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Direct xylan conversion into glycolipid biosurfactants, mannosylerythritol lipids, by *Pseudozyma antarctica* PYCC 5048^T

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ABSTRACT

Mannosylerythritol lipids (MEL) are glycolipid biosurfactants, produced by *Pseudozyma* spp., with increasing commercial interest. While MEL can be produced from D-glucose and D-xylose, the direct conversion of the respective lignocellulosic polysaccharides, cellulose and xylan, was not reported yet. The ability of *Pseudozyma antarctica* PYCC 5048^T and *Pseudozyma aphidis* PYCC 5535^T to use cellulose (Avicel®) and xylan (beechwood) as carbon and energy source has been assessed along with their capacity of producing cellulolytic and hemicellulolytic enzymes, toward a consolidated bioprocess (CBP) for MEL production. The yeasts assessed were neither able to grow in medium containing Avicel® nor produce cellulolytic enzymes under the conditions tested. On contrary, both yeasts were able to efficiently grow in xylan, but MEL production was only detected in *P. antarctica* PYCC 5048^T cultures. MEL titers reached 1.3 g/l after 10 days in batch cultures with 40 g/l xylan, and 2.0 g/l in fed-batch cultures with xylan feeding (additional 40 g/l) at day 4. High levels of xylanase activities were detected in xylan cultures, reaching 47–62 U/ml (31–32 U/mg) at 50 °C, and still exhibiting more than 10 U/ml under physiological temperature (28 °C). Total β-xylosidase activities, displayed mainly as wall-bounded and extracellular activity, accounted for 0.154 and 0.176 U/ml in *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultures, respectively. The present results demonstrate the potential of *Pseudozyma* spp. for using directly a fraction of lignocellulosic biomass, xylan, and combining in the same bioprocess the production of xylanolytic enzymes with MEL production.

1. Introduction

The industrial conversion of renewable feedstock into useful compounds, like fuels, fine chemicals and materials, is receiving increasing attention as a strategy to overcome environmental and economic concerns related to the use of non-renewable resources. The transition from an oil-based economy to a bio-economy will be mostly dependent on the use of lignocellulosic materials, which are the most abundant, ubiquitous and renewable carbon source on Earth [1,2]. Most of the research on the use of lignocellulose has been devoted to the production of second generation (2G) bioethanol [2–4]. Recently, the conversion of lignocellulosic materials into other added-value bio-based products has gained significant attention [1,5]. Several bulk and fine chemicals generated from oil refining are expected to be progressively replaced by bio-based products, including ethanol, lactic acid, succinic acid, 1,4-butanediol, sorbitol, isoprene, among others [6].

Biosurfactants are expected to reach more than USD 2 billion by 2020 [7], with industrial applications in the production of food, cosmetics, and pharmaceutics, as well as in removal of contamination by heavy metals, oils and other toxic organics [8]. The considerable interest in these bio-based products is related to their unique physical and chemical properties, biodegradability, mild production conditions and antimicrobial activity [9].

Mannosylerythritol lipids (MEL) are glycolipid biosurfactants produced by *Pseudozyma* spp., *Ustilago* spp. and related yeasts and filamentous fungi [8,10]. Soybean oil is the preferred substrate for MEL production with high yields and titers [8,9]. However, the industrial production of biosurfactants from vegetable oils may have sustainability constrains, due to the negative

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environmental impact related to the cultivation of dedicated crops for oil production, and the economic impact of the increasing prices of vegetable oils. In addition, biosurfactants recovery from the fermentation broth, where vegetable oil by-products (such as free fatty acids and mono- or di-acylglycerols) coexist, usually requires solvent-intensive processes to obtain high purity levels, leading to high product losses in downstream operations [11]. The use of lignocellulosic materials as carbon source for the production of biosurfactants might represent an upgrading on process sustainability, both at substrate and downstream levels due to their low commercial value and to hydrophilic nature, respectively.

While the production of MEL from the main lignocellulose-driven monosaccharides, as D-glucose, D-xylose and D-glucose/D-xylose mixtures, has already been demonstrated [12,13], the direct MEL production from lignocellulosic polysaccharides, cellulose and hemicellulose, has not been assessed yet. This direct conversion process, where a single microorganism combines enzyme production, enzymatic hydrolysis and bioconversion of released sugars into bio-based products, is called consolidated bioprocessing (CBP) [14]. The natural or engineered microbial capacity of producing own cellulolytic and/or hemicellulolytic enzymes can improve the economy of the lignocellulose bioconversion processes by reducing and/or optimizing the use of commercial enzyme cocktails or even completely eliminating this significant operating cost in lignocellulose biorefining. In this work, the CBP potential of *Pseudozyma antarctica* and *Pseudozyma aphidis*, to directly convert lignocellulose polysaccharides into MEL, was evaluated, and the respective enzymatic profile characterized.

2. Materials and methods

2.1. Yeast strains, maintenance and standard cultivation conditions

P. antarctica PYCC 5048^T and *P. aphidis* PYCC 5535^T were obtained from Portuguese Yeast Culture Collection (PYCC), CREM, FCT/UNL, Portugal. Yeasts were cultured for 3 days at 25 °C on yeast malt agar (YM-agar) medium (yeast extract, 3 g/l; malt extract, 3 g/l; peptone, 5 g/l; glucose, 10 g/l; agar, 20 g/l). Stock cultures were prepared by propagation of yeast cells in liquid medium as described below for the inoculum and stored (in 20% v/v glycerol aliquots) at −70 °C for later use. Inoculum was prepared by incubation of stock cultures of *P. antarctica* or *P. aphidis* at 28 °C, 140 rpm, for 48 h, in liquid medium containing glucose (40 g/l), $NaNO_3$ (3 g/l), $MgSO_4$ (0.3 g/l), KH_2PO_4 (0.3 g/l) and yeast extract (1 g/l).

