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Biomass and microbial lipids production by *Yarrowia lipolytica* W29 from eucalyptus bark hydrolysate

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ABSTRACT

Using lignocellulosic biomass hydrolysate as a renewable and abundant feedstock for microbial lipids production is a sustainable and economic high-potential approach. This study investigated the potential of the oleaginous yeast *Yarrowia lipolytica* to produce lipids-rich biomass from eucalyptus bark hydrolysate (EBH) obtained by enzymatic hydrolysis of the biomass pretreated by steam explosion. The effect of EBH concentration (undiluted and 1:3 v/v diluted) and medium supplementation (CSL and KH_2PO_4) was evaluated in Erlenmeyer flasks and lab-scale stirred tank bioreactor, respectively. Additionally, the effect of volumetric oxygen transfer coefficient (k_La) and mode of operation (batch and two-stage repeated batch) was also assessed in the bioreactor. Under the best experimental conditions (undiluted EBH, 2 g·L^{-1} CSL, 1.8 g·L^{-1} $(NH_4)_2SO_4$, and k_La of 66 h^{-1}), *Y. lipolytica* W29 grown in batch cultures accumulated 26 % (w/w) of intracellular lipids, corresponding to 5.6 g·L^{-1} of concentration. Lipids of *Y. lipolytica* were highly unsaturated and mainly composed of oleic acid (48 %), followed by palmitoleic (20 %), linoleic (17 %) and palmitic acids (14 %). This composition of *Y. lipolytica* lipids suggests their potential use as feedstock for biodiesel (a renewable biofuel). This work demonstrated the robust features of *Y. lipolytica* W29 as a potential lipids production platform to implement lignocellulose-based biorefineries.

1. Introduction

The growth of the world population is driving an upsurge in energy consumption, resulting in an increased reliance on fossil fuels and consequently intensifying environmental issues associated with their usage [1,2]. Biofuels, particularly biodiesel, hold great promise as alternative sources to address these challenges due to their biodegradability, non-toxic nature, renewability, and similar properties to conventional diesel [3]. To meet the lipids demand as biodiesel feedstock, microbial lipids have gained significant attention in the last years mainly due to their similar fatty acid composition to vegetable oils (chain length and saturation degree) [4]. Furthermore, microbial lipids do not compete with edible oils, do not increase crop fertilization nor contribute negatively to greenhouse gas emissions, and their production is not affected by the seasons [4,5].

The advantages of using yeasts as lipids producers, compared to other microorganisms, include their fast-growing rate, high lipids content, easier manipulation and cultivation in large fermenters, robustness against contamination, tolerance to operational conditions, and no need for light [6,7]. Furthermore, some yeasts have the extraordinary advantage of using a wide range of substrates, including low-cost and residual materials, to accumulate lipids through *de novo* or *ex novo* pathway [8].

The large-scale application of microbial lipids production is still hindered by the high production costs, mainly attributed to expensive raw materials for media formulation [1]. Lignocellulosic biomass is a promising and renewable carbon source for microbial processes [9]. Before utilizing complex lignocellulosic substrates, its constituent polysaccharides (cellulose and hemicellulose) need to be converted into monomeric assimilable sugars through enzymatic hydrolysis [10]. However, the rigid and compact structure of lignocellulosic materials makes their polysaccharides inaccessible to enzymes [9]. To overcome this limitation, mechanical comminution and pretreatment are required to free cellulose and hemicellulose fractions from the lignin, reduce cellulose crystallinity, and increase the porosity of the material, before an enzymatic process [11,12]. Among the different technologies

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(chemical, physical, thermochemical and biological) employed for lignocellulosic biomass pretreatment, steam explosion is one of the most mature physical-chemical technologies [9]. The biomass is treated with high-pressure saturated steam, and then the pressure is rapidly reduced (in a "blow tank"), promoting the breakdown of the lignocellulosic matrix by rupturing inter- and intra-molecular bonds [12]. Steam explosion enhances the digestibility in the subsequent enzymatic hydrolysis, operates with high dry matter contents, provides high sugar concentrations available for microorganisms [11], and generates low levels of products (phenolic compounds, furan aldehydes, and weak organic acids) with antimicrobial activity [13]. In this sense, the use of lignocellulosic biomass hydrolysates (LBH) towards a cost-effective and sustainable production of microbial lipids depends on the microorganism's ability to consume different carbon sources (hexoses and pentoses) and its tolerance to LBH-derived compounds [7].

Yarrowia lipolytica is an oleaginous yeast with an extraordinary ability to use a wide range of carbon sources [8]. Several by-products, such as waste cooking oils [14], animal fat [15], food-waste derived volatile fatty acids [16,17], and crude glycerol [8] were successfully used by *Y. lipolytica* as raw materials for microbial lipids synthesis. Moreover, it was demonstrated that biodiesel produced from *Y. lipolytica* microbial lipids is a high-quality fuel and meets the criteria set by international biodiesel standards [18]. This study aims to investigate the potential of eucalyptus bark hydrolysate (EBH), a side product from pulp and paper mills containing significant polysaccharides, as a substrate for microbial lipids production by *Y. lipolytica* W29. Medium composition (EBH concentration and supplementation), oxygenation conditions assessed through k_La, and operation mode (batch and two-stage repeated batch) were studied to enhance lipids-rich biomass production, following sustainable-friendly and circular economy approaches. To the best of our knowledge, this is the first work dealing with microbial lipids accumulation by *Y. lipolytica* W29 in EBH-based medium. In addition, the fatty acid profile of the microbial lipids accumulated was characterized to assess its suitability as feedstock for biodiesel production. Extracellular metabolites were also monitored since the co-production of intracellular lipids and organic acids, mainly citric acid [19], or polyols [20] by wild-type *Y. lipolytica* was already reported in other hydrolysates.

2. Materials and methods

2.1. Eucalyptus bark hydrolysate (EBH) preparation

Eucalyptus bark was collected in the pulp mill of Cacia (Aveiro, Portugal) from The Navigator Company. The biomass was pretreated following a proprietary non-catalyzed steam explosion technology without the addition of acids and using only high-pressure steam, initially developed by the company STEX® and since 2019 in partnership with LNEG. Steam explosion step was carried out in a 200-L reactor coupled to a 4000 L-blow tank where pretreated biomass is discharged. After pretreatment, the solid fraction was washed with water at room temperature and directly used for the enzymatic hydrolysis. The solid fraction with an initial solids' concentration of 175 $g{\cdot}L^{-1}$ (dried basis) was enzymatically hydrolyzed at 50 °C in a 600 L-stirred tank reactor for 48 h by applying Cellic® CTec3 cocktail at a dosage of 3% (w/w dried solids). The resulting EBH was centrifuged to remove the unreacted solids, frozen at – 20 °C and thawed at 4 °C overnight before being used for microbial cultivations.

