptone and yeast extract) in the same concentrations of the synthetic medium; Medium G – Cheese whey supplemented with salts and extracts in the same concentrations of the synthetic medium.

2.4. Intracellular enzyme extraction

Three different methods, reported in literature as the most used for β-galactosidase extraction, were tested to extract the intracellular enzyme:

Method 1 – Sonication: 0.2 g (wet weight) of properly washed cells was suspended in a 15 mL Falcon tube containing 2 mL of sodium-citrate buffer (50 mM pH 4.5). The cell suspensions were sonicated using an ultrasonic homogenator Cole-Parmer 750-Watt (13 mm probe tip, 35% of amplitude, 30 seconds pulse and 10 seconds pause for cooling) for different periods of time (2, 4 and 6 minutes). The tubes containing the cells were kept in ice during sonication. After that, the tubes were centrifuged at 4000 g for 15 minutes and the β-galactosidase activity in the supernatant was determined.

Method 2 – Glass beads: 0.1 g (wet weight) of properly washed cells was suspended in 1 mL of sodium-citrate buffer (50 mM pH 4.5) and 0.2 g glass beads (425-600 µm, Sigma) were added. The cell lysis was performed using a cell disruptor Fast Prep FP120 (Thermo): 6.5 m/s for 2, 4 and 6 minutes (30 seconds on and 30 seconds cooling on ice). After that, the suspensions were centrifuged at 4000 g for 15 minutes and the β-galactosidase activity in the supernatant was determined.

Method 3 – Tween-80: 0.2 g (wet weight) of properly washed cells was suspended in 2 mL of sodium-citrate buffer (50 mM pH 4.5). The cell suspensions were shaken, in an oscillating platform shaker, in the presence of 0.5, 1 and 5% (w/v) Tween-80 for 30 minutes and 1 hour at room temperature. Then, the suspensions were centrifuged at 4000 g for 15 minutes and the β-galactosidase activity in the supernatant was determined.

2.5. Enzyme precipitation

Two different methods were tested for enzyme precipitation:

Method 1 - Isopropanol precipitation: enzyme samples were combined with 100% isopropanol (1:1, v/v). After vortex mixing, the samples were placed overnight at -18 °C and then centrifuged at 10 000 g for 15 minutes at 4 °C. The supernatant was removed and the dried pellets were resuspended in

appropriate volume of sodium-citrate buffer (50 mM pH 4.5). The enzymatic activity was determined using the β-galactosidase activity assay, as described before (**Section 2.6**).

Method 2 – Trichloroacetic acid (TCA) precipitation followed by acetone washing: enzyme samples were combined with 100% TCA (4:1, v/v). The mixture was incubated at 4 °C for 20 minutes and then centrifuged at 15 000 g for 10 minutes at 4 °C. The supernatant was removed and ice-cold acetone was added. A new centrifugation was performed (using the same conditions as the ones described above), the supernatant was removed and the dried pellets were resuspended in appropriate volume of sodium-citrate buffer (50 mM pH 4.5). The enzymatic activity was determined using the β-galactosidase activity assay, as described before (**Section 2.6**).

2.6. β-galactosidase activity assay

The hydrolytic β-galactosidase activity was determined using the methodology described by Nagy et al. (Nagy, Kiss, Szentirmai, & Biró, 2001) with some modifications. Samples containing the enzyme were previously centrifuged and the supernatant (50 µL) was incubated, at 37 °C for 30 minutes, with 50 µL of *O*-nitrophenyl-β-D-galactopyranoside (ONPG, Sigma) solution (3 mM) prepared in sodium-citrate buffer (50 mM pH 4.5). The reaction was stopped by the addition of 200 µL of sodium carbonate (0.1 M). The released O-nitrophenol was determined spectrophotometrically at 415 nm. One unit (U) of enzyme was defined as the amount of enzyme that liberates 1 µmol of *O*-nitrophenol from ONPG per minute under the assay conditions.

2.7. Sugar quantification

Lactose consumption over fermentation time was determined using the DNS method (Miller, 1959). The aldehyde and ketone groups of the sugar will reduce the 3,5-dinitrosalicylic acid (DNS) to 3-amino-5-nitrosalicylic acid. Lactose solutions with different concentrations (from 0.2 to 10 g/L) were used to obtain the calibration curve. Samples from the fermentation broth (100 µL) were incubated, at 100 °C for 10 minutes, with 100 µL of DNS reagent. After samples being cooled in ice bath, 1 mL of distilled water was added. The reduced 3-amino-5-nitrosalicylic acid was detected spectrophotometrically at 540 nm.

2.8. Determination of the biomass wet weight

The fermentation broth was filtered and the biomass was conveniently washed with distilled water. In order to remove the water excess, the biomass previously filtered was transferred to a Petri plate containing a double paper filter and allowed to dry for 15-20 minutes. After that, the biomass wet weight was determined using a precision balance.

2.9. Mycotoxins production evaluation

The fermentation was conducted in synthetic medium for 9 days at 28 °C and 180 rpm and samples were collected at different time points (3, 6 and 9 days) to assess the ocratoxin A (OTA) production. The extraction of OTA was performed by mixing 2 mL of fermentation broth with 2 mL of an extraction solution containing acetonitrile:methanol:acetic acid (78:20:2, v/v). After 10 minutes, the solution was mixed in a vortex for 1 minute and filtered (0.2 µm) to an amber vial. The OTA production was evaluated by HPLC-FD with a Jasco FP-920 fluorescence detector (λ_{ex}= 333 nm and λ_{em}= 460 nm). Chromatographic separations were performed on a reverse phase C18 column YMC-Pack ODS-AQ (250 mm x 4.6 mm ID, 5 µm) fitted with a pre-column using the same stationary phase. The mobile phase composed by acetonitrile:water:acetic acid (99:99:2, v/v) was pumped at 0.8 mL/min and the injection volume was defined as 50 µL. A calibration curve was previously prepared with standards of OTA (Sigma) in the range 1 - 50 ng/mL (Soares, Calado, & Venâncio, 2012).

2.10. Sodium Dodecyl Sulfate-Polyacrylamide gel electrophoresis (SDS-PAGE)

The molecular size of the enzyme was determined by SDS-PAGE, according to the Laemmli (Laemmli, 1970) methodology. The samples were previously treated with 2x Laemmli Sample Buffer (65.8 mM Tris-HCl pH 6.8, 2.1% SDS, 26.3% glycerol, 0.01% bromophenol blue and 0.05% 2-mercaptoethanol) and then denatured at 100 °C for 10 minutes. Electrophoresis was performed using acrylamide gels (Table 7) and conducted for 60 minutes at 150 V. Gels were stained with Coomassie brilliant blue R-250. The molecular size of the β-galactosidase was determined from the relative mobility of molecular size markers (PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa, Thermo Scientific™) run simultaneously.

The hydrolytic activity of the enzyme was investigated by preparing a zymogram. The samples were previously treated with 2x Laemmli Buffer but, not thermally denatured. Electrophoresis was performed using acrylamide gels (Table 7) without SDS and conducted for 60 minutes at 150 V. Gels were properly washed with water and then placed in a 0.5% (v/v) Triton X-100 solution prepared in sodium-citrate buffer (50 mM pH 4.5) for 20 minutes. The gels were then incubated with 20 mL of X-gal (1 mg/mL).

Table 7. Composition of the running and stacking gels.

	Running gel (10%)	Stacking gel (4%)
Acrylamide/Bis-acrylamide	3 mL	0.4 mL
Tris–HCl 0.5 M pH 6.8	–	1 mL
Tris–HCl 1.5 M pH 8.8	3 mL	–
Ultrapure water	5.3 mL	2.35 mL
SDS 10%	120 μL	40 μL
TEMED	6 μL	3 μL
APS 10%	400 μL	200 μL

2.11. Enzyme characterization

The effect of temperature, pH, metal ions, additives, detergents and sugars on the extracellular enzyme activity was evaluated.

The temperature effect on the enzyme activity was evaluated in a 50 mM sodium-citrate buffer (pH 4.5) at various temperatures (15, 25, 35, 40, 45, 50, 55 and 60 °C). For each temperature, the enzymatic activity was determined using the β-galactosidase activity assay, as described before (Section 2.6).

The effect of pH on the β-galactosidase activity was evaluated at 37 °C in buffer with different pH (3.0, 3.5, 4.5, 5.5, 6.5 and 7.5). The following buffers were used: 50 mM sodium-citrate buffer (pH 3.0–5.5) and 50 mM phosphate-citrate buffer (pH 6.5 and 7.5). For each pH, the enzymatic activity was determined using the β-galactosidase activity assay, as described before (Section 2.6).

To study the effect of different metal ions, additives, detergents and sugars, the 50 mM sodium-citrate buffer (pH 4.5) was supplemented with the several compounds. For metal ions, the buffer was mixed with 10 mM of the following metal ion sources: $CaCl_2$, $BaCl_2$, $MgCl_2$, KCl, NaCl, $ZnSO_4$, $MnCl_2$, $FeCl_2$, LiCl, $CoCl_2$ and $CuSO_4$. The effect of the detergents was studied by preparing 10 mM SDS, 1% (w/v) Tween-40 or 1% (w/v) TritonX-100 in 50 mM sodium-citrate buffer (pH 4.5). For the additives, 10 mM

phenylmethanesulfonyl fluoride (PMSF), 10 mM EDTA or 10 mM ascorbic acid solutions were prepared in 50 mM sodium-citrate buffer (pH 4.5). Finally, for the effect of sugars, different concentrations of fructose, glucose and galactose (5, 25, 75 and 100 mM) were prepared in 50 mM sodium-citrate buffer (pH 4.5). The enzymatic activity was determined using the β-galactosidase activity assay, as described before (Section 2.6).

2.12. Prebiotics production

The prebiotic production was investigated using three different approaches:

Method 1 – The fermentation was performed for 6 days, at 28 $^{\circ}$C and 180 rpm, using the synthetic medium (Medium A). After that, different volumes of 250 g/L substrate solutions (lactose and fructose for lactulose production; lactose and sucrose for lactosucrose production) were added to the medium. Different enzyme/substrates ratios were studied by the addition of different substrate concentrations (10:10, 20:20, 30:30 g/L of lactose to fructose or sucrose) to the fermentation broth. Different substrate ratios were also studied by addition of different substrates proportions (10:20, 15:15, 20:10 g/L of lactose to fructose or sucrose) to the fermentation broth. The prebiotic production was evaluated for 3 days and samples were taken each day.

Method 2 – The fermentation was performed for 6 days, at 28 $^{\circ}$C or 37 $^{\circ}$C and 180 rpm, using the synthetic medium (Medium A). After that, the biomass was collected and placed in 40 mL of 150 g/L solution of substrates (lactose and fructose for lactulose production; lactose and sucrose for lactosucrose production). The prebiotic production was evaluated for 3 days and samples were taken each day.

Method 3 – The fermentation was performed for 9 days, at 28 $^{\circ}$C and 180 rpm, using the synthetic medium (Medium A). After that, the biomass was removed by filtration and the fermentation broth with β-galactosidase activity was used to study the prebiotic production. The enzymatic synthesis was performed at 37 $^{\circ}$C for 6 hours by mixing 5 mL of the enzyme extract with 5 mL of a sugar solution (300 g/L lactose+300 g/L fructose for lactulose production, and 300 g/L lactose+300 g/L sucrose for lactosucrose production) prepared in sodium-citrate buffer (50 mM pH 4.5). Samples were taken each hour to evaluate the prebiotic production along time.