Xylose, glucose, commercial grade beechwood xylan and Avicel® cellulose (40 g/l) were directly used as carbon source for cultivation of *P. antarctica* and *P. aphidis* and cultivation media were supplemented with $MgSO_4$ (0.3 g/l), KH_2PO_4 (0.3 g/l) and yeast extract (1 g/l). Potassium phthalate (50 mM) was used as buffer at pH 5.5. Culture medium was inoculated with 10% v/v of inoculum and incubated at 28 °C, 140 rpm, for 10–14 days. Two pulsed feeding strategies were applied: feeding strategy 1, with xylan (40 g/l) pulse; feeding strategy 2, with a pulse of xylan (40 g/l) and $NaNO_3$ (3 g/l). All experiments were carried out, at least, in a biological duplicate. Samples were taken periodically and analyzed for yeast growth, sugar profile, MEL (and fatty acid) production and enzyme activities. One milliliter culture sample was taken and, after centrifugation (at 13,000 rpm for 10 min, at 4 °C), the supernatants were stored at −20 °C for sugar and enzyme assays. For MEL (and fatty acid) quantification, a 3 ml-culture broth sample was lyophilized and weighted for further analysis.

2.2. Yeast growth

Yeast growth was determined either by cell protein quantification or by optical density [OD] measurement at 640 nm. For determination of cell protein, 1 ml of culture broth was centrifuged for 10 min at 13,000 rpm. The supernatant was discarded and the pellet was washed with demineralized water. The pellet was then ressuspended and the cells were disrupted with Y-PER™ (Yeast Protein Extraction Reagent, Pierce, Thermo Scientific, USA). The protein content of the supernatant was determined using Pierce™ BCA protein assay kit (Thermo Scientific, USA).

2.3. Substrate and product quantification

Supernatants aliquots were filtered through a 0.45 μm-pore-size filter and analyzed for glucose, xylose and erythritol quantification in high performance liquid chromatography (HPLC) system (Merck Hitachi, Darmstadt, Germany) equipped with a refractive index detector (L-7490, Merck Hitachi, Darmstadt, Germany) and an Aminex HPX-87H column (300 mm × 7.8 mm, Bio-Rad), at 50 °C. Sulfuric acid (5 mM) was used as mobile phase at 0.4 ml/min. Xylan and cellulose contents were determined through quantitative hydrolysis with sulphuric acid in two stages (the first step with 72% w/v acid at 30 °C for 1 h; and the second with 4% w/v acid for 1 h at 121 °C) according to the method described by Browning [15]. The quantification of the monosaccharides obtained was carried out by HPLC, as described above. The acid insoluble residue was considered as Klason lignin, after correction for the acid insoluble ash [16].

MEL were quantified as previously described, through GC analysis of methyl esters generated by methanolysis of freeze-dried biological samples (3 ml) [12].

2.4. Enzymatic activity assays

2.4.1. Cellulolytic activity assays

Cellulase activity was assessed according to Ghose [17], as filter paper activity (FPase), by measuring the release of reducing sugars from Whatman number 1 filter paper. Reducing sugars were estimated by the dinitrosalicylic acid (DNS) method [18]. The assay was scaled-down according to King et al. [19] by using filter paper cylinders (2 × 2.2 mg). A few modifications were further introduced, such as the use of potassium phthalate buffer (50 mM, pH 5.5), a temperature of 28 °C, and a total reaction volume of 125 μl. Filter paper unit (FPU) is defined as the amount of enzyme required to release 1 μmol of glucose reducing equivalent per minute, under the conditions previously defined [17].

β-Glucosidase activity was assayed in a reaction mixture (0.3 ml) containing 5 mM p-nitrophenyl-β-D-glucoside (pNPG, Sigma, USA), 50 mM potassium phthalate buffer pH 5.5, and the appropriately diluted sample. After incubation at 28 °C for 60 min, 0.15 ml of 1 M Na_2CO_3 was added to stop the reaction [20]. The p-nitrophenol (pNP) was quantified, by spectrophotometry, at 405 nm. One unit (U) of β-glucosidase activity was defined as the amount of enzyme required to release 1 μmol of pNP per minute.

2.4.2. Xylanolytic activity assays

Endo-1,4-β-xylanase activity was determined by measuring the release of reducing sugars (as xylose equivalents) from xylan using 3,5-dinitosalicylic acid (DNS) method as described by Miller [18]. The standard assay mixture contained 0.1 ml of appropriately diluted sample supernatant and 1% w/v beechwood xylan solution (in phthalate buffer pH 5.5). After incubation at 28 °C, for 30 min, the reaction was stopped with 0.6 ml of DNS reagent. The mixture was then boiled for 5 min and cooled to room temperature, and reducing sugars were determined, by spectrophotometry, at 550 nm, against a standard curve with different D-xylose concentrations processed with the same procedure as the samples. Each reaction and its control were run in quadruplicate. One unit (U) of xylanase activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar equivalent per minute under the assay conditions.