2.2. Erlenmeyer flask experiments

The ability of *Y. lipolytica* W29 (ATCC 20460) to grow on EBH was first evaluated in batch cultures carried out in 250-mL Erlenmeyer flasks filled with 100 mL of culture medium. The effect of EBH concentration (undiluted and 1:3 v/v diluted) on biomass and lipids production was studied. Yeast cells grew overnight in YPD medium [21] were centrifuged and resuspended to an initial concentration of 0.5 $g{\cdot}L^{-1}$ in a medium composed of EBH (diluted or undiluted), 0.5 $g{\cdot}L^{-1}$ corn steep liquor (CSL) and 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ to obtain a C/N ratio of 75.

2.3. Bioreactor batch experiments

Yarrowia lipolytica W29 batch cultures were performed in a 2-L DASGIP Parallel Bioreactor System (Eppendorf, Hamburg, Germany) filled with 400 mL of EBH medium (undiluted EBH, 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$, 0.5 $g{\cdot}L^{-1}$ or 2 $g{\cdot}L^{-1}$ CSL, C/N ratio 75). The supplementation of EBH medium with 0.3 $g{\cdot}L^{-1}$ KH_2PO_4 was also studied. The experiments were conducted at 27 °C, 600 rpm, and 2 vvm (volume of air per volume of medium per minute). The pH was automatically maintained at 5.5 ± 0.5, adding 4 M HCl or 4 M NaOH.

Additional experiments were carried out to study the effect of k_La by varying the agitation rate (600 rpm, 800 rpm) and the specific airflow rate (2 vvm, 3 vvm) in a medium composed of undiluted EBH, 2 $g{\cdot}L^{-1}$ CSL and 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$.

2.4. Bioreactor two-stage repeated batch experiments

A two-stage repeated batch (TSRB) strategy was applied to enhance the final biomass concentration and microbial lipids concentration. In the 1st stage, cells were cultured for 56 h in undiluted EBH, 2 $g{\cdot}L^{-1}$ CSL and 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ (C/N ratio 75). After this, cells were harvested, centrifuged (8000 rpm, 10 min), and resuspended in fresh medium (undiluted EBH, 2 $g{\cdot}L^{-1}$ CSL, 1.34 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ to attain a C/N ratio of 100) for the 2nd cultivation stage (88 h). The experiments were conducted at 800 rpm and 2 vvm.

2.5. Measurement of volumetric oxygen transfer coefficient

A static gassing-out technique, as described by Ferreira et al. [22], was employed to calculate the experimental volumetric oxygen transfer coefficient (k_La) at different agitation and aeration conditions in the bioreactor experiments. Briefly, after a preliminary gassing-out with nitrogen to remove oxygen from the medium, air was introduced, and the dissolved oxygen concentration in the liquid was monitored. The values of k_La were determined from the slope of the straight line obtained from a plot of ln ($C^* - C_L$) vs time (where C^* is the saturated dissolved oxygen concentration and C_L is the concentration of dissolved oxygen in the medium).

2.6. Analytical methods

Lignocellulosic biomass – feedstock and pretreated biomass – were gravimetrically analyzed for water (by oven drying at 105 °C to constant weight) and ashes content (by applying NREL/TP-510-42622 protocol) [23]. Polysaccharides (hemicellulose and cellulose) and lignin contents were assayed by quantitative hydrolysis with sulphuric acid, based on NREL/TP-510-42618 protocol [24]. The acid-insoluble residue was considered as Klason lignin after correction for the acid-insoluble ashes.

Glucose, xylose, arabinose, acetic acid, 5-HMF, formic acid, and citric acid were quantified by HPLC using an Aminex HPX-87H column (300 mm × 7.8 mm, 8 μm particle size) and RI and UV detectors. The column temperature was maintained at 60 °C, and a mobile phase (H_2SO_4 5 mM) was used at a flow rate of 0.7 mL min^{-1}.

Total phenols were quantified by the Folin–Ciocalteu method (Commission Regulation (EEC) No. 2676/90) using gallic acid as standard. Cuvette test kits LCK 914, LCK 338, and LCK 350 (Hach-Lange GmbH, Germany) were used for the spectrophotometric determination of chemical oxygen demand, total nitrogen, and phosphorus in EBH, respectively. Total solids corresponded to the weight of a hydrolysate residue after evaporation in an oven at 105 °C in a weighed crucible and dried to constant weight. Total suspended solids coincided with the portion of total solids retained by a filter and dried to a constant weight

at 105 °C [25].

The biomass concentration was determined by cell counting using a Neubauer counting chamber in a binocular bright-field microscope and converted to cell dry weight ($g{\cdot}L^{-1}$) using a conversion factor.

Intracellular lipids were quantified in lyophilized cells using the phospho-vanillin colorimetric method according to Lopes et al. [15]. Results were expressed as microbial lipids content (ratio between lipids concentration and lyophilized biomass concentration) and microbial lipids concentration (calculated by multiplying lipids content by biomass concentration in the cultivation medium). Microbial lipids composition was analyzed in lyophilized cells by measuring fatty acid methyl esters (FAMEs), according to the protocol described by Lopes et al. [14].

2.7. Statistical analysis

Data were subjected to one-way analysis of variance (ANOVA), followed by Tukey's multiple range test to identify significant differences among means ($p < 0.05$) using *IBM SPSS Statistics* 27 (IBM, NY, USA).

3. Results and discussion

3.1. Hydrolysate composition

The high polysaccharides' content (60.1%) on eucalyptus bark feedstock (Table 1) confirmed the attractive potential of this residue to be used for sugar platform biorefineries. By carrying out a mass balance to steam explosion pretreatment step, 85% (dry weight basis) of the biomass was recovered, with partial (61%) solubilization of xylan and complete or near-complete solubilization of arabinan and acetyl groups (Table 1), respectively. Similar findings were reported in previous studies for eucalyptus woodchips subjected to steam explosion [26]. Acetyl groups, bound to xylan in the native eucalyptus biomass, were also totally removed during steam explosion treatments conducted in the Chiarello et al. [27] study. The glucan content found in treated samples was higher than that in untreated biomass because cellulose remained mostly unaffected during the treatment (only 3% of solubilization), while hemicellulose components (xylan, arabinan, and acetyl groups) were removed from the solid fraction [26]. Depending on the extent of the treatment, steam explosion might also remove minor amounts of lignin. However, in this study, the lignin content increased after pretreatment.