The lactulose conversion and the prebiotic yield were calculated using the following equations, where C_i and C_f are the initial and final concentration of lactose, respectively, and C_p is the concentration of prebiotic.

$$lactose\ conversion = \frac{c_i - c_f}{c_i} \times 100 \qquad\qquad (1)$$

$$prebiotic\ yield = \frac{c_p}{c_i} \times 100 \qquad\qquad (2)$$

2.13. HPLC analysis of the reaction products from prebiotic synthesis

Substrates and products of reactions were analyzed by High Performance Liquid Chromatography (HPLC) using a Prevail Carbohydrate ES column and an Evaporative Light Scattering Detector (ELSD). A mixture of acetonitrile and water (75:25, v/v) pumped at 0.9 mL/min was used as mobile phase. The injection volume was defined as 20 µL.

2.14. β-fructofuranosidase activity assay

The β-fructofuranosidase (FFase) activity was determined by measuring the amount of glucose released from sucrose (Yoshikawa, Amachi, Shinoyama, & Fujii, 2006). The reaction was performed by mixing 100 µL of fermentation extract with 500 µL of sucrose solution (600 mM) prepared in sodium acetate buffer (100 mM pH 5.0) in a total volume of 1 mL. The mixture was incubated for 20 minutes at 30 °C and then the reaction was stopped by boiling for 5 minutes. After cooling, the amount of glucose released was measured by HPLC, using the same conditions described in **Section 2.13**. One U of the FFase activity was defined as the amount of enzyme that releases 1 µmol of glucose per minute from sucrose.

3. RESULTS AND DISCUSSION

3.1. Preliminary study to validate *A. lacticoffeatus* as a β-galactosidase producer

One of the goals of this project was to validate and optimize the β-galactosidase production by *A. lacticoffeatus*. Initial chromogenic tests (**Figure 4**) performed in agar plates containing X-gal suggested that this microorganism is able to produce the enzyme. X-gal is a soluble colorless compound consisting of a galactose linked to a substitute indole. β-galactosidase has high affinity for the galactose part, which leads to the hydrolysis of X-gal and the release of the indole molecule. This molecule dimerizes and originates an insoluble blue product (Juers, Matthews, & Huber, 2012). Thus, on agar plates containing X-gal, it is possible to identify potential β-galactosidase producers by the blue color of the medium.

Figure 4. X-gal test performed in agar plates with the fungus *Aspergillus lacticoffeatus*.

Therefore, two preliminary fermentations under submerged conditions were performed to evaluate the enzyme production. The effect of the temperature and the nature of the enzyme produced (intra or extracellular) was also studied. *A. lacticoffeatus* was grown on agar plates and then inoculated on synthetic medium at 28 °C and 37 °C under 200 rpm agitation. Fermentations were performed for 19 days.

To assess the production of β-galactosidase under submerged conditions, the enzymatic activity was measured along the fermentation time using the ONPG assay previously described (**Section 2.6**). The results obtained confirmed that *A. lacticoffeatus* effectively produces the enzyme. β-galactosidase was produced at both temperatures (28 and 37 °C) and the enzymatic activity increased during the fermentation time to reach a maximum around 16–19 days of fermentation (**Figure 5**).
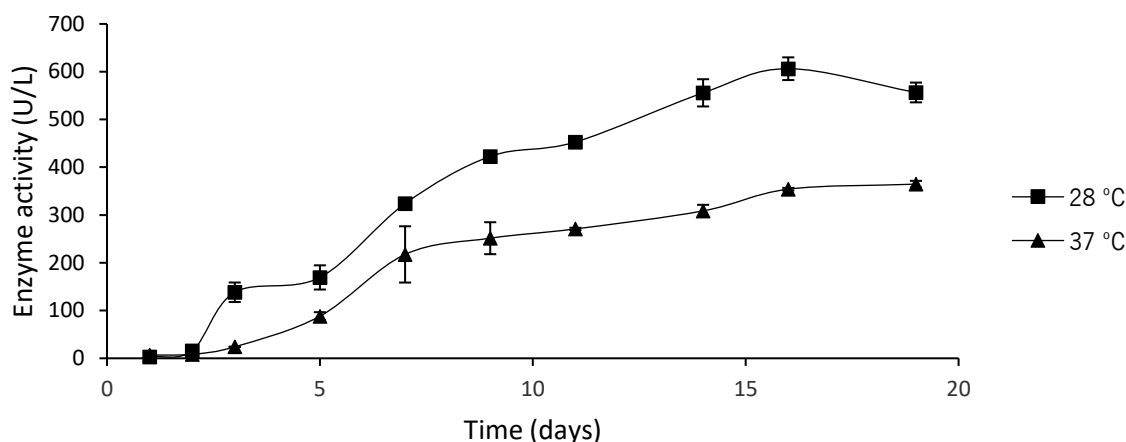
Figure 5. Extracellular β-galactosidase activity from *Aspergillus lacticoffeatus* along a fermentation conducted with culture medium A. Results are shown as mean ± SD of one independent experiment with three replicates.

The consumption of lactose was found to be related with the increase of enzymatic activity (**Figure 6**), which is in accordance with typical batch fermentations. A similar behavior has already been reported for a fermentation conducted with *Saccharomyces cerevisiae* expressing a β-galactosidase from *A. niger* (Domingues, Teixeira, Penttilä, & Lima, 2002).

At 28 °C, *A. lacticoffeatus* started to metabolize lactose at day 2 and took 5 days to consume all the substrate present in the medium, since at day 7 the lactose concentration was almost zero (**Figure 6 – A**). On the other hand, at 37 °C, the microorganism took 9 days to consume all the substrate present in the fermentation medium (**Figure 6 – B**).
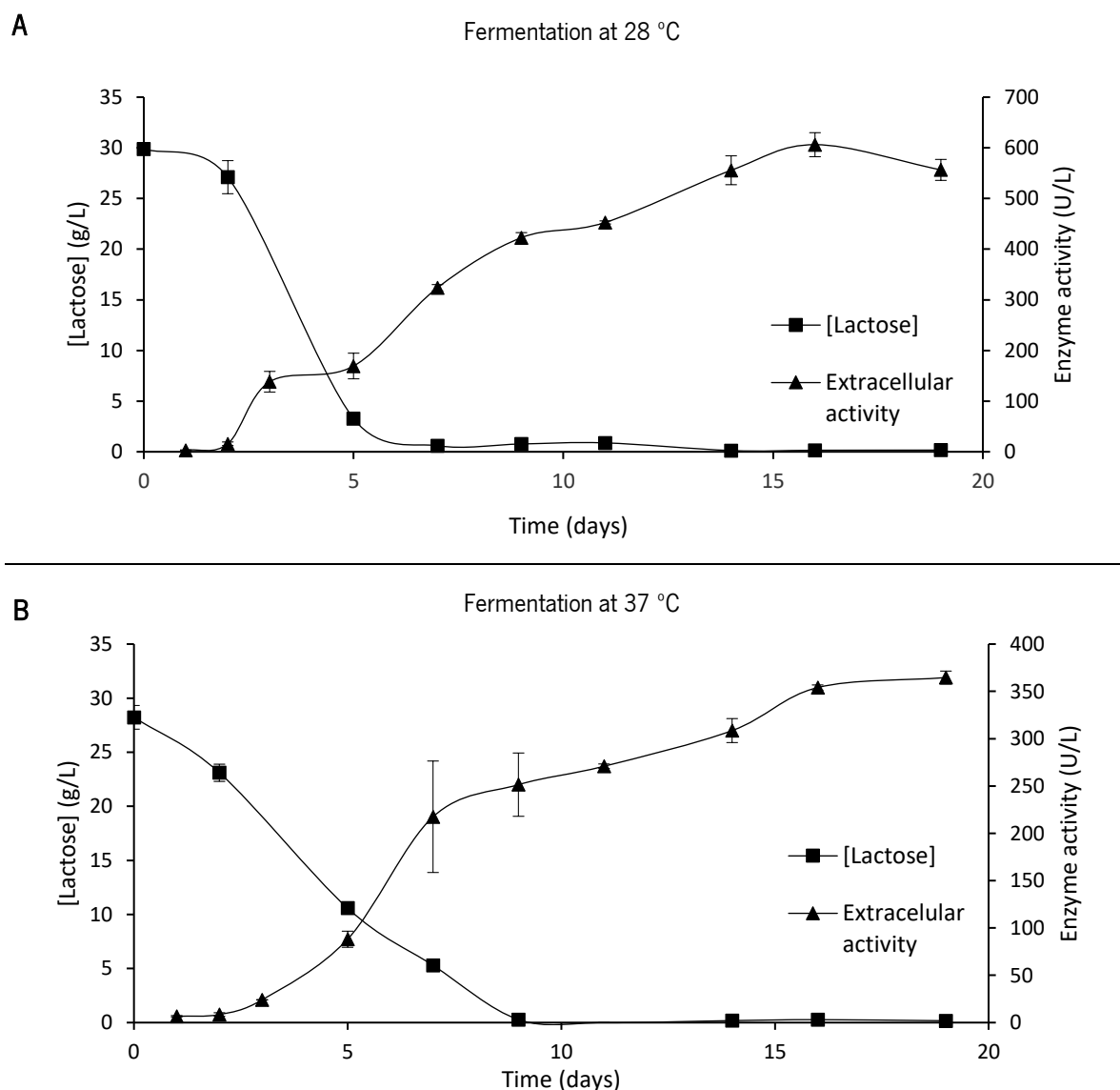
Figure 6. Concentration of lactose and extracellular β-galactosidase from *Aspergillus lacticoffeatus* along a fermentation conducted with culture medium A. (A) Fermentation at 28 °C; (B) Fermentation at 37 °C. Results are shown as mean ± SD of one independent experiment with three replicates.

Besides the enzyme secreted to the fermentation medium, some β-galactosidase retained in the cells (intracellular) may also have been produced. Therefore, intracellular and extracellular activities of the enzyme produced by *A. lacticoffeatus* in the culture medium A at 28 °C and 37 °C were measured and compared (**Table 8**). The intracellular enzyme was determined after the sonication of 0.2 g of biomass (**Section 2.4**) to promote the lysis of the cell walls and the release of the enzyme. The results showed that the extracellular activity was about 10 times higher than the intracellular one for both temperatures studied.

Comparing both temperatures, the results showed that β-galactosidase activity was higher when the enzyme was produced at 28 °C (**Figure 5** and **Table 8**). Similar results have already been reported for several β-galactosidases from different microorganisms like *A. alliaceus* (Sen, Ray, & Chattopadhyay, 2012), *K. marxianus* (Panesar, 2008) and *Enterobacter cloacae* (Ghatak, Guha, & Ray, 2010).

These preliminary results validated *A. lacticoffeatus* as a β-galactosidase producer and led to the further studies of production, characterization and application of the enzyme.

Table 8. Intracellular and maximal extracellular activity obtained for β-galactosidase from *Aspergillus lacticoffeatus* in fermentations conducted in culture medium A during 19 days. Results are shown as mean ± SD of one independent experiment with three replicates.

	Temperature	
	28 °C	37 °C
Intracellular activity (U/L)	66.5 ± 7.5	38.7 ± 9.2
Extracellular activity (U/L)	606.2 ± 23.8	364.6 ± 6.9

3.2. Enzyme extraction

Since the preliminary study showed the production of some intracellular β-galactosidase activity, the first step was to optimize the β-galactosidase extraction from *A. lacticoffeatus* cells.