Extracellular β-xylosidase activity was determined as previously described [21], in a reaction mixture (0.3 ml) containing 5 mM p-nitrophenyl-β-D-xyloside (pNPX) (Sigma, USA), 50 mM potassium phthalate buffer pH 5.5, and the appropriately diluted supernatant sample. After incubation at 28 °C, for 30 min, the reaction was stopped with 0.15 ml of 1 M Na_2CO_3. Cell wall-bounded and intracellular β-xylosidase activities were determined from samples of 10 ml culture broth. Samples were centrifuged at 13,000 rpm (for 10 min, at 4 °C) and washed three times with demineralized water. For determination of cell wall-bounded β-xylosidase activity, washed intact cells were directly assayed as described above (in a reaction mixture of 0.6 ml), but under continuous magnetic stirring to avoid cell sedimentation. After incubation at 28 °C for 30 min, the reaction was stopped with 0.3 ml of 1 M Na_2CO_3. For determination of intracellular activity, washed cells were incubated with Y-PER™ (Yeast Protein Extraction Reagent, Pierce, Thermo Scientific, USA), and the cell crude extract (free of cell debris) was used in enzymatic assay as described above. The p-nitrophenol (pNP) was quantified, by spectrophotometry, at 405 nm. One unit (U) of β-xylosidase activity was defined as the amount of enzyme required to release 1 μmol of pNP per minute.

2.5. Statistical analysis

Statistics were performed by analysis of variance (one-way ANOVA) and *p*-values of the differences between groups were corrected for simultaneous hypothesis testing according to Tukey's method. The level of significance was set at $p < 0.05$.

3. Results

3.1. Cellulose and xylan assessed as carbon and energy source for *Pseudozyma spp.*

The ability of *Pseudozyma* spp. to grow in cellulose and xylan, which constitute the two main polysaccharides present in most lignocellulosic materials, was evaluated. Yeast cells were directly inoculated in media containing cellulose or xylan as carbon and energy source and the results were compared with reference cultures containing D-glucose and D-xylose. During 7 days, cell

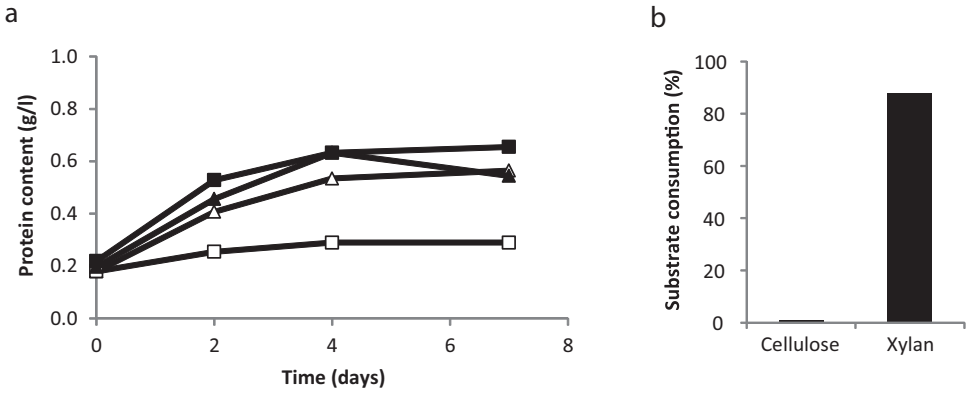

Fig. 1. Cultivation of *P. antarctica* PYCC 5048^T in polysaccharides (xylan or cellulose) or in monosaccharides (D-xylose or D-glucose) at 28 °C, 140 rpm, for 7 days. (a) Yeast biomass assessed through intracellular protein content of cells grown in 40 g/l of D-glucose (black square), D-xylose (black triangle), cellulose (white square) or xylan (white triangle) medium. (b) Total polysaccharide consumption (expressed as the percentage of initial content) after 7-days incubation in 40 g/l cellulose (Avicel®) or xylan (beechwood) medium.

growth was monitored by the determination of intracellular protein content (Fig. 1a), which had a linear correlation with cell dry weight (data not shown). Either in D-glucose or in D-xylose, the intracellular protein content reached 0.6 g/l after 4 days. In xylan medium, biomass (measured as intracellular protein content) reached approximately 90% of that obtained in D-glucose or D-xylose reference cultures. On contrary, in Avicel® the biomass production was residual (Fig. 1a).

The insoluble oligosaccharides and polysaccharides remaining after 7-days incubation in both Avicel® and xylan media were assessed by quantitative acid hydrolysis (Fig. 1b). In Avicel® cultures, virtually all the substrate remained in the media after 7-days incubation, which indicated that yeasts were unable to use cellulose as carbon and energy source. Conversely, the insoluble xylan measured after 7-days incubation was only 13% of the initial amount supplied, implying that this substrate was almost entirely consumed. The residual biomass production observed in Avicel® cultures resulted, most probably, from the consumption of carbon source present in yeast extract and/or of sugars carried-over with the inoculum (containing less than 3 g/l of glucose). Therefore, under the conditions tested, yeasts revealed to own endogenous mechanisms for the hydrolysis of xylan, but not of cellulose.