The enzymatic hydrolysis of pretreated biomass produced a hydrolysate with 64.5 $g{\cdot}L^{-1}$ of total sugars, consisting of 86% glucose and 14% xylose (Table 2). This composition corresponds to hydrolysis yields of 61% and 44% for the conversion of cellulose and xylan fractions, respectively, into glucose and xylose. Martín-Sampedro et al. [26] reported lower hydrolysis yields (25% for glucose and 36% for xylose) with eucalyptus bark subjected to the same process stages. However, Chiarello et al. [27] achieved a conversion of glucans up to 95% and a maximum glucose concentration of 125 $g{\cdot}L^{-1}$ through stepwise substrate addition (fed-batch feeding) and efficient mechanical agitation (shaft with multiple stirrers). In other studies, employing different pretreatment methods (e.g., hydrothermal, mechanochemical, and green liquor) before enzymatic hydrolysis, total sugars concentration in EBH ranged between 45 $g{\cdot}L^{-1}$ and 108 $g{\cdot}L^{-1}$ with different proportions of glucose and xylose [28–30]. This variability is highly influenced by the specific experimental conditions applied in the pretreatment procedures, as well as the chemical composition of the eucalyptus species.

Table 1
Chemical characterization (expressed in mass percentage of the oven-dried sludge) of Eucalyptus bark - as received and after pretreatment.

Component	Content in Eucalyptus bark (%)	
	As received	Pretreated
Glucan	41.0	46.3
Xylan	19.1	8.8
Arabinan	1.2	0.0
Acetyl groups	3.2	0.4
Klason Lignin	23.2	30.3
Ash	8.5	12.9
Others	3.8	1.3

Table 2
Main composition of eucalyptus bark hydrolysate obtained by steam explosion followed by enzymatic hydrolysis. Data are the average ± standard deviation of two independent analyses.

	Concentration ($g{\cdot}L^{-1}$)
Glucose	55.7 ± 1.0
Xylose	8.8 ± 0.6
Arabinose	0.010 ± 0.005
Acetic acid	6.2 ± 0.3
Formic acid	0.8 ± 0.1
5-HMF	0.22 ± 0.04
Total Phenols	0.66 ± 0.2
Chemical Oxygen Demand (COD)	92.3 ± 0.2
Total nitrogen	0.43 ± 0.01
Total phosphorus	0.011 ± 0.001
Total solids	68.4 ± 1.3
Total suspended solids	28.5 ± 1.7

The growth of *Y. lipolytica* was not inhibited in a glucose medium with similar concentrations of acetic acid, formic acid, and 5-HMF [21] as those obtained in the present EBH.

Despite the low nitrogen (<0.5 $g{\cdot}L^{-1}$) and phosphorus (<0.02 $g{\cdot}L^{-1}$) content, EBH has high levels of organic load (expressed as the COD content) and sugars, justifying its use as a substrate for microbial growth.

3.2. Erlenmeyer flask experiments

The ability of *Y. lipolytica* W29 to grow in real LBH was evaluated in undiluted and diluted EBH (Fig. 1).

*Yarrowia lipolytic*a grew in undiluted EBH supplemented with a low concentration of CSL and ammonium sulfate. Although the final biomass obtained was equal regardless of EBH concentration, a slightly higher cellular growth rate was attained in the diluted EBH (0.080 h^{-1} ± 0.004 h^{-1}) compared to the undiluted EBH (0.065 h^{-1} ± 0.004 h^{-1}). Despite the presence of compounds that can be considered toxic to yeasts, no

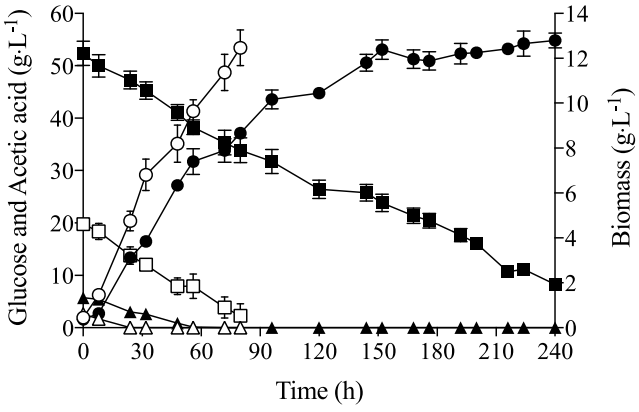

Fig. 1. Biomass (●, ○), glucose (■, □), and acetic acid (▲, △) concentration obtained in *Y. lipolytica* W29 batch cultures carried out in Erlenmeyer flasks with diluted (open symbols) and undiluted (closed symbols) eucalyptus bark hydrolysate medium supplemented with 0.5 $g{\cdot}L^{-1}$ CSL and 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$. The error bars represent the standard deviation of two independent replicates.

latency phase was observed for the undiluted EBH medium (Fig. 1). Acetic acid, a carbon source assimilated by *Y. lipolytica* [16,17], was completely consumed after 24 h (diluted EBH) and 56 h (undiluted EBH). *Yarrowia lipolytica* W29 assimilated all formic acid after 48 h and 72 h in diluted and undiluted EBH, respectively. 5-HMF was not consumed in both conditions, but its presence in the EBH medium did not negatively affect cell proliferation. A study by Dias et al. [21] reported that *Y. lipolytica* W29 grew in a medium with 5-HMF up to 1 $g{\cdot}L^{-1}$. Moreover, yeast cells assimilated 40%–70% of 5-HMF in a synthetic medium mimicking LBH [21]. This ability of *Y. lipolytica* to tolerate and/or consume these compounds (acetic acid, formic acid, and 5-HMF) is strain-dependent. For instance, *Yarrowia lipolytica* ATCC 8661 growth was inhibited by certain concentrations of acetic acid, formic acid, 5-HMF, and furfural in spruce wood chips hydrolysate, but halving the concentrations of these compounds alleviated the inhibitory effects [31]. Tsigie et al. [32] reported that *Y. lipolytica* Po1g growth in non-detoxified sugarcane bagasse hydrolysate was limited owing to the presence of furfural and 5-HMF. Due to the inhibitory effect of some compounds on cellular metabolism, the need for a sequential adaptation of yeasts using different ratios of hydrolysates was reported. Indeed, bioethanol productivity improved in *S. cerevisiae* cultures previously adapted to wheat straw hydrolysate [33].

Glucose was consumed at similar uptake rates in diluted (0.21 $g\ L^{-1}\ h^{-1} \pm 0.02\ g\ L^{-1}\ h^{-1)}$ and undiluted ($0.18\ g\ L^{-1}\ h^{-1} \pm 0.01\ g\ L^{-1}\ h^{-1}$) EBH, remaining 8 $g{\cdot}L^{-1}$ glucose at the final culture medium in the undiluted EBH for the chosen operation time. Regardless of EBH concentration, xylose was not consumed throughout the cultivation time. The conditions of experiments in Erlenmeyer flasks and the time of experiments may have not be adequate to trigger the xylose utilization pathway. Although it was not possible to observe a sequential metabolism of glucose and xylose [7], xylose concentration in the medium did not inhibit the consumption of other carbon sources or cell growth.