Sonication, glass beads and Tween-80 extraction protocols (**Section 2.4**), using different conditions, were evaluated regarding the β-galactosidase activity obtained after cell disruption. Among the methodologies tested, sonication was found to be the best method to extract the enzyme from *A. lacticoffeatus* cells (**Figure 7**), providing the highest values of enzymatic activity. It was expected that longer sonication times would lead to higher amounts of released enzyme. However, since the results are presented as enzyme activity instead of the quantity of released enzyme, it is not possible to confirm this fact. Nevertheless, it is important to ensure that the enzyme remained properly active and this aspect was considered in this work. The best condition was 2 minutes of sonication (~ 6.5 U/g biomass). The decrease observed in the β-galactosidase activity for the longer sonication times could be due to the heat generated during the sonication process which could possibly denature the enzyme. Regarding the glass beads method, similar thermal denaturation may also occurred since the higher activity values were found after 2 minutes of rupture (~ 5 U/g biomass), contrary to the expected. Although this method seems to release a properly active enzyme, it has a significant disadvantage related to the limited amount of

biomass that could be submitted to disruption in each experiment. The Tween-80 method proved to be inadequate to promote the release of β-galactosidase, since the values obtained were significantly lower than those observed using the other methods. These results could possibly be justified by the fact that the Tween-80 is a detergent that may promote the denaturation of the enzyme. Comparing the six conditions tested for this detergent, no significant differences were found neither in concentration or exposure time.

Since β-galactosidases from *Aspergillus* spp. are generally extracellular enzymes (Nakayama & Amachi, 1999), there are no studies reporting the best extraction method. However, some studies report sonication as the best method to extract this enzyme from *Kluyveromyces* and *Lactobacillus* spp. (Becerra et al., 2001; Özbek & Ülgen, 2000; D. Wang & Sakakibara, 1997).
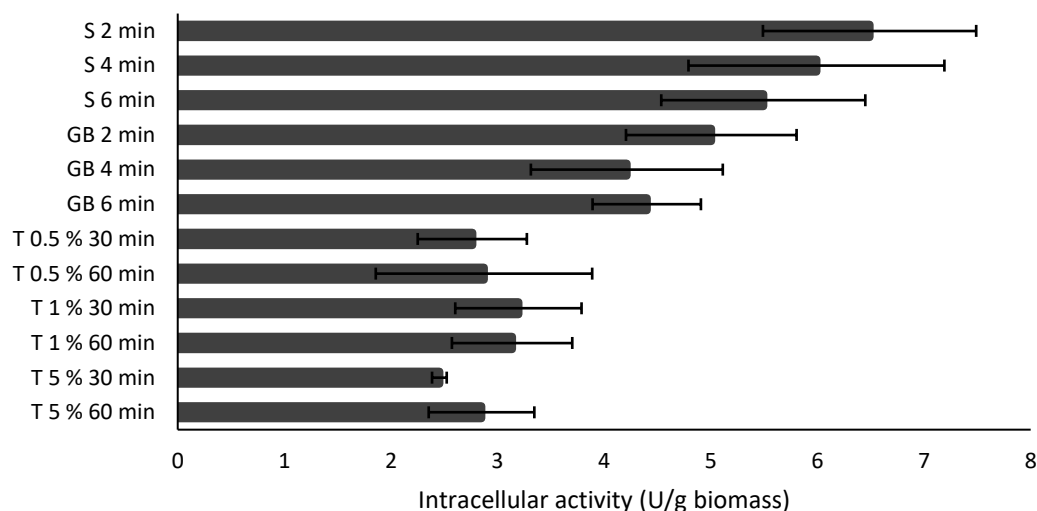
Figure 7. Extraction methods and conditions to extract β-galactosidase from *Aspergillus lacticoffeatus*. Symbols: S – sonication; GB – glass beads (425-600 µm); T – Tween-80. Results are shown as mean ± SD of one independent experiment with three replicates.

3.3. Enzyme production

3.3.1. Fermentation media

A synthetic medium and six alternative media containing the by-products cheese whey or CSL were used to evaluate the *A. lacticoffeatus*'s growth, as well as the production of intra and extracellular β-galactosidase (**Section 2.3**). In all cases, the fermentations were conducted for 6 days and performed in duplicate.

Regarding the amount of biomass produced, no significant differences were observed between the alternative media and the synthetic medium, except for the condition B, which seems to exhibit lower biomass production (**Figure 8**). The results show that the fungus was able to adapt to the different media and grow properly. However, the way *A. lacticoffeatus* grew in the several fermentation media was not similar (**Figure 9**). Although the growth occurred, in all conditions, in the form of pellets, the pellets' size was different when it was grown in the cheese whey medium (**Figure 9 – D**) and cheese whey with salts (**Figure 9 – E**). In these cases, smaller pellets were observed. This difference in the biomass appearance was observed for the media where no supplementation of nitrogen was used (extracts or CSL), which possibly means that this nutrient is related with the biomass production. This result is in accordance with some studies reported in literature for *A. terreus* and *A. oryzae*, that shows that the nitrogen source has effects on microorganism growth and metabolite production (Casas López et al., 2003; Pedersen & Nielsen, 2000).
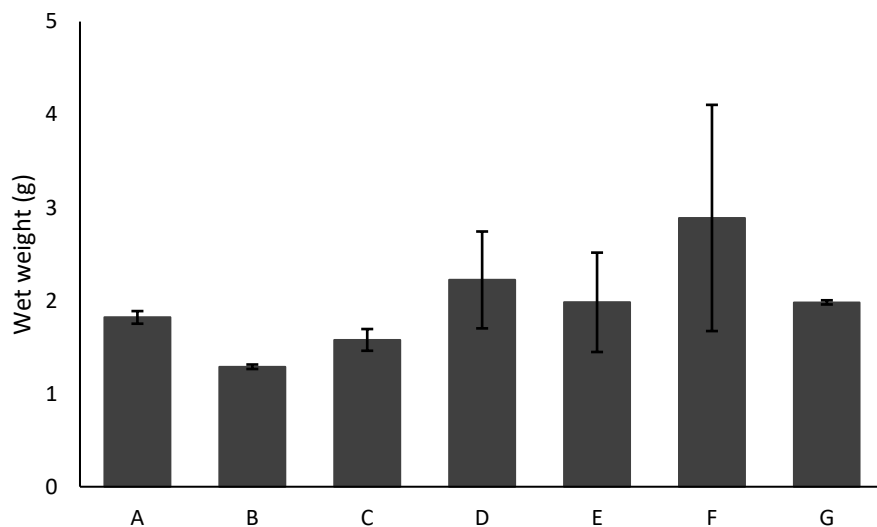
Figure 8. Biomass production (wet weight), after 6 days of fermentation of *Aspergillus lacticoffeatus* in different culture media. (A) – Synthetic medium; (B) – 8 g/L CSL medium; (C) – 20 g/L CSL medium; (D) – Cheese whey medium; (E) – Cheese whey + salts medium; (F) – Cheese whey + extracts medium; (G) – Cheese whey + salts + extracts medium. Results are shown as mean $\pm$ SD of duplicate experiments.
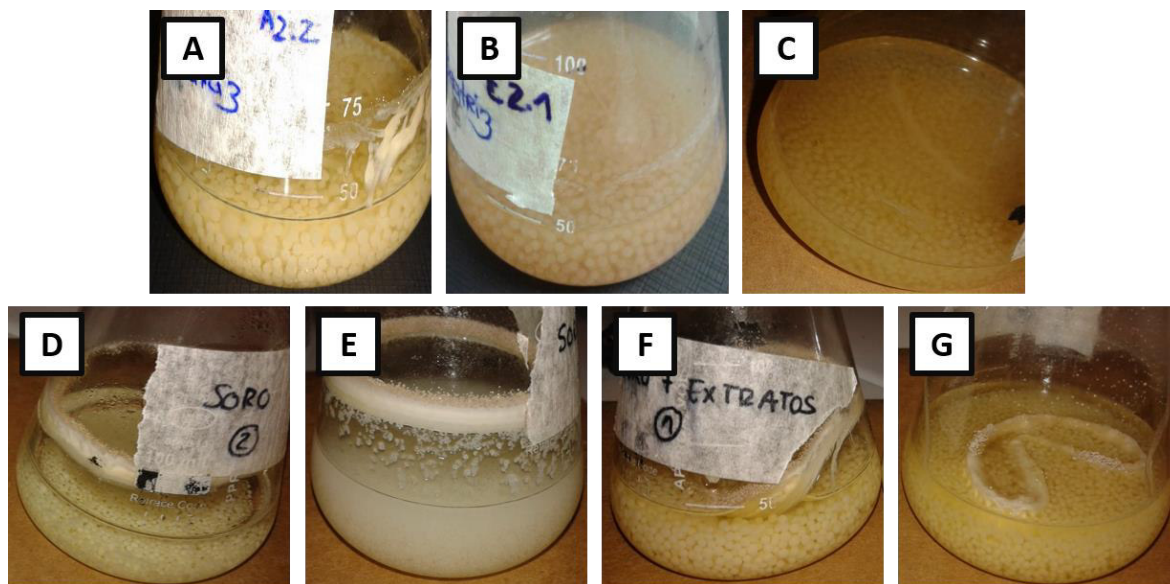
Figure 9. *Aspergillus lacticoffeatus* growth after 6 days fermentation in different culture media. (A) – Synthetic medium; (B) – 8 g/L CSL medium; (C) – 20 g/L CSL medium; (D) – Cheese whey medium; (E) – Cheese whey + salts medium; (F) – Cheese whey + extracts medium; (G) – Cheese whey + salts + extracts medium.

Regarding the intracellular activity, some differences were observed between the media prepared with the by-products and the synthetic medium (**Figure 10**). Both media composed by CSL showed less intracellular activity than the synthetic medium and the activity levels were higher at 20 g/L concentration of CSL than at 8 g/L. As mentioned before, some studies have already report the effect of the nitrogen source in metabolites and enzyme production, which could possibly justify these results (Casas López et al., 2003; Pedersen & Nielsen, 2000). These data are also in accordance with those shown in **Figure 8** for the biomass production, since the enzyme production is directly related with the biomass growth. On the other hand, there were no significant differences between the media with cheese whey and the synthetic medium, except for the cheese whey + salts medium (E) (**Figure 10**), where the intracellular activity was considerably higher. These results could be related with the way the fungus grew in this medium (**Figure 9**). However, it is important to highlight that in the conditions where the fungus grew in presence of cheese whey, the disruption of the pellets was very difficult and it seems that the microorganism became more resistant to the ultrasound treatment. Therefore, in these cases the treatment time was increased (from 2 to 6 minutes), which could have some negative effects on the intracellular activity due to enzyme denaturation.
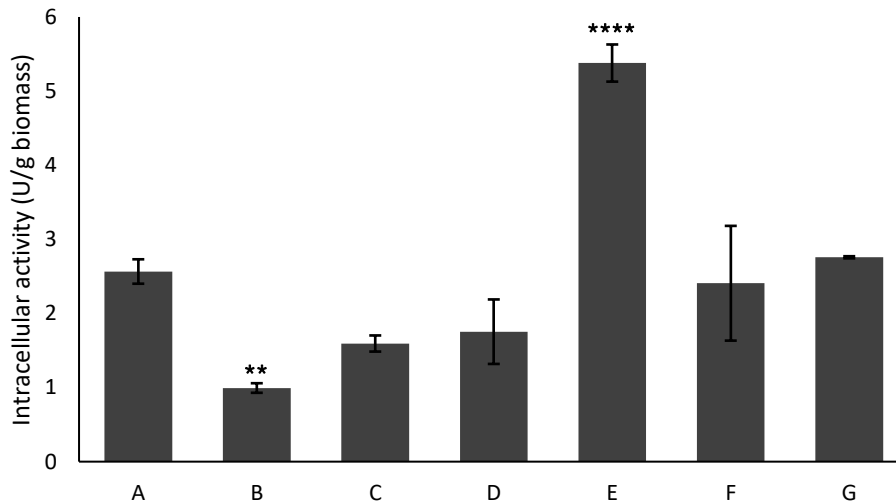
Figure 10. Intracellular activity of β-galactosidase produced by *Aspergillus lacticoffeatus*, after 6 days fermentation, in the media studied. (A) – Synthetic medium; (B) – 8 g/L CSL medium; (C) – 20 g/L CSL medium; (D) – Cheese whey medium; (E) – Cheese whey + salts medium; (F) – Cheese whey + extracts medium; (G) – Cheese whey + salts + extracts medium. Results are shown as mean ± SD of duplicate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