3.2. Direct conversion of xylan into MEL by Pseudozyma yeasts

3.2.1. Batch process for direct xylan conversion into MEL

P. antarctica and *P. aphidis* are described as MEL producers from hexoses (D-glucose) [13], D-xylose and D-xylose/D-glucose mixtures [12]. Since the *Pseudozyma* strains tested were able to use xylan, but not cellulose, as carbon and energy source, the ability of *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T to directly convert xylan into MEL was evaluated (Fig. 2b and d, Table 1). The experiments were performed during 10 days of incubation at 28 °C and 150 rpm, using xylan (40 g/l) as substrate, and compared with D-xylose (40 g/l) cultures under similar conditions. Both yeasts were able to grow in xylan, but slower than in D-xylose. Also, both yeasts produced lower biomass when grown in xylan than in D-xylose cultures ($OD_{640\,nm}$ of 11.0 and 14.2, respectively, for *P. antarctica* PYCC 5048^T; $OD_{640\,nm}$ of 19.2 and 22.1, respectively, for *P. aphidis* PYCC 5535^T) (Fig. 2). D-Xylose consumption rate was also higher for *P. aphidis* PYCC 5535^T than for *P. antarctica* PYCC 5048^T (Fig. 2a and c), which is in agreement with the previously reported values of 0.32 and 0.28 g/l/h, respectively [12].

In xylan cultures, xylobiose and xylose accumulated in the first 2 days. Xylobiose accumulated up to 2.5 and 3.3 g/l, in *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultures, respectively; while xylose accumulated up to 2.7 and 1.3 g/l in *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultures, respectively. Those maximal xylobiose and xylose concentrations were reached at day 1 and day 2, respectively, and became virtually zero from day 4 until the end of the incubation period. The results obtained suggest that the two yeast strains tested have efficient mechanisms for xylan assimilation, with *P. aphidis* PYCC 5535^T showing a slightly better catabolic performance. In both cultures, the accumulation of xylobiose and xylose was transient, revealing a first stage with higher rate of xylan hydrolysis and a second stage of higher rate of xylose consumption, as a consequence of continuous biomass increase. The production of xylanolytic enzymes in both *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T was then characterized (see Section 3.3).

The direct MEL production from xylan was observed with *P. antarctica* PYCC 5048^T (Fig. 2b). After 10 days, at 28 °C and 150 rpm, *P. antarctica* PYCC 5048^T reached 1.3 ± 0.2 g/l of MEL from xylan

Table 1
MEL titers, yields and productivities and biomass production in 10-days batch cultivations in D-xylose and xylan (40 g/l) for *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T.

	P. antarctica PYCC 5048^T		*P. aphidis* PYCC 5535^T	
	D-Xylose	Xylan	D-Xylose	Xylan
MEL (g/l)	3.2 ± 0.3	1.3 ± 0.2	1.2 ± 0.2	0.0 ± 0.0
$Biomass_{max}$ ($OD_{640\,nm}$)	14.2 ± 1.1	11.0 ± 1.4	20.9 ± 2.2	19.2 ± 1.4
q_{MEL} (g/l/h)	0.014 ± 0.001	0.006 ± 0.001	0.007 ± 0.001	0.000 ± 0.000
(at day)	(10)	(7)	(7)	
$Y_{MEL/S}$ (g/g)	0.08 ± 0.01	0.03 ± 0.01	0.03 ± 0.00	0.00 ± 0.00

$Biomass_{max}$ – maximum biomass ($OD_{640\,nm}$).
q_{MEL} – maximum MEL productivity (g/l/h).
$Y_{MEL/S}$ – MEL yield (g MEL/g substrate).