In diluted EBH, *Y. lipolytica* accumulated 9% (w/w) of intracellular lipids and secreted 5.7 $g{\cdot}L^{-1}$ of citric acid. On the other hand, 4% (w/w) of intracellular lipids and 12 $g{\cdot}L^{-1}$ of citric acid were produced in undiluted EBH. Since intracellular lipids were evaluated only at the end of the experiments, the low lipids content in these conditions may have been a result of lipids mobilization (consumption of accumulated lipids). Indeed, *Y. lipolytica* also metabolized lipids previously accumulated during yeast growth in switchgrass hydrolysate [20] and sugarcane bagasse hydrolysate [32] when total sugars were depleted or almost depleted.

Nitrogen limitation is a common factor required for microbial lipids and citric acid production. *Yarrowia lipolytica* prioritizes microbial lipids production under nitrogen-limited conditions but can also trigger the production of low-molecular-weight metabolites, such as citric acid [34]. Despite citric acid production is often more pronounced after nitrogen depletion, its accumulation is also affected by operational conditions (e.g., pH, aeration, and cultivation mode) [35]. Although the citric acid/substrate mass yields obtained were equal in diluted (0.30 ± 0.01) and undiluted (0.27 ± 0.01) EBH, the highest citric acid production in undiluted EBH may be associated with the entry into the stationary growth phase (depletion of nitrogen) [35], which was not observed in the batch culture with the diluted EBH.

Considering that *Y. lipolytica* was not inhibited by undiluted EBH, biomass, and valuable compounds production were further studied in stirred tank bioreactors (STR).

3.3. Bioreactor experiments

3.3.1. Effect of medium composition

Regardless of medium composition, biomass production, sugars (glucose and xylose), and other compounds (acetic acid, formic acid, and 5-HMF) consumption were faster in the STR-bioreactor experiments (Fig. 2) than in the Erlenmeyer flask experiments (Fig. 1).

Nitrogen is essential for yeast growth and metabolism since it plays a key role in sugar transport and compound biosynthesis, including proteins [36]. Phosphorus is another essential nutrient for lipid, coenzymes, and nucleic acids biosynthesis pathways [37]. CSL is a by-product from the corn steeping process composed of organic nitrogen, free amino acids, reducing sugars, important vitamins, trace elements, and lactic acid [38]. In this sense, CSL addition may promote yeast growth by providing "unidentified growth factors" [38], and simultaneously reduce the economic and environmental impact of value-added compound production [35].

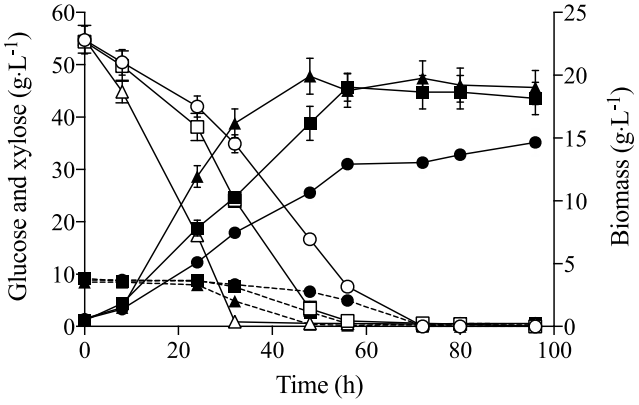

Fig. 2. Biomass (closed symbols, solid line), glucose (open symbols), and xylose (closed symbols, dashed line) concentration obtained in *Y. lipolytica* W29 batch cultures carried out in STR lab-scale bioreactor with undiluted eucalyptus bark hydrolysate supplemented with 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ and 0.5 $g{\cdot}L^{-1}$ CSL (●,○); 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$ and 2 $g{\cdot}L^{-1}$ CSL (■,□); 1.8 $g{\cdot}L^{-1}$ $(NH_4)_2SO_4$, 2 $g{\cdot}L^{-1}$ CSL and 0.3 $g{\cdot}L^{-1}$ KH_2PO_4 (▲,△). The error bars represent the standard deviation of two independent replicates.

In this work, increasing CSL concentration from 0.5 $g{\cdot}L^{-1}$ to 2 $g{\cdot}L^{-1}$ led to an approximately 1.5-fold improvement in specific growth rate and biomass concentration at the end of the exponential growth phase (Fig. 2). Furthermore, the addition of 2 $g{\cdot}L^{-1}$ CSL to EBH also led to faster consumption of glucose and xylose. Acetic acid, formic acid, and 5-HMF were consumed at similar rates regardless of CSL concentration.

Although the specific growth rate and sugars (and acetic acid - data not shown) consumption rates were higher in the medium containing 0.3 $g{\cdot}L^{-1}$ KH_2PO_4 compared to cultures without phosphorus supplementation, final biomass at the stationary cellular growth phase and biomass yield were statistically equal. The results support that phosphorus supplementation, which increases the costs associated with fermentative medium and overall bioprocess, is unnecessary in this case.

Sugar consumption patterns were correlated with biomass production - total glucose and xylose assimilation coincided with the beginning of the stationary growth phase. Xylose began to be consumed after 24 h in bioreactor experiments when glucose concentration was already below 25 $g{\cdot}L^{-1}$ (0.5 $g{\cdot}L^{-1}$ CSL and 2 $g{\cdot}L^{-1}$ CSL) or 35 $g{\cdot}L^{-1}$ (2 $g{\cdot}L^{-1}$ CSL + 0.3 $g{\cdot}L^{-1}$ KH_2PO_4), being consumed in 24 h–48 h. Furthermore, acetic acid, formic acid, and 5-HMF were completely consumed after 24 h in all conditions.

Yarrowia lipolytica W29 efficiently assimilated sugars and other compounds of EBH without needing previous detoxification or adaptation steps. Tsigie et al. [32] reported that *Y. lipolytica* Po1g completely metabolized the mixture of glucose and xylose of sugarcane bagasse hydrolysate without needing extensive adaption periods and with no diauxic growth. The simultaneous consumption of glucose and acetic acid indicates that the assimilation mechanisms of these substrates in *Y. lipolytica* are independent [39]. In the last years, xylose-degrading enzymes and a cryptic xylose utilization pathway were discovered in the *Y. lipolytica* genome [40,41]. Some studies on *Y. lipolytica* growth in LBH reported simultaneous consumption of glucose and xylose [19,32], while others related a sequential consumption pattern [41,42] due to

allosteric competition for sugar transporters and glucose repression of xylose metabolism [7]. In this work, the high cell density and the low glucose concentration after 24 h/32 h may have reduced the competition for protein transporters and/or repression of catabolism, explaining *Y. lipolytica* W29 glucose and xylose uptake behavior.

Higher lipids and citric acid content were reached in the bioreactor experiments compared to the flask experiments, possibly due to total xylose consumption combined with mechanical agitation, pH-controlled, and forced aeration. In the case of *Y. lipolytica*, a strictly aerobic yeast, the forced aeration and mechanical agitation rate achieved in the STR are particularly important to improve oxygen mass transfer rate (OTR) by volumetric mass transfer coefficient (k_La) enhancement [43].