Regarding the extracellular activity, the synthetic medium presented a significantly higher activity level than the other 6 media studied (**Figure 11**). These results showed that, although the microorganism grew considerably in all the media studied, the production and secretion of a properly active form of the enzyme was favored in the synthetic medium.
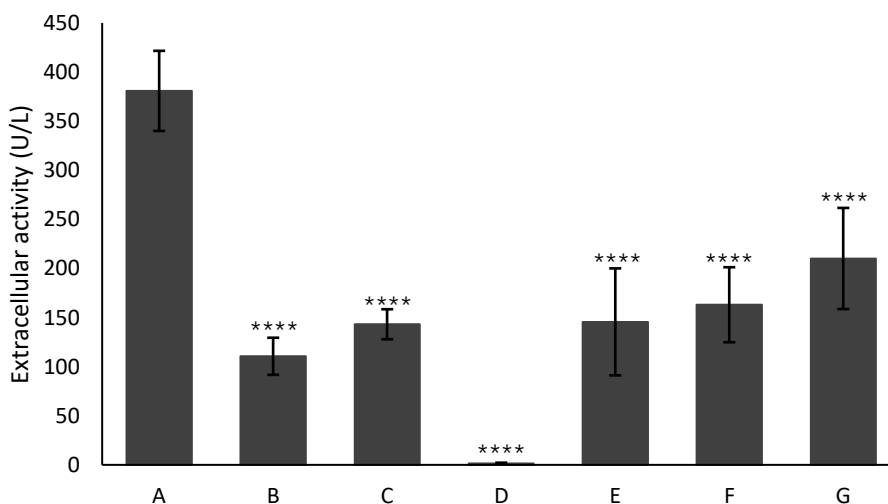
Figure 11. Extracellular activity of β-galactosidase produced by *Aspergillus lacticoffeatus*, after 6 days fermentation, in the media studied. (A) – Synthetic medium; (B) – 8 g/L CSL medium; (C) – 20 g/L CSL medium; (D) – Cheese whey medium; (E) – Cheese whey + salts medium; (F) – Cheese whey + extracts medium; (G) – Cheese whey + salts + extracts medium. Results are shown as mean ± SD of duplicate experiments. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

According to the results obtained for the several fermentation media studied, it is possible to conclude that the synthetic medium is the best option to grow *A. lacticoffeatus* as it is the medium that provides the highest production of extracellular β-galactosidase. However, the use cheese whey and CSL as low cost source of lactose and nitrogen, respectively, also provided interesting values of β-galactosidase activity and they can present recognized economic advantages to the process. Therefore, a further detailed study of β-galactosidase production using these residues could possibly improve the results and significantly reduce the costs of the process.

Since the desired application for this enzyme is the prebiotic production, it is crucial to have some information about the presence of mycotoxins in the fermentation broth. As it has been previously reported that *A. lacticoffeatus* is an OTA producer (Ostry, Malir, & Ruprich, 2013; Perrone et al., 2007; Samson, Houbraken, Kuijpers, Frank, & Frisvad, 2004), it is important to understand if this mycotoxin is produced at a harmful level in the fermentation conditions used in this work. Therefore, a fermentation experiment was conducted in synthetic medium to evaluate the potential mycotoxin production along the fermentation time (**Section 2.9**).

3.3.2. OTA production evaluation

The profile of OTA production for 9 days fermentation is presented in **Figure 12**. It is possible to observe that OTA is not detected after 3 days of fermentation, then it reaches a maximum (4.9 ng/mL) in 6 days and after that its concentration decreases to 1.46 ng/mL at the end of the fermentation. This reduction could be due to the OTA degradation promoted by light exposure, since some studies have reported that OTA produced by some *Penicillium* and *Aspergillus* spp. are photosensitive toxins (Schmidt-Heydt et al., 2011, 2012) and/or from the action of some proteolytic enzymes (Abrunhosa, Paterson, & Venâncio, 2010; Abrunhosa, Santos, & Venâncio, 2006). Although there is no legal information about the concentrations accepted in fermentations or for enzymatic extracts used in the synthesis of prebiotics, there are already some legal limits established for drink and food products such as wines and grape juices (2 ng/mL) (el Khoury & Atoui, 2010), processed cereals and infantile food (500 ng/kg) and instant coffees and raisins (10 000 ng/kg) (Köppen et al., 2010).
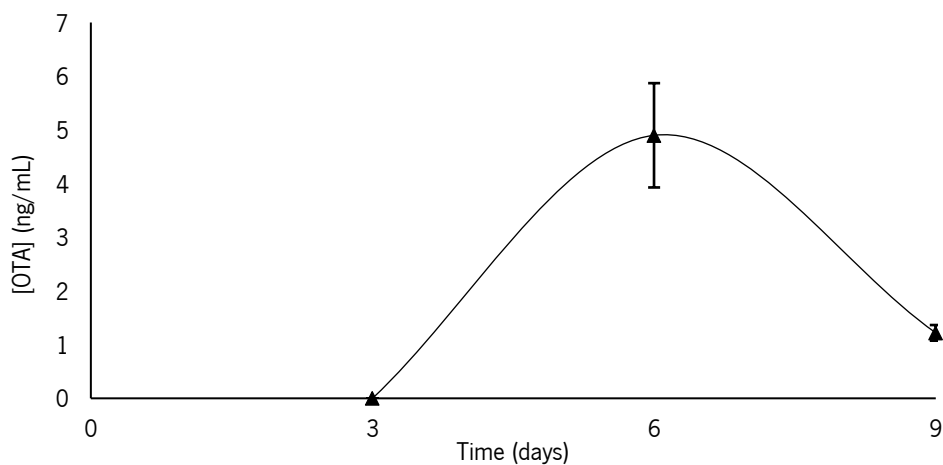
Figure 12. Ochratoxin A production by *Aspergillus lacticoffeatus* along a fermentation conducted at 28 °C in the culture medium A. Results are shown as mean ± SD of triplicate experiments.

According to the results obtained, it was considered that 9 days was a suitable period of time to conduct the fermentation, since the enzyme is properly active (**Figure 5**) and the OTA concentration is below the legal comparable value for wine and grape juices (**Figure 12**). Therefore, enzyme characterization and application in prebiotic synthesis was performed using the fermentation broth after 9 days.

3.4. Enzyme characterization

3.4.1. Optimal temperature and pH

The influence of temperature and pH on the activity of β-galactosidase (hydrolytic activity using ONPG as substrate) was studied and the results are shown in **Figure 13**. Regarding the temperature, it was possible to conclude that this enzyme has its maximum activity in the range between 50 °C and 55 °C (**Figure 13 – A**). Furthermore, the enzyme remained active for temperatures between 40 °C and 60 °C and lost its activity for lower temperatures (**Figure 13 – A**). These results are similar to those obtained for other β-galactosidases from *Aspergillus* spp. (**Table 9**) and the differences observed may be due to the fact that the enzyme herein studied was not purified. Some studies report that purification improves thermal stability which is strongly related with the enzyme activity (Braga et al., 2013).

Figure 13 – B presents the results obtained for the effect of the several pH values in the enzymatic activity. It was observed that the optimal pH for this β-galactosidase is in the range between 3.5 and 4.5,

and the enzymatic activity decreased significantly for higher pH values. In **Table 9**, the optimal pH reported for β-galactosidase from other *Aspergillus* spp. is compared with the value obtained in this study for *A. lacticoffeatus* and it can be seen that the values presented are similar.
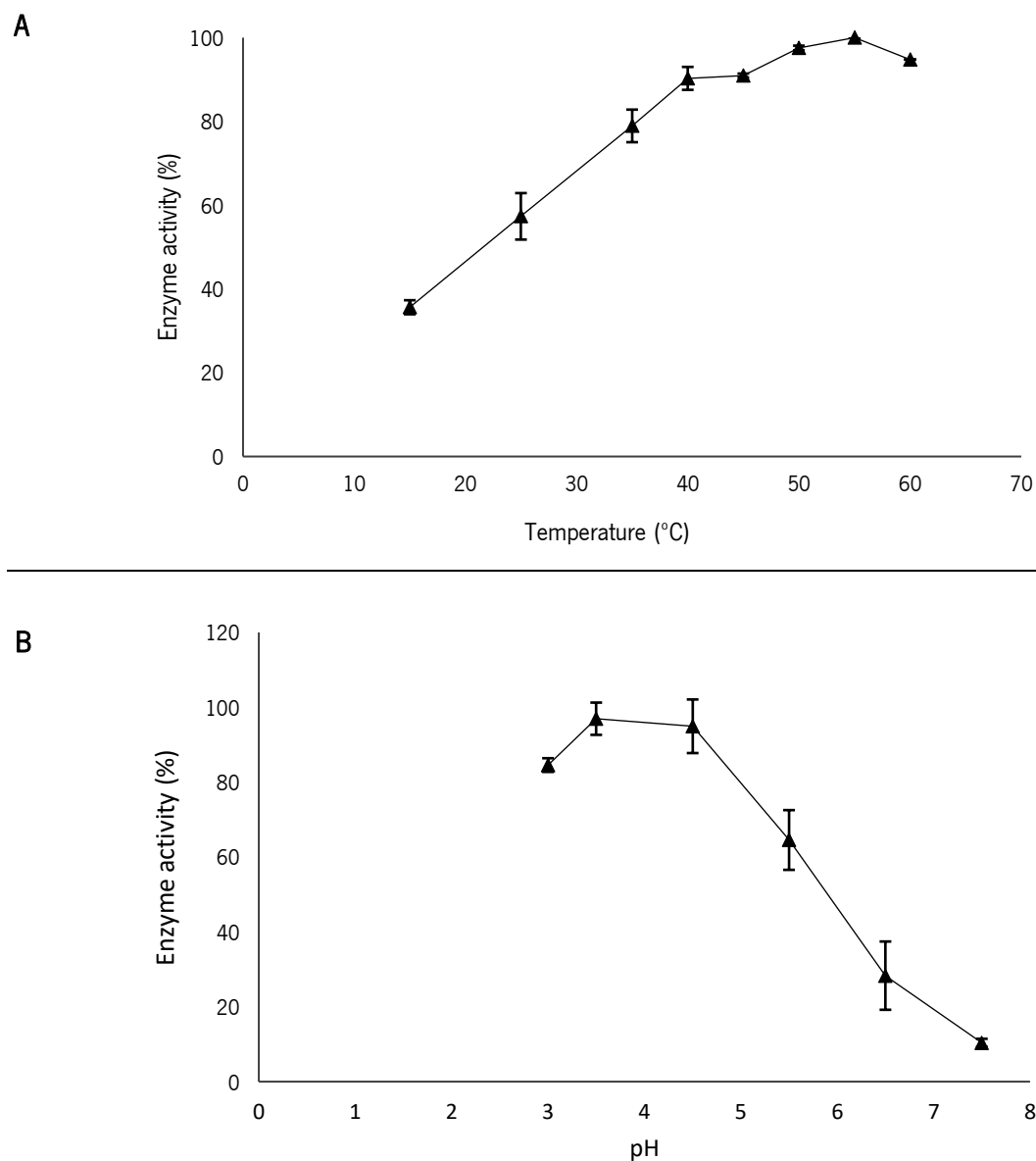
Figure 13. Optimal temperature (A) and pH (B) for the extracellular β-galactosidase produced by *Aspergillus lacticoffeatus* in a fermentation conducted with culture medium A at 28 °C. Results are shown as mean ± SD of two independent experiment with three replicates.