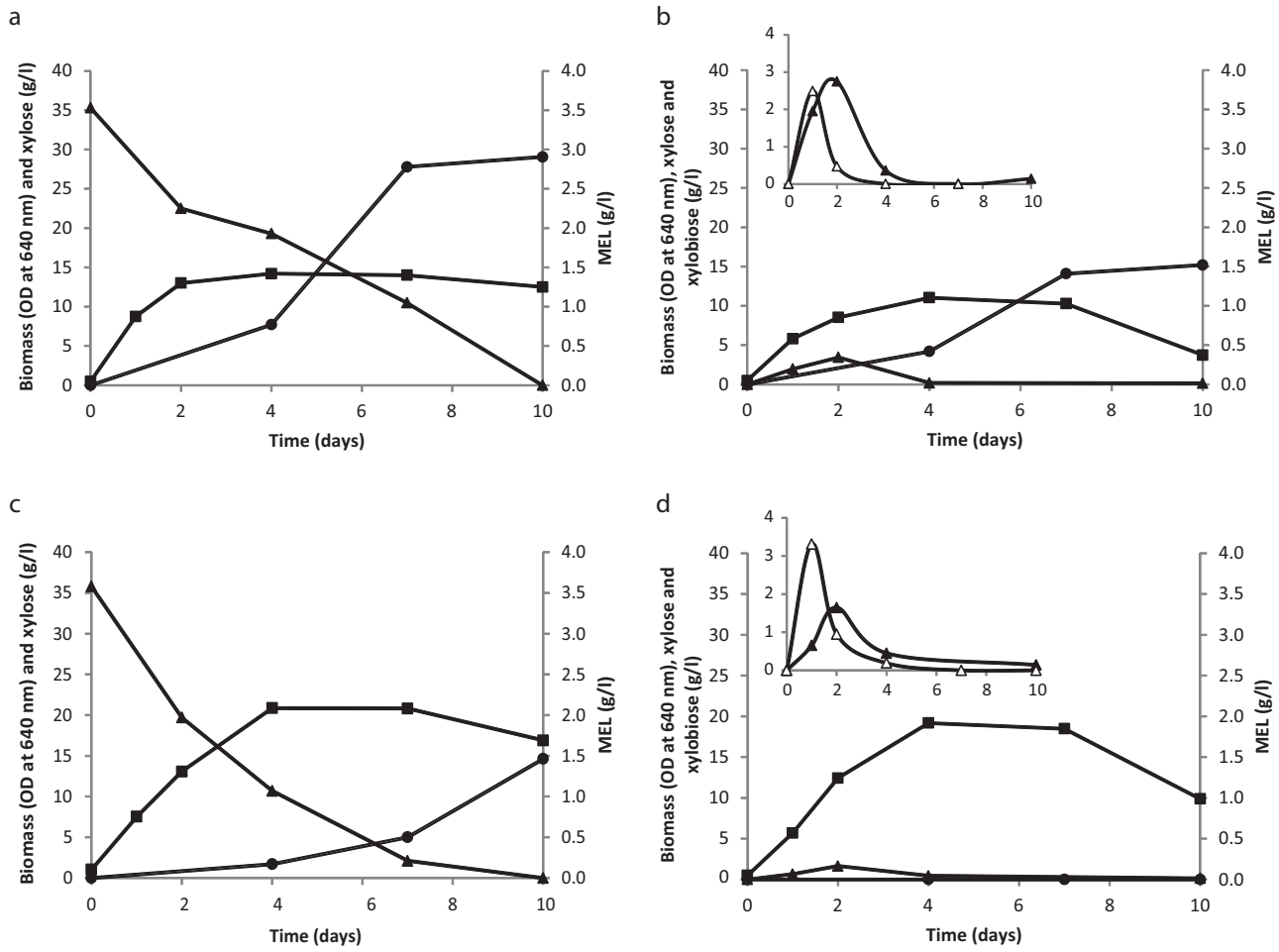

Fig. 2. Cultivation of *P. antarctica* PYCC 5048^T (a and b) and *P. aphidis* PYCC 5535^T (c and d) in D-xylose (40 g/l) (a and c) or beechwood xylan (40 g/l) (b and d) medium. Biomass (square), D-xylose (dark triangle), xylobiose (white triangle) and MEL (circle).

(40 g/l) (Fig. 2b, Table 1), against 3.2 ± 0.3 g/l from D-xylose (40 g/l) (Fig. 2a, Table 1), which corresponded to around 40% of MEL yield and productivity from the polysaccharide when compared to those obtained from the respective monosaccharide (Table 1). *P. aphidis* PYCC 5535^T produced 1.2 ± 0.2 g/l of MEL from xylose (Fig. 2c, Table 1), but, under the conditions used, did not generate detectable amounts of MEL from xylan (Fig. 2d, Table 1). The carbon chain length of MEL acyl groups obtained using xylan was similar to the ones found when D-glucose [13], D-xylose and D-xylose/D-glucose mixtures [12] were used. The longer acyl groups associated to MEL are mainly composed of C10:n and C12:n. Such profile is slightly different from that found in MEL produced from soybean oil, where C8:0 and C10:n are usually reported [22].

3.2.2. *Fed-batch process for direct xylan conversion into MEL by P. antarctica PYCC 5048^T*

Under the experimental set-up applied in this work (10% v/v of 48-h inoculum, 40 g/l substrate), biomass achieved a maximum between days 2 and 4 and it was kept relatively steady until day 10; moreover, the production of MEL from monosaccharides, such as D-xylose, was firstly detected at day 4 and MEL accumulated until days 10–14 (Fig. 2a) [12]. However, when *P. antarctica* PYCC 5048^T was cultivated on xylan, the biomass decreased earlier than on D-xylose cultures (Fig. 2b), which can result from an earlier xylose depletion in xylan cultures. Since MEL was mainly produced after day 4, it would be advantageous to have non-limiting carbon source for MEL production. Therefore, a fed-batch strategy was conducted, with xylan feeding, as a pulse, at day 4. This approach also considers that after 4-days incubation, part of the initial xylan has been hydrolyzed, which means that the xylanolytic system has already been induced (see Section 3.3), and takes into account previous results using D-xylose as substrate, which have demonstrated that fed-batch strategies and nitrate supplementation can be crucial to obtain higher MEL production [12]. Accordingly, two feeding strategies were tested in a fed-batch process with *P. antarctica* PYCC 5048^T (Fig. 3):

(i) Feeding strategy 1: pulse of xylan (40 g/l) at day 4. This strategy resulted in a continuous xylose accumulation from day 4 until day 7, reaching 10.2 g/l and 15.6 g/l at days 5 and 7, respectively (Fig. 3a). The maximum xylobiose accumulation was observed at day 5 (3.5 g/l), but kept at relatively low concentrations. These results revealed an efficient enzymatic system of *P. antarctica* PYCC 5048^T for the hydrolysis of xylan into soluble sugars, with low xylobiose accumulation, which can be explained by high β-xylosidase activity and low inhibition by the product, D-xylose. The xylan feeding at day 4 also resulted in a continuous increase in biomass until day 10, whereas in the batch process (in the absence of a xylan pulse) a decrease in biomass was observed from day 7 to day 10. Therefore, higher ($p < 0.05$) biomass values were obtained for the fed-batch process than for the batch process ($OD_{640\,nm}$ of 13.2 and 11.0, respectively), which confirms the carbon source limitation in the batch process. Under the fed-batch process, MEL production reached 2.0 g/l (from a