Medium composition affected microbial lipids accumulation. Although maximum lipids content obtained at 96 h was statistically equal independent of CSL and KH_2PO_4 concentration (Fig. 3A), the values were higher in all sampling times for the medium with 2 g·L^{-1} CSL without phosphorous supplementation than for the other conditions. This, associated with the cell growth profile, led to a 2.7-fold improvement in lipids concentration reaching around 3 g·L^{-1} obtained at 48 h of operation. *Yarrowia lipolytica* lipids accumulation was lower in KH_2PO_4-supplemented EBH for all sampling times (Fig. 3A) in comparison with non-phosphorus-supplemented EBH, particularly with 2 g·L^{-1} CSL. However, no significant mobilization of lipids occurred during the monitored operation time.

The accumulation of citric acid was highest for the condition with the lowest CSL concentration (Fig. 3B). Citric acid production followed different patterns depending on medium composition: (a) for 0.5 g·L^{-1} CSL, citric acid continuously increased, achieving the maximum value of 26 g·L^{-1} at 72 h and keeping it till 96 h, and that was the highest citric acid titter attained in all experiments; and (b) for 2 g·L^{-1} CSL (with or without KH_2PO_4), citric acid reached the maximum after 48 h, decreasing afterward. The consumption of the citric acid produced is possibly explained by the depletion of all carbon sources in the culture medium, that were completely consumed during the first 48 h. CSL contains approximately 45% (w/w) of organic nitrogen, of which about half is present in free amino acids form [38]. Nitrogen depletion in the culture medium is fundamental for citric acid accumulation since NH_4^+ down-regulates citrate synthase activity, which is responsible for citric acid production in the tricarboxylic acid cycle [35]. The lower levels of nitrogen contained in 0.5 g·L^{-1} CSL compared to 2 g·L^{-1} CSL probably led to faster nitrogen depletion conditions, channeling the metabolism towards an accentuated citric acid production.

It is worth noticing that, in these experiments, lipids accumulation patterns were negatively correlated with citric acid production. Since nutrient-limited conditions (nitrogen and phosphorus) are required simultaneously for microbial lipids and citric acid production [34], lipids production can shift metabolism away from citric acid synthesis and vice versa. Some studies have reported a shift of the metabolic pathways of *Y. lipolytica* towards citric acid synthesis, while others found no direct relation between lipids and citric acid production in the glucose-based nitrogen-limited medium [44,45]. Zhang et al. [45] suggest that other mechanisms (e.g., pH and medium composition) are involved as well. Furthermore, it was also verified in the current study, that the conditions that favor yeast growth or lipids accumulation are not the same. As occurs for nitrogen, the depletion of other compounds like phosphorus or magnesium associated with the excess of available carbon sources triggers metabolite production instead of biomass growth [37,46]. Therefore, the following experiments were conducted without phosphorus supplementation.

3.3.2. *Effect of volumetric oxygen transfer coefficient (k_La)*

In the case of strictly aerobic yeasts such as *Y. lipolytica*, the dissolved oxygen concentration in the cultivation medium is a key factor for its metabolism and lipids production [15]. Therefore, the effect of k_La on biomass and lipids production by *Y. lipolytica* was studied, in undiluted EBH supplemented with 2 g·L^{-1} CSL, by varying the agitation and aeration rates, obtaining values of k_La equal to 44 h^{-1} (600 rpm, 2 vvm), 54 h^{-1} (800 rpm, 2 vvm), and 66 h^{-1} (800 rpm, 3 vvm). As expected, the increase of agitation rate from 600 rpm to 800 rpm and the aeration rate from 2 vvm to 3 vvm led to 24% and 22% enhancement in k_La, respectively.

Increasing k_La from 44 h^{-1} to 66 h^{-1} did not affect the specific growth rate (0.095 h^{-1} to 0.103 h^{-1}) and the final biomass (Fig. 4A). However, the condition of k_La of 66 h^{-1} had a clear positive effect on biomass concentration at 48 h and reached the stationary growth phase 8 h earlier than in lower k_La conditions. Furthermore, regardless of oxygenation conditions, glucose, and xylose consumption (Fig. 4A) and biomass yield (values ranged between 0.27 g g^{-1} and 0.32 g g^{-1}) were similar, with sugars (glucose and xylose) and other compounds (acetic acid, formic acid, and 5-HMF) being completely consumed after 56 h and 24 h, respectively.

Due to the aerobic feature of *Y. lipolytica*, it was expected to enhance cellular growth by raising the oxygen mass transfer. Indeed, the enhancement of the k_La or OTR had a positive effect on biomass concentration of *Y. lipolytica* W29 when cultivated in a mixture of acetate, propionate, butyrate, and glucose [17], waste cooking oils [14], and pork lard [15]. The increase in total air pressure had a clear positive

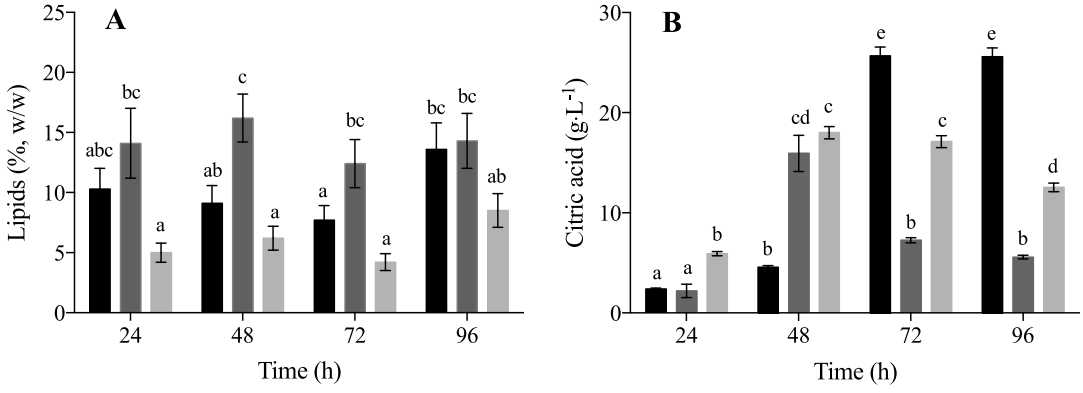

Fig. 3. Microbial lipids content (**A**) and citric acid concentration (**B**) obtained in *Y. lipolytica* W29 batch cultures carried out in STR lab-scale bioreactor with undiluted eucalyptus bark hydrolysate supplemented with 1.8 g·L^{-1} $(NH_4)_2SO_4$ and 0.5 g·L^{-1} CSL (black); 1.8 g·L^{-1} $(NH_4)_2SO_4$ and 2 g·L^{-1} CSL (dark grey); and 1.8 g·L^{-1} $(NH_4)_2SO_4$, 2 g·L^{-1} CSL and 0.3 g·L^{-1} KH_2PO_4 (light grey). The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