Table 9. Optimal temperature and pH reported for β-galactosidases from *Aspergillus* spp. (Guerrero et al., 2015).

Source	Optimal temperature (ºC)	Optimal pH
Aspergillus lacticoffeatus (<u>this work</u>)	50–55	3.5–4.5
Aspergillus aculeatus	60	3.5
Aspergillus niger	65	3.5
Aspergillus oryzae	65	4.5

3.4.2. Molecular size

The molecular size of the enzyme was investigated by electrophoresis. In order to obtain good quality results from electrophoresis, the enzyme was previously precipitated and concentrated. Isopropanol and TCA precipitation protocols were tested, as mentioned before (**Section 2.5**), in order to find the best precipitation method. Enzymatic activity values (both extra and intracellular) were significantly higher when the isopropanol precipitation was used (**Table 10**). TCA precipitation followed by acetone wash seemed to be a very ineffective method to concentrate the enzyme in a properly active form. Considering the aim of this project, the isopropanol precipitation was found to be the best method to precipitate β-galactosidase.

Table 10. Comparison of the two methods evaluated to precipitate β-galactosidase from *Aspergillus lacticoffeatus* in a fermentation conducted with culture medium A at 28 ºC. The enzyme was concentrated 20x.

	Extracellular enzyme 3 days (U/L)	Extracellular enzyme 6 days (U/L)	Intracellular enzyme (U/L)
Isopropanol	648.91	791.46	858.43
TCA	9.40	3.52	35.64

After enzyme precipitation and concentration, a SDS-PAGE electrophoresis was performed to determine the molecular size of the enzyme and also a zymogram was conducted to prove that the enzyme is actually a β-galactosidase with hydrolytic activity towards X-gal.

Figure 14 illustrates the SDS-PAGE gel analysis (**Figure 14 – A**) and the zymogram (**Figure 14 – B**). In **Figure 14 – A** it is possible to observe the run of the molecular size marker (M), a commercial preparation of β-galactosidase from *A. niger* (Maxilact A4, DSM) and the concentrated enzyme after isopropanol treatment. Considering the commercial β-galactosidase band (**Figure 14 – A**, lane 1, between

100 kDa and 150 kDa) it was possible to identify a comparable band in the enzyme lane 2 and also an additional band between 70 kDa and 100 kDa. Therefore, considering the electrophoresis results, this β-galactosidase from *A. lacticoffeatus* could have a molecular size between 100 and 150 kDa, comparable to the similar enzyme from A. *niger* with 124 kDa (Mahoney, 1997), or between 70 and 100 kDa, which is close to the β-galactosidase from *A. oryzae* with 90 kDa (Mahoney, 1997). Furthermore, considering the zymogram (**Figure 14 – B**, lane 2), it was also possible to identify a band that corresponds to a β-galactosidase, since it was a positive reaction with the X-gal.
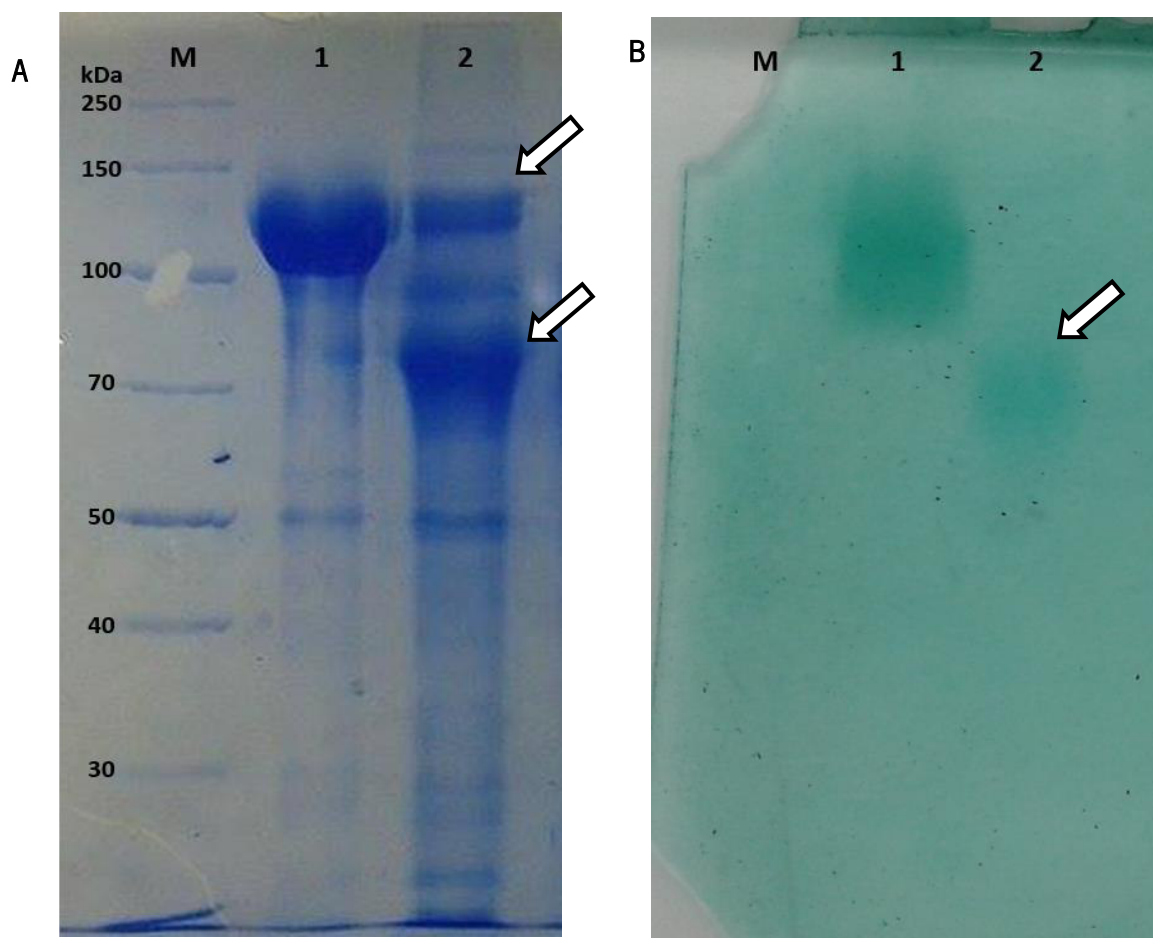
Figure 14. Electrophoretic analysis. (A) - SDS-PAGE gel; (B) - Zymogram; lane M: molecular size marker; lane 1: commercial enzyme from *Aspergillus niger*; lane 2: 20x concentrated β-galactosidase from *Aspergillus lacticoffeatus* produced in a fermentation conducted with culture medium A at 28 °C.

3.4.3. Metallic ions

Some studies reported that monovalent and divalent cations can affect the β-galactosidase activity (Liu, Zhao, Deng, Huang, & Liu, 2015). Therefore, metallic ions were added to the enzymatic extract, as mentioned in **Section 2.11**, in order to study their effect on the enzymatic activity of the β-galactosidase produced by *A. lacticoffeatus*. The results obtained are presented in **Figure 15**, except those for Ca^{2+} in which salt precipitation occurred and it was not possible to perform the enzymatic activity test.

None of the studied ions promoted a significant enhancement of the hydrolytic activity and Ba^{2+} and Fe^{2+} ions negatively affected the enzyme, resulting in a decrease on the enzymatic activity of 21% and 43%, respectively. The way that metallic ions affect β-galactosidase may be strongly dependent on the enzyme source, since many studies report different results. For example, Liu *et al.* concluded that all studied ions positively affected an enzyme from a bacterial consortium (Liu et al., 2015). However, in other cases, metal ions such as Ca^{2+}, Cn^{2+}, Mn^{2+} and Cu^{2+} were reported to promote a decrease on the enzymatic activity of β-galactosidases from *Lactobacillus delbruekii* (Intanon et al., 2014) and *Thermotoga naphthophila* (Kong, Wang, Cao, Gao, & Xie, 2014). Moreover, some studies reported that metal ions had no effect on enzymes from *Bacillus coagulans* (Batra, Singh, Banerjee, Patnaik, & Sobti, 2002) and *Sulfolobus solfataricus* (Pisani et al., 1990).
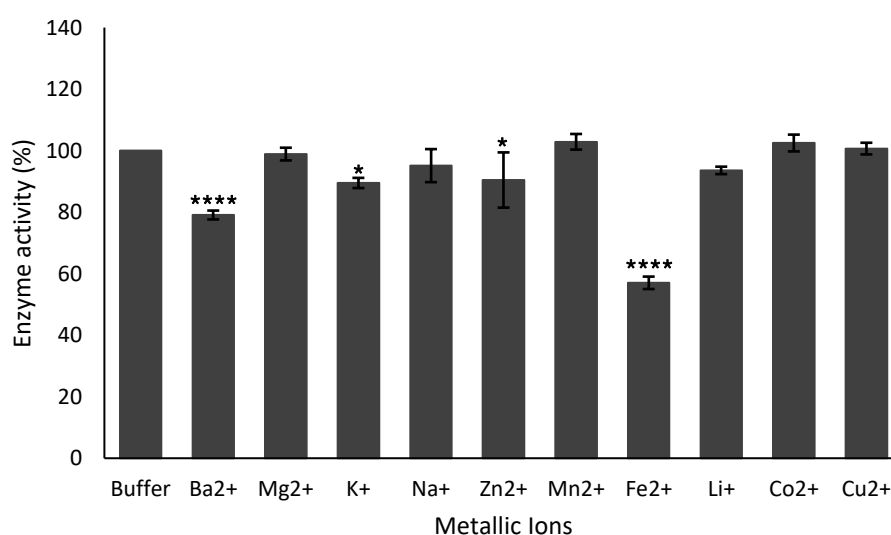
Figure 15. Effect of some metallic ions on the extracellular β-galactosidase activity produced by *Aspergillus lacticoffeatus* in the culture medium A at 28 °C. Results are shown as mean ± SD of one independent experiment with three replicates. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

3.4.4. Additives and detergents

The effect of some additives and detergents was also studied. From **Figure 16**, it is possible to observe that the enzymatic activity was enhanced by all the additives and detergents used, except for ascorbic acid that was found to decrease the enzymatic activity in about 15%. The studies found in the literature for the effect of this additive on the β-galactosidase activity reported both its negative effect on the hydrolytic activity of β-galactosidase from *E. cloacae* (Ghatak et al., 2010; Sen et al., 2012), and on the contrary the enhancement of the enzymatic activity of β-galactosidase from Almond (*Amygdalus communis*) (Pal, Lobo, & Khanum, 2013). As ascorbic acid is a reducing agent, its action may be related with the reduction of the amino acids residues, that could have a positive or negative effect on the enzyme activity (Pal et al., 2013).

Regarding the SDS addition, it was expected that this detergent had a negative effect on the β-galactosidase activity, since it has been reported as a promoter of enzyme denaturation (Laemmli, 1970; Nowakowski, Wobig, & Petering, 2014). However, the results obtained showed a positive effect with about 13% increase in the enzymatic activity. Similarly, Muga and collaborators (Muga, Arrondo, Bellon, Sancho, & Bernabeu, 1993) reported a slight activation of β-galactosidase from *E. coli* by the presence of SDS in concentrations up to 2%. The addition of Triton X-100 and Tween led to an enhancement of the enzymatic activity of 6% and 33%, respectively, which is in accordance with the results from Liu *et al.* (Liu et al., 2015).