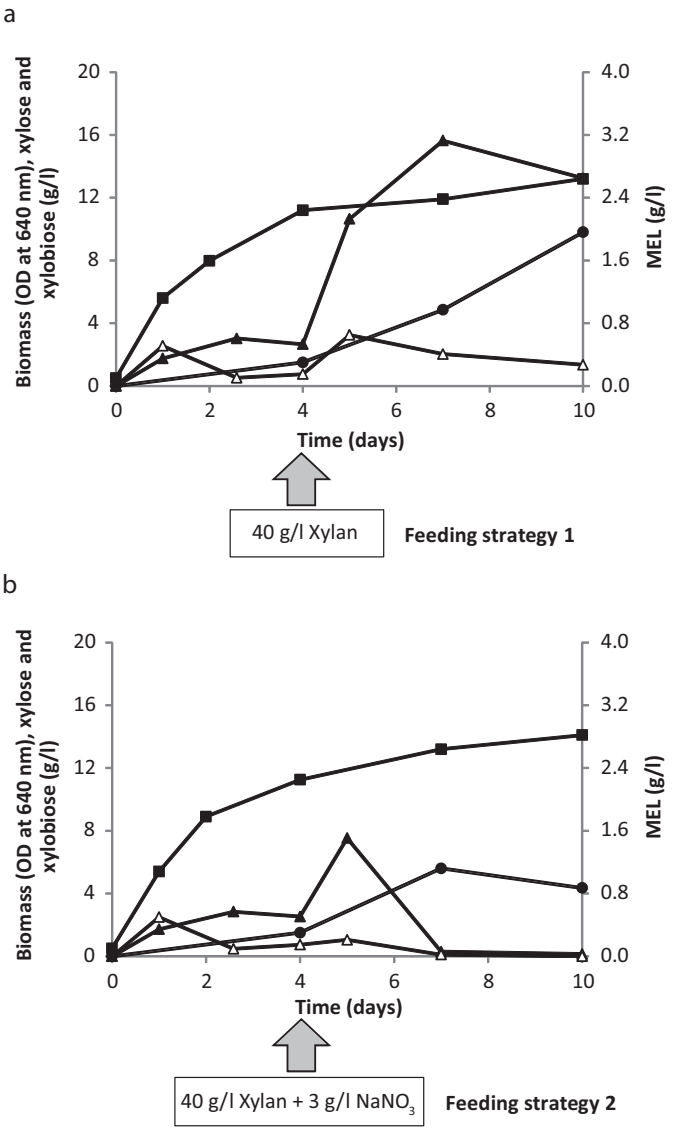

Fig. 3. Fed-batch CBP strategies for the production of MEL from xylan with *P. antarctica* PYCC 5048^T. Biomass (square), D-xylose (dark triangle), xylobiose (white triangle) and MEL (circle). (a) Feeding strategy 1:40 g/l xylan feeding at day 4. (b) Feeding strategy 2:40 g/l xylan and 3 g/l $NaNO_3$ feeding at day 4.

total of 80 g/l xylan), which represented a significant increase ($p < 0.05$), 1.5-fold, of MEL titer when compared to the batch process (1.3 g/l, from a total of 40 g/l xylan).

(ii) Feeding strategy 2: pulse of xylan (40 g/l) and $NaNO_3$ (3 g/l) at day 4. The simultaneous nitrate supplementation, along with xylan, promoted a lower xylose accumulation in the medium when compared to feeding strategy 1, with xylose increasing only up to day 5, to a concentration of 7.5 g/l, and steeply decreasing in the subsequent period reaching a value of virtually zero at day 7. Apparently, the supplementation of nitrogen source at day 4 improved sugar consumption. This result is in agreement with the trend previously observed for *P. antarctica* PYCC 5048^T when cultivated in xylose medium containing nitrate (3 g/l) [12]. In this feeding strategy 2, xylobiose was kept residual, lower than in feeding strategy 1. With nitrate supplementation, along with apparent higher sugar consumption rate, biomass attained the highest value ($OD_{640\,nm}$ = 14.1). However, the maximum MEL concentration of 1.1 g/l did not represent an improvement when compared to the batch experiments (1.1 g/l at day 7 and 1.3 g/l at day 10). Apparently, under this condition

Table 2
Volumetric xylanase and β-xylosidase activities and protein content. Enzyme volumetric activities (U/ml) were determined at 50 °C, after 10-days incubation in (xylan) medium, at 28 °C, for *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T.

	P. antarctica PYCC 5048^T	*P. aphidis* PYCC 5535^T
Xylanase (U/ml)	46.6 ± 4.4	61.5 ± 7.0
β-Xylosidase (U/ml)	0.14 ± 0.03	0.09 ± 0.01
Total protein (mg/ml)	1.5 ± 0.3	1.9 ± 0.2

(Fig. 2b), the supplementation of the nitrogen source, sodium nitrate, conducted to a faster depletion of the carbon source in the medium toward biomass formation and other metabolic routes than those leading to MEL biosynthesis.

3.3. Characterization of hydrolytic enzyme profiles of *P. antarctica PYCC 5048^T* and *P. aphidis PYCC 5535^T*

3.3.1. *P. antarctica PYCC 5048^T* and *P. aphidis PYCC 5535^T* as producers of cellulase-free xylanases

The hydrolytic potential of *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T were firstly evaluated in terms of extracellular cellulolytic and xylanolytic activities (FPase, β-glucosidase, xylanase and β-xylosidase) at 50 °C. Under cellulose or glucose induction, both cellulolytic and xylanolytic activities were not detected. When cultured in xylose or in xylan, both strains revealed xylanase and β-xylosidase activities (Table 2, data not shown for D-xylose induction), but no cellulase (FPase) and β-glucosidase activities. Such results confirm that these yeasts own mechanisms to hydrolyze xylan, but not cellulose (see Fig. 1), i.e. are producers of cellulase-free xylanases. The tested *Pseudozyma* strains have produced xylanolytic enzymes with volumetric activities far superior to those previously reported in other yeasts genera (0.1–10 U/ml) [23] and at the level of those from many filamentous fungi (10–100 U/ml) (additional information on xylanase production and volumetric activities can be found elsewhere [24]).