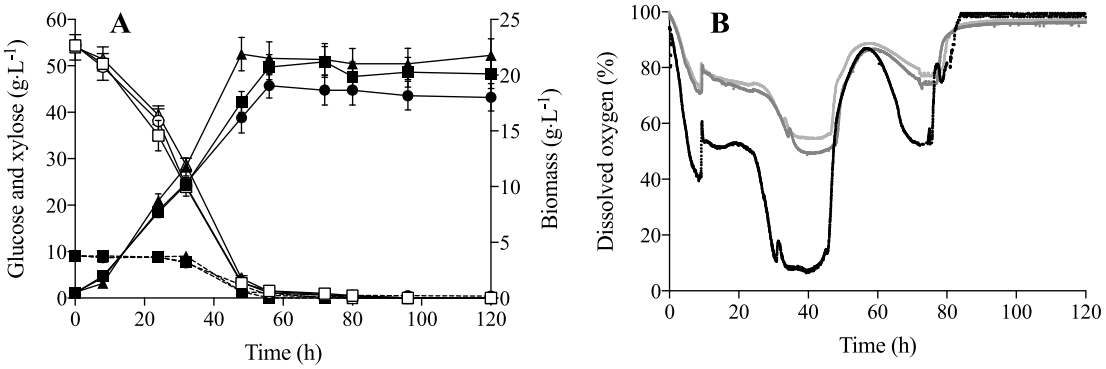

Fig. 4. Biomass (closed symbols, solid line), glucose (open symbols), and xylose (closed symbols, dashed line) concentration (**A**) and dissolved oxygen concentration (% of saturation) (**B**) obtained in *Y. lipolytica* W29 batch cultures carried out in lab-scale bioreactor with undiluted eucalyptus bark hydrolysate, 2 g·L^{-1} CSL and 1.8 g·L^{-1} $(NH_4)_2SO_4$ at different k_La conditions: 44 h^{-1} (●, ○, black line); 54 h^{-1} (■,□, dark grey line); and 66 h^{-1} (▲,△, light grey line). The error bars represent the standard deviation of two independent replicates.

effect on *Y. lipolytica* W29 growth in glucose [47], but no significant effect was observed in crude glycerol [48]. Despite previous studies demonstrating that high aeration and agitation rates significantly increase biomass production by *Candida tropicalis* in cassava residue hydrolysate [49] and *Lipomyces starkeyi* in corn stover hydrolysate [50], this is the first work reporting the effect of k_La on *Y. lipolytica* metabolism in LBH. In the current work, the lack of improvement in final biomass, substrate consumption, and kinetic parameters with the increase in k_La may have been due to the narrow range of k_La studied (44 h^{-1} – 66 h^{-1}). In agreement, the dissolved oxygen concentration (DOC) profiles demonstrate that no total depletion oxygen conditions were reached (Fig. 4B). Only in the experiment with k_La of 44 h^{-1}, near-zero oxygen conditions were attained since dissolved oxygen dropped to 7% of saturation during the first hours of yeast cultivation (exponential growth phase). In all experiments, DOC increased during the stationary phase of growth and remained at 100% until the end of the experiments (Fig. 4B).

Despite the insignificant effect of k_La on *Y. lipolytica* final biomass production, increasing the k_La value from 44 h^{-1} to 66 h^{-1} enhanced microbial lipids content. The higher lipids content attained in these experiments was observed at 72 h (23%, w/w) and 48 h (25%, w/w) in 54 h^{-1} and 66 h^{-1} k_La conditions, respectively (Fig. 5A). Furthermore, a clear positive effect on lipids concentration was also observed at 48 h due to the higher biomass concentration (Fig. 4A). Lipids concentration increased from 2.6 g·L^{-1} ± 0.5 g·L^{-1} to 5.6 g·L^{-1} ± 0.9 g·L^{-1}, by increasing k_La from 44 h^{-1} to 66 h^{-1}, corresponding to approximately 2.2-fold improvement. Notably, the highest k_La condition achieved a 1.4-fold increase in lipids productivity.

Lipids accumulation followed different patterns for the k_La conditions studied: (a) for a k_La of 44 h^{-1}, lipids content remained constant throughout cultivation; and (b) for a k_La of 54 h^{-1} and 66 h^{-1}, lipids content reached a maximum value, decreasing after 72 h or 48 h, respectively. Microbial lipids accumulated by *Y. lipolytica* in sugarcane bagasse hydrolysate medium also reached a maximum on the 3rd day [32]. The author attributed this result to nitrogen source exhaustion and a better adaptation of the enzymes to the environment (low concentration of inhibiting compounds). The mobilization of synthesized lipids (lipid turnover) observed from 72 h or 48 h is correlated with the depletion of carbon sources in the culture medium at the beginning of the stationary growth phase, a pattern already reported for *Y. lipolytica* cultured in different LBH [20,32].

Several authors have recognized the importance of oxygen in lipids production by *Y. lipolytica*. Increasing the oxygen mass transfer in experiments carried out with pork lard [15], waste cooking oils [14], and a mixture of volatile fatty acids and glucose [17] resulted in a clear enhancement in lipids accumulation by *Y. lipolytica* W29. Under high

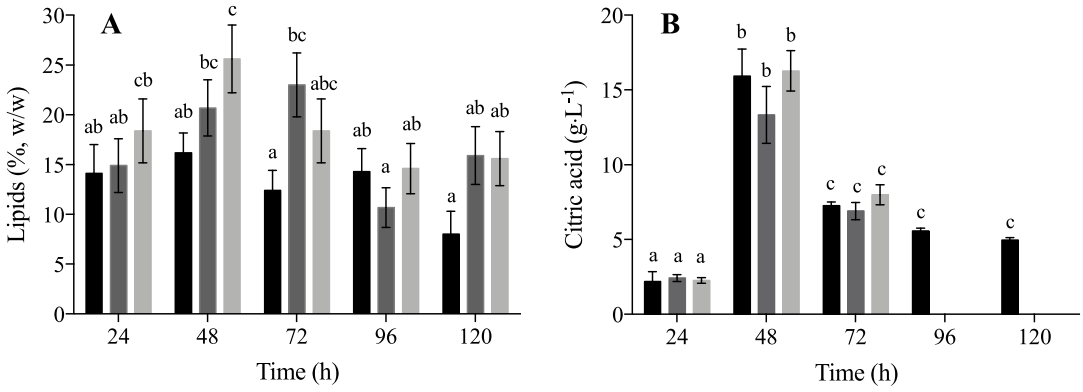

Fig. 5. Microbial lipids content (**A**) and citric acid concentration (**B**) obtained in *Y. lipolytica* W29 batch cultures carried out in lab-scale bioreactor with undiluted eucalyptus bark hydrolysate, 2 g·L^{-1} CSL and 1.8 g·L^{-1} $(NH_4)_2SO_4$ at different k_La conditions: 44 h^{-1} (black bars); 54 h^{-1} (dark grey bars); and 66 h^{-1} (light grey bars). The error bars represent the standard deviation of two independent replicates. Bars with the same letter do not present statistically significant differences ($p \geq 0.05$).