Guven *et al.* (Guven, Kaplan, Guven, Matpan, & Dogru, 2011) reported the inhibitory effect of PMSF (about 72% inhibition) on the enzymatic activity of β-galactosidase from *Alicyclobacillus acidocaldarius*. However, in the current study, PMSF was found to promote the enzymatic activity in about 30% (**Figure 16**). This could be explained by the fact that PMSF is a protease inhibitor (James, 1978), thus it may had inhibited the proteolytic enzymes possibly present in the sample which could promote the degradation of the β-galactosidase.

Several studies reported the negative effect of EDTA on the enzymatic activity of β-galactosidase (Ghatak et al., 2010; Liu et al., 2015; Pal et al., 2013; H. Wang et al., 2014). However, in this study, this additive exhibited a positive effect on the enzymatic activity (an increase around 18%). This result is in accordance with the report from Guven *et al.* (Guven et al., 2011) that found a slight enhancement on the enzymatic activity (20%) of β-galactosidase from *Alicyclobacillus acidocaldarius*. The increase obtained could be due to the fact that EDTA, as a chelating agent, probably complexed some metal ions

present in the fermentation medium which could be negatively affecting the enzyme. In addition, this result could suggest that this β-galactosidase does not require metal ions for its hydrolytic activity, which is also corroborated by the results present in **Figure 15** since none of the metal ions studied was found to significantly enhance the enzyme activity. Similar results for a β-galactosidase from *A. acidocaldarius* were reported by Guven and collaborators (Guven et al., 2011).
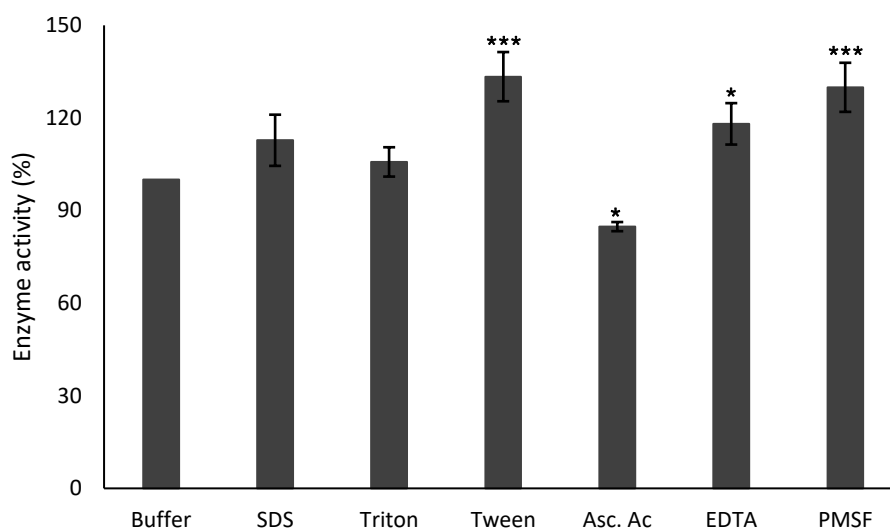
Figure 16. Effect of some additives and detergents on the extracellular β-galactosidase activity produced by *Aspergillus lacticoffeatus* in the culture medium A at 28 °C. Results are shown as mean $\pm$ SD of one independent experiment with three replicates. * $p<0.05$; ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$.

3.4.5. Sugars

As glucose and galactose are the end products of the lactose hydrolysis, their effect on enzyme activity was also studied. According to the results presented in **Figure 17**, glucose seems to enhance the enzyme activity in all the concentrations studied, except for 25 mM, where it became slightly inhibitory. For galactose, it was possible to verify that its presence strongly inhibited the enzyme, for all the concentrations studied. This result was expected and it is in accordance with the literature, where it has been reported that galactose competitively inhibits the β-galactosidase (Pal et al., 2013) and also that acidic enzymes are more susceptible to that inhibition (Boon et al., 2000).

Regarding fructose, it seems that this sugar has no significant effect on the enzyme activity, except for 5 mM, where a slight enhancement of the enzymatic activity was observed. Similar results

were reported for a β-galactosidase from *A. acidocaldarius*, where fructose at 10 mM and 100 mM barely affected the enzymatic activity (Guven et al., 2011).
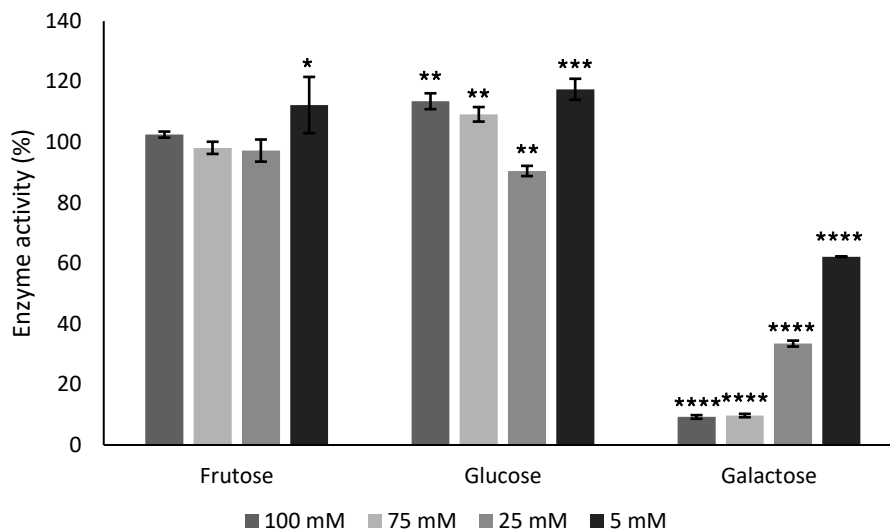
Figure 17. Effect of some sugars on the extracellular β-galactosidase activity produced by *Aspergillus lacticoffeatus* in the culture medium A at 28 °C. Results are shown as mean ± SD of one independent experiment with three replicates. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

3.5 – Enzyme application

Another important goal of this work was the possible application of the β-galactosidase produced under submerged fermentation in the synthesis of lactose-derived prebiotics, namely lactulose and lactosucrose. Therefore, three different methods were tested to assess the production of prebiotics, as mentioned in **Section 2.12**. Both the substrate conversion and product formation were followed by HPLC. Considering the HPLC conditions used in this study, the retention times observed for the different sugar molecules are summarized in **Table 11**. The method 1, which consisted in the addition of the substrates to the ongoing fermentation, was proven to be a very ineffective method, since no prebiotic was detected, in any condition.

Table 11. Retention times for the different sugars analyzed by HPLC.

Sugar molecule	Retention time (minutes)
Fructose	7.6
Glucose/Galactose	9.2
Lactulose	11.6
Sucrose	12.3
Lactose	13.9
Lactosucrose	19.2

The method 2, which consisted in the transfer of the biomass to a solution containing 150 g/L of the substrates (lactose and fructose for the production of lactulose; lactose and sucrose for the production of lactosucrose) was more effective. In this case, the production was conducted for 72 hours at 28 and 37 °C. The results obtained for both temperatures were very similar. **Figures 18** and **19** show the results obtained for the production of lactulose (retention time ~ 11 minutes), where it is possible to observe the production of this prebiotic after 72 hours (1.36 g/L and 1.71 g/L, for 28 and 37 °C, respectively) (blue chromatograms). The presence of lactulose was detected after 24 hours of reaction and the maximum prebiotic yield obtained at 28 °C was 1.6%, with a lactose conversion of 38.4% (after 72 hours). For the reaction conducted at 37 °C, the lactose conversion was 32.9% and the maximum prebiotic yield was 1.9% (after 72 hours). **Figures 18** and **19** also show the production of a possible GOS (*not quantified*), with a retention time between 22 and 23 minutes, in both temperatures.

Figures 20 and **21** illustrate the results obtained when using lactose and sucrose as substrates. The enzyme was unable to catalyze the production of lactosucrose (peak around 19 minutes), in both temperatures. However, the production of a possible GOS (peak between 22 and 23 minutes) was detected after 24 hours, with a maximum concentration after 72 hours (*not determined in this work*). The results were similar in both temperatures.