3.3.2. Characterization of xylanase activity profile

The xylanolytic enzyme assays were also performed at 28 °C to allow the direct correlation between enzyme activities and yeast metabolism. After 10 days in xylan medium, *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T produced xylanases with volumetric activities of 11.1 and 13.4 U/ml (at 28 °C), respectively (Fig. 4), which corresponds to approximately 5-fold lower activity than those measured at 50 °C, typically the optimal temperature of these enzymes and the temperature usually used in standard xylanase quantification methods [25]. In the current study, cell growth took place up to 2–4 days (Fig. 2), but extracellular xylanolytic activities continuously increased until day 10, although with a different profile for each strain tested (Fig. 4). In *P. antarctica* PYCC 5048^T, the enzyme activity profile was linear between days 1 and 10, which means that xylanase activity increased not only during growth phase, but also during stationary phase. However, the xylanase production by *P. aphidis* PYCC 5535^T boosted from days 2 to 4, reaching more than 85% of maximum activity, while, at the same time point (day 4), *P. antarctica* PYCC 5048^T cultures presented less than 50% of its maximum xylanase activity (day 10).

3.3.3. Characterization of β-xylosidase activity profile

The extracellular β-xylosidase volumetric activities measured in *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultures reached 0.07 and 0.06 U/ml (measured at 28 °C), respectively, at day 10 (Fig. 4). These values are up to 2-fold lower than those measured at 50 °C (Table 2), which is in agreement with the differences observed for other yeast β-xylosidases [23]. Whereas the overall profiles observed for xylanase activity were different for the two

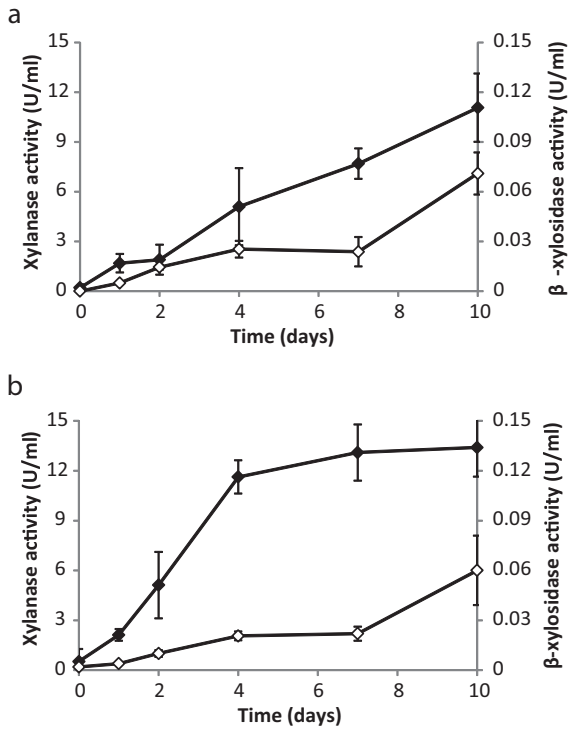

Fig. 4. Extracellular xylanolytic profile determined in *P. antarctica* PYCC 5048^T (A) and *P. aphidis* PYCC 5535^T (B) cultured in xylan. Xylanase (dark diamond) and β-xylosidase (white diamond) activities at 28 °C.

strains tested, the profile of extracellular β-xylosidase was similar, increasing until day 4, kept steady from day 4 up to 7 and increasing again from day 7 to day 10. Despite of the apparently low extracellular β-xylosidase activities (Fig. 4), the decrease in xylobiose concentration (Fig. 2) was fairly fast. Therefore, the presence of additional β-xylosidase mechanisms was investigated, both as intracellular and cell wall-bounded β-xylosidase activities, in samples collected after 10-days incubation in xylan. Cell wall-bounded activities were estimated to represent 47% and 63% of total β-xylosidase activity for *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultures, respectively (Fig. 5). Total β-xylosidase volumetric activity accounted for 0.154 and 0.176 U/ml for *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T, respectively, with the extracellular and cell wall-bounded activities accounting for more than 90%

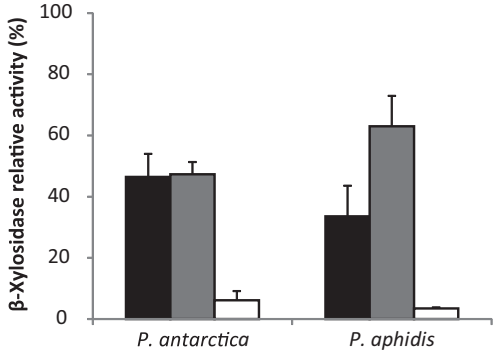

Fig. 5. Localization of β-xylosidase activity of *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T cultivated in xylan. Extracellular (black), wall-bounded (gray) and intracellular (white) β-xylosidase activities were determined (at 28 °C) for yeasts cultivated in 40 g/l xylan medium for 10 days, at 28 °C. Total β-xylosidase activity equivalent to 0.154 and 0.176 U/ml for *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T, respectively, corresponding to 100% of activity.

(Fig. 5), which means that complete xylan hydrolysis (incl. xylobiose) was mainly, or exclusively, performed outside the cell.