oxygenation conditions, the activity of enzymes involved in lipids synthesis is upregulated, improving lipids production [51]. However, above certain levels of DOC, lipids accumulation by *Y. lipolytica* is hampered by increased acyl-CoA oxidase activity, which directs the carbon source toward the production of lipid-free biomass, instead of lipids synthesis [52] (a behavior that was not observed in the current study). Although no studies concerning the effect of k_La on lipids production by *Y. lipolytica* from LBH were found in the literature, Walker et al. [20] observed that *Y. lipolytica* CBS7504 was able to accumulate 18% (w/w) of lipids growing in switchgrass hydrolysate at 900 rpm and 1 vvm. Similarly, *Y. lipolytica* Pod1 achieved the same lipids content (18 %) in agave bagasse hydrolysate at 750 rpm and 1 vvm [19]. It is important to highlight that the lipid concentration achieved by *Y. lipolytica* W29 in this study is similar or even higher than those accumulated by other wild-type or genetically modified *Y. lipolytica* strains in lignocellulosic hydrolysates (Table 3). Kommoji et al. [53] achieved high lipids concentration (10.8 $g{\cdot}L^{-1}$) with MTCC 9519 strain but the grass hydrolysate was supplemented with 0.4 % (w/v) sodium *n*-octanoate as a surfactant to enhance lipids production. In eucalyptus hydrolysates, *R. toruloides* CCT 7815 accumulated a lipid content of 50 % (w/w) in Erlenmeyer flasks, corresponding to 3 $g{\cdot}L^{-1}$ of lipids [2]. *Ashbya gossypii* accumulated 11 % (w/w) of lipids in a bioreactor experiment at 500 rpm and 2 vvm [54]. These results underscore the potential of utilizing *Y. lipolytica* W29 for microbial lipids accumulation from eucalyptus hydrolysates.

Yarrowia lipolytica produced similar amounts of citric acid regardless of the k_La condition. Citric acid reached a maximum value after 48 h, decreasing afterward, indicating the mobilization of produced metabolites for cellular metabolic activities after carbon sources depletion. Previous studies demonstrated that increasing k_La or OTR enhanced citric acid synthesis in *Y. lipolytica* W29 from crude glycerol, but the narrow range of k_La (44 h^{-1} – 66 h^{-1}) studied in the current study may explain the similar amounts of citric acid obtained.

3.3.3. Two-stage repeated batch experiments

Two-stage cultivation strategies were tested in yeast lipids production to overcome challenges associated with achieving high productivities in traditional batch cultivations [6]. A TSRB strategy, consisting of a 1st stage in which the cells are cultivated in a nutrient-rich medium to obtain high cell densities, followed by the cultivation in a nutrient-limited medium to enhance lipids accumulation (2nd stage) [59], was carried out. According to the literature, *de novo* lipid synthesis in *Y. lipolytica*, which occurs from hydrophilic substrates (e.g., glucose and acetic acid), requires carbon excess and nitrogen limitation (high C/N ratio) [8]. In the 2nd stage, biomass concentration (Fig. 6A) increased 51% due to the addition of fresh EBH, but with a lower specific growth rate (0.025 h^{-1} ± 0.002 h^{-1}) compared to the 1st stage (0.10 h^{-1} ± 0.01 h^{-1}), possibly due to the increase in the C/N ratio from 75 to 100. High nitrogen concentration and consequently a low C/N ratio are generally associated with high biomass production by *Y. lipolytica* [60]. Glucose and xylose were completely consumed after 56 h in the 1st stage but the uptake rates were greatly enhanced in the 2nd stage, being glucose and xylose consumed after 40 h and 24 h, respectively. Acetic acid, formic acid, and 5-HMF were also consumed more rapidly in the 2nd stage. Biomass yield was similar in the 1st (0.30 $g\ g^{-1}$ ± 0.02 $g\ g^{-1}$) and 2nd stage (0.29 $g\ g^{-1}$ ± 0.02 $g\ g^{-1}$) of the experiment. These results indicate that *Y. lipolytica* ability to assimilate xylose was slightly accelerated in the 2nd stage due to previous cultivation in xylose-containing medium. The higher cell density existing at the beginning of the 2nd stage may also have contributed to this xylose consumption behavior, as well as to the consumption of other carbon sources.

Although a 1.6-fold increase in biomass production was observed, no improvement in intracellular lipids content was achieved in the TSRB culture (Fig. 6B) compared to the traditional batch culture (Fig. 5A) under the same agitation and aeration conditions. The maximum lipids accumulation occurred at the end of the 1st stage (48 h), followed by a subsequent decrease until the end of the experiment. The metabolization of lipids combined with the total consumption of sugars and other compounds suggested that the carbon source added in the 2nd stage was channeled for biomass production, synthesis of other metabolites, and cellular maintenance, instead of lipids synthesis. Interestingly, even though lipids content was lower in the 2nd stage, lipids concentration obtained at 96 h was equal in TSRB (2.3 $g{\cdot}L^{-1}$ ± 0.2 $g{\cdot}L^{-1}$) and traditional batch culture (2.2 $g{\cdot}L^{-1}$ ± 0.3 $g{\cdot}L^{-1}$), as a result of higher biomass concentration. The potential enhancement of microbial lipids production by a C/N ratio increase to 100 [61] was not observed in this study.

Citric acid production reached its maximums at 56 h (15 $g{\cdot}L^{-1}$) and 96 h (18 $g{\cdot}L^{-1}$) at the end of the exponential growth phase in the 1st and

Table 3
Lipids content, lipids concentration, and fatty acids composition obtained in several oleaginous yeast cultures growing in lignocellulosic biomass hydrolysates.