Figure 18. HPLC profile for lactulose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 2 conducted at 28 °C (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (72 hours); Red chromatogram – Lactulose pattern (1 g/L).
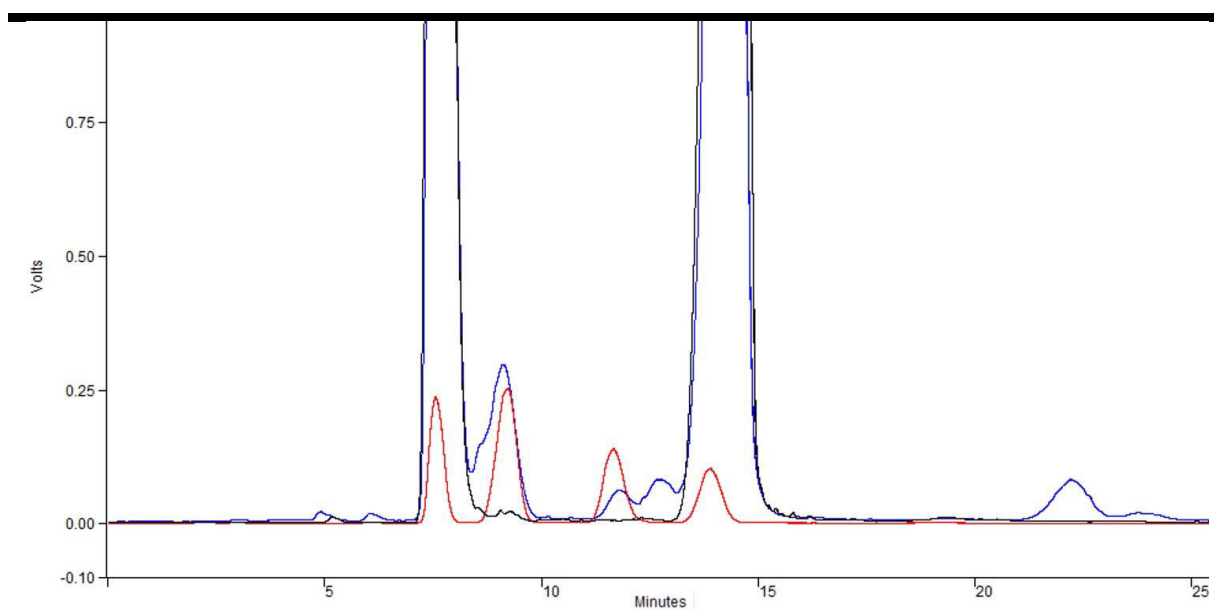
Figure 19. HPLC profile for lactulose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 2 conducted at 37 °C (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (72 hours); Red chromatogram – Lactulose pattern (1 g/L).
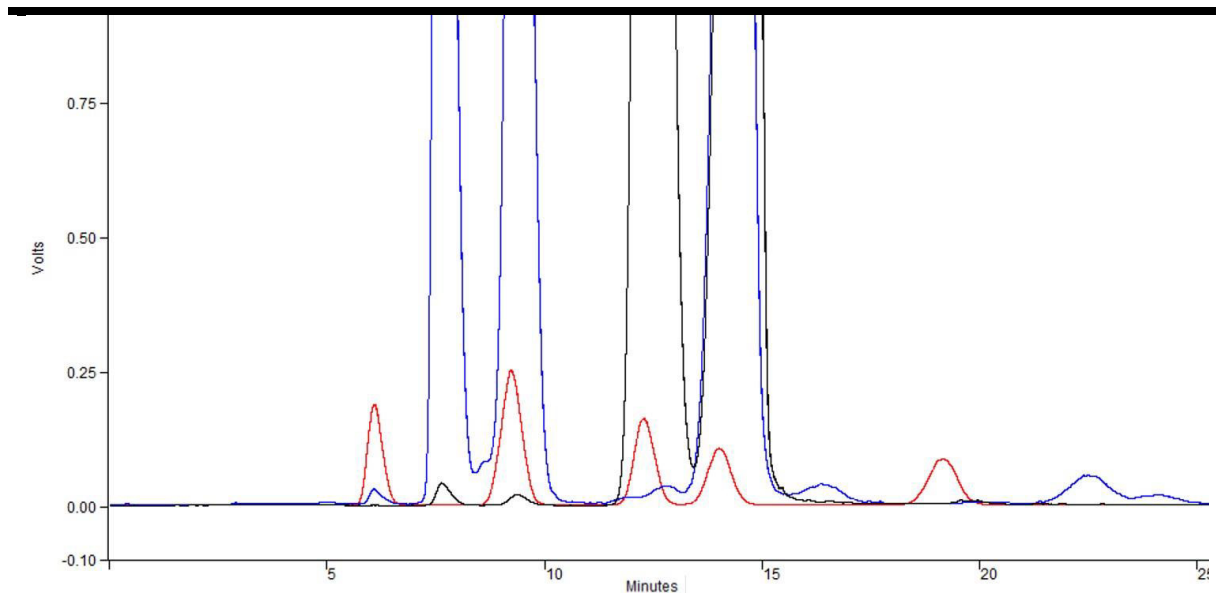
Figure 20. HPLC profile for lactosucrose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 2 conducted at 28 °C (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (72 hours); Red chromatogram – Lactosucrose pattern (1 g/L).
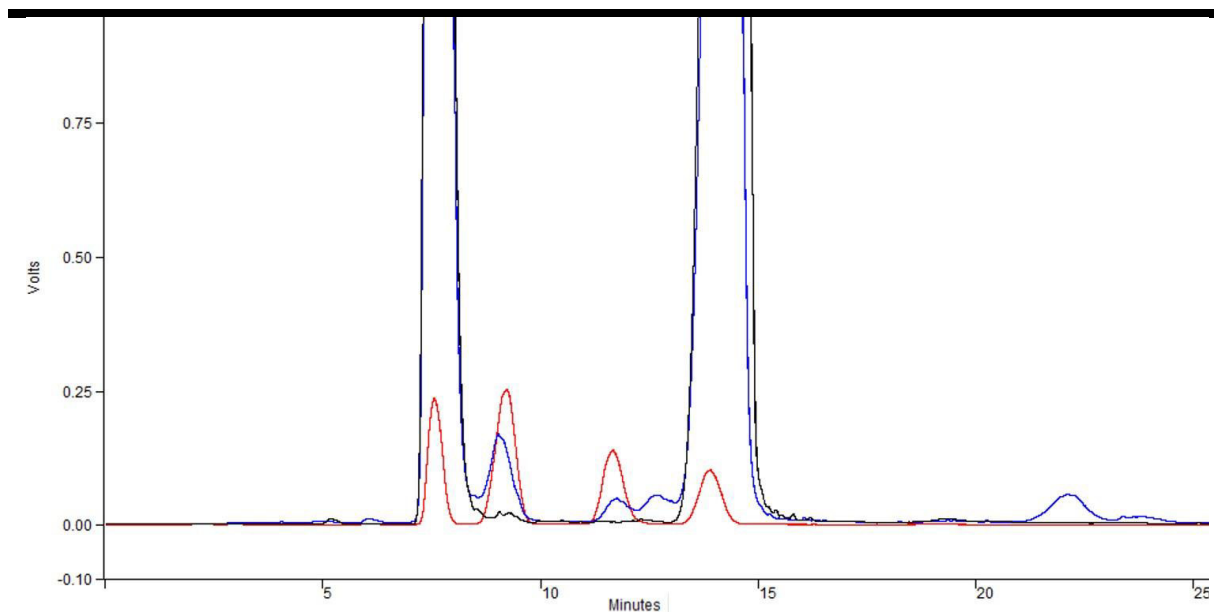

Figure 21. HPLC profile for lactosucrose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 2 conducted at 37 °C (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (72 hours); Red chromatogram – Lactosucrose pattern (1 g/L).
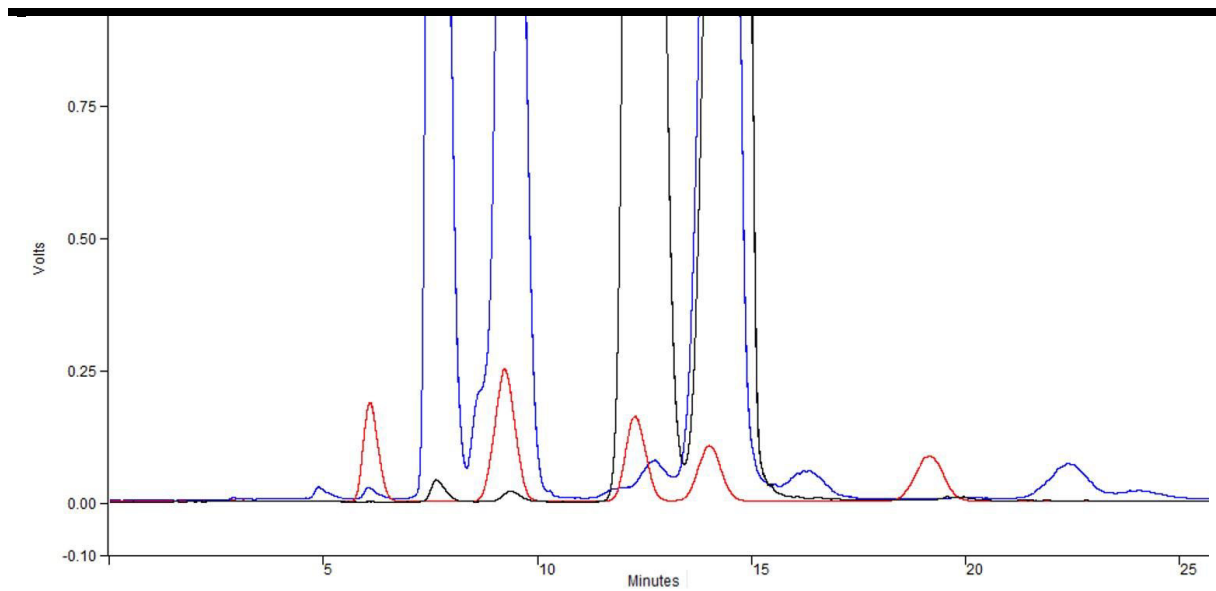
The results for method 3 are presented in **Figures 22** and **23**. This approach consisted in the use of the fermentation broth with β-galactosidase activity to perform an enzymatic synthesis, adding the adequate substrates and following the reaction for 6 hours, at 37 °C. As it is possible to see in **Figure 22**, this reaction led to the production of lactulose (retention time ~ 11 minutes), that was detected after 6 hours and the maximum yield was 1.7% with a lactose conversion of 10.3%. This condition also led to the production of a GOS with a retention time around 23 minutes (*not quantified*). **Figure 23** shows the results obtained for the substrates lactose and sucrose. Lactosucrose was not produced. On the other hand, it was detected the presence of a GOS (peak at 22-23 minutes, *not quantified*) and also kestose, a FOS, with a retention time of 20 minutes (*not quantified in this work*). Both oligosaccharides were detected after 1 hour of reaction and the maximum concentration was obtained after 6 hours. The production of this FOS possibly means that there is another enzyme, probably a FFase, present in the medium. Therefore, an activity test for this enzyme was performed to confirm this assumption (results are show further in this discussion).

Figure 24 shows the chromatograms obtained for methods 2 and 3. It is possible to conclude that the GOS produced and mentioned in each condition is the same for all the conditions, and has a retention time between 22 and 23 minutes.

The results obtained for the production of prebiotics are summarized in **Table 12**. As it is possible to see, the lactose conversion and prebiotic yields are significantly low, which could possibly be a result of the use of a crude extract, once it has already been reported that purification steps usually led to an enhancement of enzyme activity (Benavente et al., 2015; Raol et al., 2015). Low yields are also usually attributed to low values of transgalactosylation activity (Hua et al., 2013), which could be improved by the decrease of water activity, that can be achieved with higher initial concentrations of substrates and/or the use of organic solvents (Giacomini, Irazoqui, Gonzalez, Batista-viera, & Brena, 2002; Hua et al., 2013; Lee, Kim, & Oh, 2004). Additionally, the prebiotic yields have already been reported as dependent on several factors such as: the source of β-galactosidase, the initial concentration of substrates, the reaction temperature and pH, the reaction time and the operation model (H. Wang et al., 2013). Studies present in the literature generally present significantly higher prebiotic yields and concentrations. However, this prebiotics production is commonly performed with purified enzymes and under optimized conditions. The authors reported that high substrates concentrations (200–600 g/L) and high temperatures are preferable, as well as a neutral or weak acidic pH and a low water activity environment (Hua et al., 2013;