Yeast	Hydrolysate	Culture conditions	Lipid production		Fatty acid content (%, w/w)					Ref.
			% (w/w)	$g{\cdot}L^{-1}$	C16:0	C16:1	C18:0	C18:1	C18:2	
Y. lipolytica W29 (wild-type)	Eucalyptus bark	Batch (B) 800 rpm 3 vvm	26 ± 3	5.6 ± 0.9	14.3 ± 0.1	20.2 ± 0.2	–	48.4 ± 0.5	17.1 ± 0.1	This study
Y. lipolytica CBS7504 (wild-type)	Switchgrass	Batch (B) 900 rpm 1 vvm	18	2.1	NA	NA	NA	NA	NA	[20]
Y. lipolytica Pod1 (wild-type)	Agave bagasse	Batch (B) 750 rpm 1 vvm	18	2.0	NA	NA	NA	NA	NA	[19]
Y. lipolytica A101 (wild-type)	Oat bran	Batch (EF)	11	1.0	8.7	3.5	6.4	50.8	11.9	[55]
Y. lipolytica (wild-type)	Pine wood sawdust	Batch (EF)	29	3.8	NA	NA	NA	NA	NA	[56]
Y. lipolytica MTCC 9519 (wild-type)	Grass	Batch (B) 100 rpm	54	10.8	21.8	5.6	39.6	18.6	14.4	[53]
Y. lipolytica NRRL 63746 (wild-type)	Wood	Batch (EF)	15	7.4	52.0	–	30.0	10.0	–	[57]
Y. lipolytica Po1g (modified)	Rice bran	Batch (EF)	48	5.2	20.5	11.4	5.4	59.9	–	[58]
Y. lipolytica Po1g (modified)	Sugarcane bagasse	Batch (EF)	59	6.7	17.8	14.1	4.4	55.6	–	[32]
Y. lipolytica A101 (modified)	Rye straw	Batch (EF)	37	2.2	10.5	5.0	10.6	63.8	7.0	[40]
Y. lipolytica Pod1 (modified)	Agave bagasse	Batch (B) 750 rpm 1 vvm	67	16.5	NA	NA	NA	NA	NA	[19]
R. toruloides CCT 7815 (wild-type)	Eucalyptus chips	Batch (EF)	50	3.0	NA	NA	NA	NA	NA	[2]
A. gossypii A877 (modified)	Eucalyptus bark	Batch (B) 500 rpm 2 vvm	11	1.4	NA	NA	NA	NA	NA	[54]

NA: not available; B: bioreactor; EF: Erlenmeyer flask.

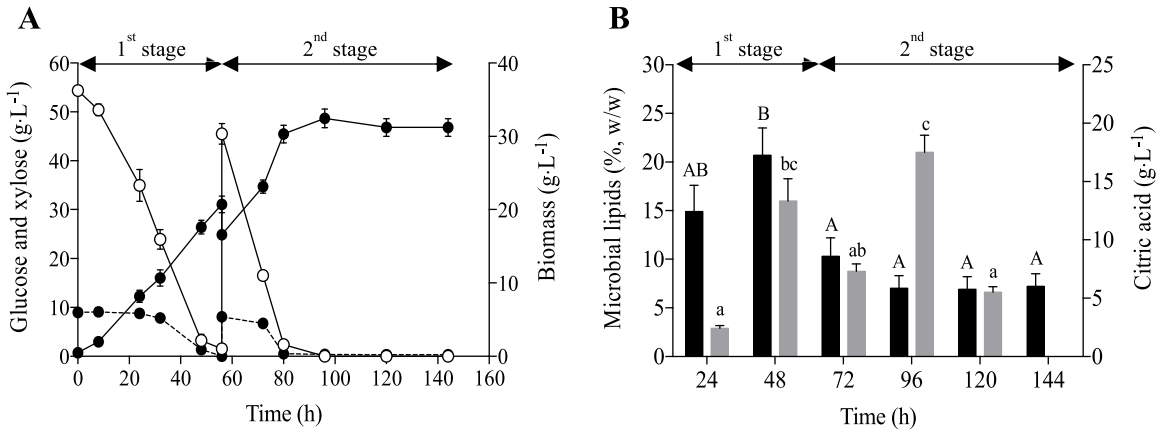

Fig. 6. Biomass (closed symbols, solid line), glucose (open symbols) and xylose (closed symbols, dashed line) concentration (**A**); microbial lipids content (black bars) and citric acid (grey bars) concentration (**B**) obtained in *Y. lipolytica* W29 two-stage repeated batch cultures carried out in lab-scale stirred tank bioreactor with undiluted eucalyptus bark hydrolysate, 2 g·L^{-1} CSL and 1.8 g·L^{-1} $(NH_4)_2SO_4$ for C/N ratio 75 – 1st stage – or 1.34 g·L^{-1} $(NH_4)_2SO_4$ for C/N ratio 100 – 2nd stage. The error bars represent the standard deviation of two independent replicates. Lowercase letters represent differences through time for citric acid production and uppercase letters indicate differences through time for microbial lipids content ($p < 0.05$).

2nd stages, respectively, followed by a decay in concentration (Fig. 6B). This pattern is consistent with previous experiments in traditional batch mode, in which the maximum production was also attained at the end of the exponential growth phase.

3.3.4. Lipids composition

The composition of microbial lipids is a crucial factor in assessing their suitability for application in biofuel industries. Lipids produced by *Y. lipolytica* in the batch bioreactor experiments at k_La of 66 h^{-1} (the highest lipids concentration obtained in this work) were mainly composed of oleic acid, followed by palmitoleic, linoleic, and palmitic acids (Table 3). The profile of the lipids accumulated in this study was similar to those obtained in previous works for *Y. lipolytica* growing in LBH. Tsigie et al. [32,58] reported that the main fatty acids of *Y. lipolytica* lipids produced from sugarcane bagasse or rice bran hydrolysates follow the order: oleic acid > palmitic acid > palmitoleic acid > stearic acid. Linoleic acid was also accumulated by *Y. lipolytica* in grass hydrolysate [53]. It is worth noticing that the high content of unsaturated fatty acids (86%) relative to saturated fatty acids (14%) observed in this study suggests that lipids produced could serve as a promising alternative feedstock for biodiesel production in place of vegetable oils [62]. Lipids of *Y. lipolytica* NCYC 2904, produced from crude glycerol and a mixture of volatile fatty acids, were extracted and successfully transesterified into high-quality biodiesel [18].

4. Conclusions

The results highlight the potential of using undetoxified and undiluted bark eucalyptus hydrolysate as a cost-effective substrate for *Y. lipolytica* growth and microbial lipids accumulation. The significance of medium composition (EBH concentration and supplementation), dissolved oxygen concentration (assessed through k_La), and mode of operation as key parameters for both biomass and lipids production were also demonstrated. The most significant factor in improving lipids production by *Y. lipolytica* W29 was the increase of k_La, given the aerobic feature of this yeast. Lipids of *Y. lipolytica* represent a potential feedstock for the biodiesel industry owing to its similar composition to vegetable oils and content of unsaturated fatty acids. This integrated bioprocess, which combines the reuse and valorization of abundantly generated lignocellulosic materials with the attainment of *Y. lipolytica* lipids-rich biomass, represents a circular bioeconomy approach that supports the development of a sustainable biorefinery.

CRediT authorship contribution statement

Bruna Dias: Formal analysis, Investigation, Methodology, Writing – original draft. **Marlene Lopes:** Conceptualization, Formal analysis, Supervision, Writing – review & editing. **Helena Fernandes:** Formal analysis, Investigation, Methodology, Writing – original draft. **Susana Marques:** Methodology, Writing – review & editing. **Francisco Gírio:** Methodology, Writing – review & editing. **Isabel Belo:** Conceptualization, Supervision, Writing – review & editing.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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