Lee et al., 2004). Furthermore, recent reviews show that the main problem associated with enzymatic production of prebiotics is thermal intolerance, so β-galactosidase should be improved by structural modification, improving the enzyme functionality (Kim et al., 2006; Panesar & Kumari, 2011; H. Wang et al., 2013). Other strategies to promote the enhancement of prebiotics production is the use of immobilized enzymes with circulation of substrates solutions (H. Wang et al., 2013).
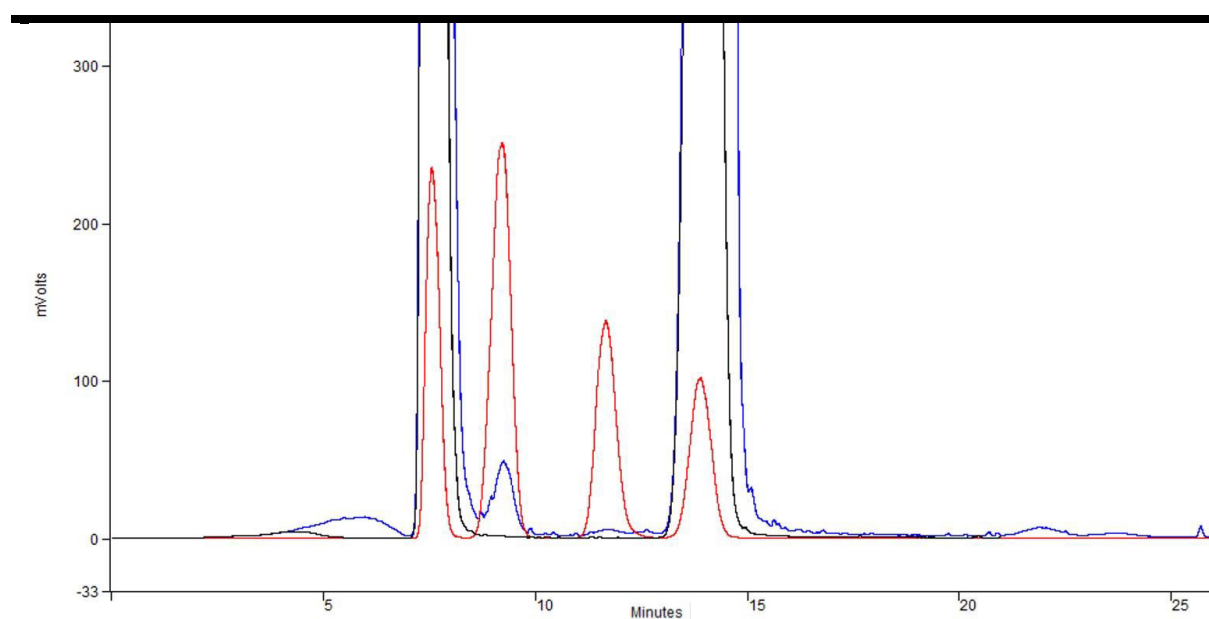
Figure 22. HPLC profile for lactulose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 3 (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (6 hours); Red chromatogram – Lactulose pattern (1 g/L).
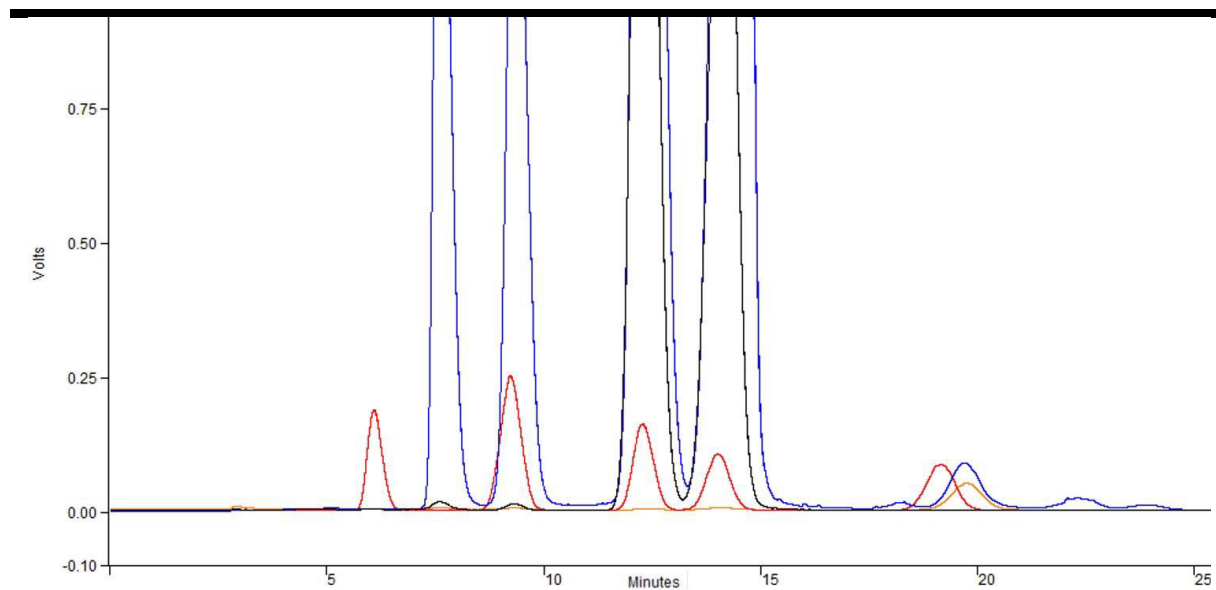
Figure 23. HPLC profile for lactosucrose production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the Method 3 (Section 2.11). Black chromatogram – Initial sample; Blue chromatogram – Final sample (6 hours); Red chromatogram – Lactosucrose pattern (1 g/L); Orange chromatogram – FOS Kestose pattern (1 g/L).
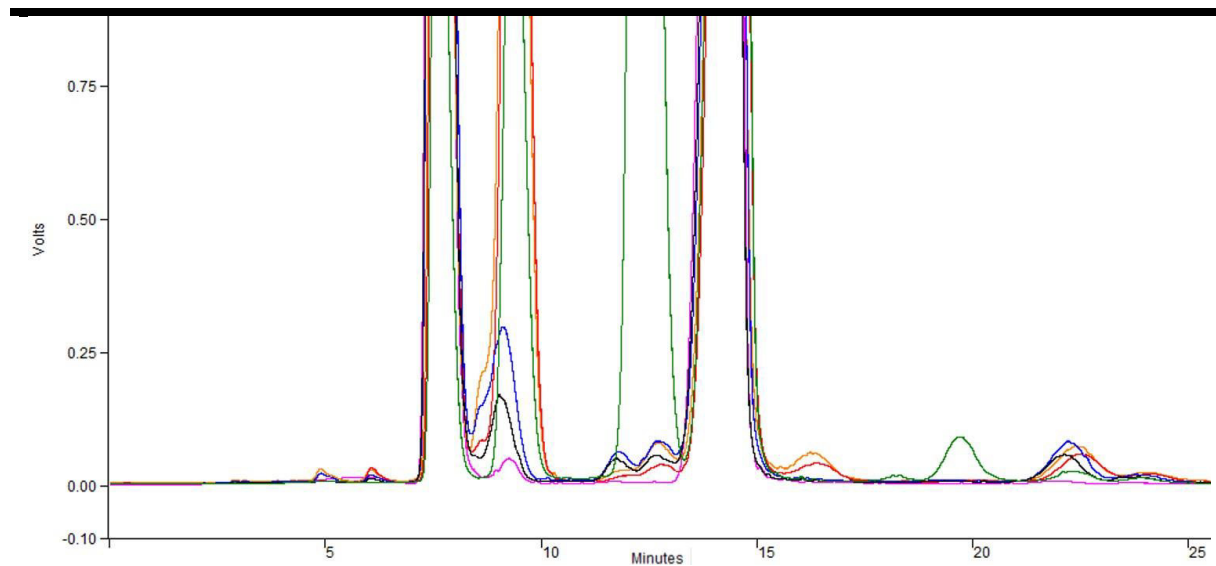
Figure 24. HPLC profile for prebiotics production using the β-galactosidase from *Aspergillus lacticoffeatus*. Black chromatogram – Lactulose production through the Method 2 conducted at 28 °C (Section 2.11). Blue chromatogram – Lactulose production through the Method 2 conducted at 37 °C. Red chromatogram – Lactosucrose production through the Method 2 conducted at 28 °C. Orange chromatogram – Lactosucrose production through the Method 2 conducted at 37 °C. Pink chromatogram – Lactulose production through the Method 3 (6 hours). Green chromatogram – Lactosucrose production through the Method 3 (6 hours).

Table 12. Summary of the prebiotic production using the β-galactosidase from *Aspergillus lacticoffeatus*, through the methods 2 and 3.

Condition	Lactose conversion (%)	Prebiotic produced	Prebiotic yield (%)
Method 2, lactose + fructose. 28 °C	38.4	Lactulose (1.36 g/L)	1.6
		GOS	(*not quantified*)
Method 2, lactose + fructose. 37 °C	32.9	Lactulose (1.71 g/L)	1.9
		GOS	(*not quantified*)
Method 2, lactose + sucrose. 28 °C	26.8	GOS	(*not quantified*)
Method 2, lactose + sucrose. 37 °C	33.9	GOS	(*not quantified*)
Method 3, lactose + fructose. 37 °C	10.3	Lactulose (1.75 g/L)	1.7
		GOS	(*not quantified*)
Method 3, lactose + sucrose. 37 °C	28.9	FOS Kestose, GOS	(*not quantified*)

As mentioned before, an activity test for FFase was conducted (**Table 13**). The test was performed using the fermentation broth from 3, 6 and 9 days. The results obtained clearly suggest that FFase is present in the medium, with a maximum activity of 16.42 U/mL. Thus, this enzyme was the probable responsible for the hydrolysis of sucrose (observe in chromatogram present in **Figure 23**) and subsequent synthesis of kestose.

Table 13. Extracellular β-fructofuranosidase activity along a fermentation conducted with *Aspergillus lacticoffeatus* in culture medium A at 28 °C.

Condition	Enzyme activity (U/mL)
Fermentation broth 3 days	16.35
Fermentation broth 6 days	15.76
Fermentation broth 9 days	16.42

4. GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

The main aim of this project was the production of β-galactosidase from *A. lacticoffeatus* and the evaluation of its further potential application in the production of lactose-based prebiotics. For that purpose, several fermentations were performed to assess and optimize the enzyme production and then, three different approaches were used to study the prebiotics production.

A preliminary fermentation performed under submerged conditions confirmed that *A. lacticoffeatus* is a β-galactosidase producer, being the highest values of enzymatic activity obtained at 28 °C. It was also concluded that this microorganism produces both extra and intracellular enzyme. The use of ultrasounds for 2 minutes was found to be the best method to extract the intracellular β-galactosidase from *A. lacticoffeatus*.

The *A. lacticoffeatus*'s growth and β-galactosidase production (intra and extracellular) were evaluated under submerged fermentation conditions using a synthetic medium and six alternative media containing the by-products cheese whey or CSL. Regarding the biomass production, the results showed that the fungus was able to adapt to the different media and grow properly. Although there were no significant differences between the media studied, the pellets' size was smaller when it was grown in the cheese whey medium and cheese whey with salts. In respect to the intracellular enzyme production, the best results were obtained when the fermentation was conducted with the cheese whey + salts medium. Concerning the extracellular enzyme, synthetic medium presented a significant higher activity level. Concluding, from all the fermentation media studied, the synthetic medium was the best to grow *A. lacticoffeatus* and produce extracellular β-galactosidase.

The optimal pH and temperature for the extracellular β-galactosidase present in the enzymatic extract was found in the range 3.5–4.5 and 50–55 °C, respectively. However, regarding the molecular size, the results were not conclusive, resulting in a range of molecular weight between 70 and 150 kDa.

The behavior of the enzyme with the addition of metallic ions shows that this β-galactosidase probably does not require metallic ions for its hydrolytic activity. All the detergents tested promoted an enhancement in the enzyme activity, as well as the additives EDTA and PMSF. The ascorbic acid was found to be an enzyme inhibitor. Regarding the effect of the sugars in the enzyme activity, galactose showed a negative effect, as expected, promoting inhibition at all the concentrations tested. Fructose and glucose had no significant effects on the enzyme hydrolytic activity.

Two of the three methods studied for the synthesis of prebiotics using β-galactosidase from *A. lacticoffeatus* resulted in the formation of lactose-based prebiotics. The enzyme was able to catalyze the

transfer reactions involved in the formation of lactulose (maximum concentration 1.75 g/L) and a galacto-oligosaccharide with a retention time between 22 and 23 minutes. This β-galactosidase was unable to catalyze the formation of lactosucrose under the experimental conditions studied. The formation of a fructo-oligosaccharide, namely kestose, was also detected, due to the presence of a β-fructofuranosidase with a maximum activity of 16.42 U/mL in the enzymatic extract obtained at 9 days fermentation.

In conclusion, this work validated *A. lacticoffeatus* as a β-galactosidase producer and proved that this enzyme is able to catalyze the production of lactose-based prebiotics. To improve the results, a purification step could be performed, since purification usually provides more active extracts and eliminates contaminants. Therefore, better results could be achieved regarding the molecular size of the enzyme produced and also its optimal pH and temperature. Additionally, the methods for the production of lactose-based prebiotics could be optimized to obtain higher product yields and prebiotics concentrations. This optimization could be achieved through several parameters like reaction temperature and pH, substrates and enzyme concentrations and reaction media. As final remark, this β-galactosidase from *A. lacticoffeatus* seems to have a great potential for the production of prebiotics by transgalactosylation. However, for an industrial application, more detailed studies should be done in order to evaluate and understand its catalytic action, and then a further optimization of the process of synthesis so that becomes more attractive and competitive commercially. Finally, *A. lacticoffeatus* also demonstrated potential for the production of other interesting prebiotics, namely FOS, and their synthesis could be investigated in additional studies.

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