4. Discussion

Biosurfactants still represent a small share of the total surfactants market [26], but as other bio-based products their market share is increasing, following the tendency of a transition from the oil-based economy to a bio-economy era. Biosurfactant (MEL) production by *Pseudozyma* spp. is already commercially deployed using vegetable oil as substrate (Toyobo, USA, Inc.). However, the sustainable MEL production will probably benefit from the use of lignocellulosic materials as substrates.

In this work, the ability of *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T to assimilate lignocellulosic polysaccharides, cellulose and xylan, was investigated. These basideomycetous yeasts were able to grow in xylan, but not in cellulose, as sole carbon and energy source. Filamentous fungi are generally the best cellulolytic and hemicellulolytic microorganisms, but several yeasts were already described as displaying xylanolytic enzymes. In fact, some of basidiomycetous and ascomycetous yeasts were identified as able to efficiently grow in xylan, displaying xylanase and β-xylosidase activities [23]. However, those are lower than the measured for the *Pseudozyma* spp. assessed. The xylanase volumetric activities inhere reported are significantly higher (20-fold higher) than the best reported for *Cryptococcus laurentii* UFMG-HB 48 [24]. However, the xylanase specific activity is only 2–5-fold higher for the *Pseudozyma* spp. assessed than for such yeast, indicating that the higher volumetric activity is mainly a result of higher protein production by the former. The total β-xylosidase activity of the *Pseudozyma* spp. assessed is approximately 5-fold higher than the best reported in that study [24], with *Sugiyamaella smithiae* UFMG-HM 80.1 also performing xylan hydrolysis outside the cell (extracellular and wall-bounded activities). Among the *Pseudozyma* spp. assessed, the higher xylanase volumetric activity found for *P. aphidis* PYCC 5535^T was apparently related with higher protein production, since *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T displayed similar xylanase specific activities (31.1 and 32.4 U/mg, respectively).

In sum, the xylanase production by yeasts from the genus *Pseudozyma* (in this work and elsewhere [27]) is superior than in any other yeasts (0.1–10 U/ml) [23] and comparable to (or even higher than) those described for filamentous fungi (10–100 U/ml) [28]. The xylanolytic activities of *Pseudozyma* spp. crude extracts should be tested in lignocellulose conversion processes (alone or in supplementation of cellulolytic cocktails), aiming at the production of biofuels and other bio-based products (e.g. biosurfactants). Moreover, such xylanolytic capacity can be further explored in the development of own or heterologous CBP systems. In this work, the endogenous capacity of *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T to directly convert xylan into MEL was explored. *P. antarctica* and *P. aphidis* were already described as MEL producers [10,29], able to use the most abundant lignocellulosic monosaccharides D-glucose [13] and D-xylose, as well as from D-glucose/D-xylose mixtures, for production of this biosurfactant [12]. The later study reported higher volumetric MEL production by *P. antarctica* PYCC 5048^T, when compared with *P. aphidis* PYCC 5535^T, for cultivations carried out under the same conditions.

The results obtained in this study revealed that the xylan assimilation capacity of *P. aphidis* PYCC 5535^T is higher than that of *P. antarctica* PYCC 5048^T, as denoted by higher xylanase activity (particularly between days 1 and 4, Fig. 4), slightly higher total β-xylosidase activity (Fig. 5) and higher D-xylose consumption rate (Fig. 2a and c). However, the direct MEL production from xylan was only observed in *P. antarctica* PYCC 5048^T cultures (Fig. 2b and

d). This is, for the best of our knowledge, the first report of MEL production directly from xylan.

The benefits of a CBP, where the product is obtained by direct bioconversion, in a single step, without the need for enzyme supplementation, are often accompanied by the reduction of product yield when compared to SHF and SSF systems due to carbon flux diversion toward enzyme synthesis [14]. Here, after 10 days incubation, MEL production from xylan (1.3 g/l) was 50% lower than that obtained from the same concentration of D-xylose (3.2 g/l), which most probably resulted from a significant fraction of carbon source being used for enzyme production, as denoted by the extracellular total protein found in xylan cultures (1.5 g/l, against 1.1 g/l in xylose cultures).

MEL production has previously been reported to be favored under nitrogen limitation [9]. However, previous fed-batch experiments with D-xylose revealed that the C/N ratio should be re-balanced, through supplementation of nitrate, toward a higher D-xylose consumption rate and maximal MEL titers [12]. In this work, fed-batch experiments with xylan (pulse feeding at day 4) were performed with and without supplementation of nitrate. The supplementation of nitrate in the fed-batch experiment resulted, as expected, on higher D-xylose consumption rate. Apparently, nitrate supplementation also favored protein synthesis (including the production of xylanolytic enzymes). This feature could be beneficial for the CBP system, but under these specific experimental conditions, the carbon flux toward protein synthesis was apparently too high and limited MEL biosynthesis. On contrary, in the fed-batch experiment with a single xylan pulse (at day 4), the CBP system has benefited from the existing xylanolytic system in-place, reaching a MEL titer of 2.0 g/l, after the same10-days incubation. Although MEL yield was not improved in comparison to the batch experiment, a 50% increase in overall productivity was obtained. This approach can be further explored in the context of MEL production from lignocellulosic materials containing xylan, with a first step of biomass and enzyme production, followed by a second step dedicated to MEL production, at low nitrate levels.

5. Conclusions

This work reported, for the first time, the direct MEL production from xylan by one yeast strain, *P. antarctica* PYCC 5048^T. Moreover, both *P. antarctica* PYCC 5048^T and *P. aphidis* PYCC 5535^T revealed to be efficient producers of cellulase-free xylanases. The complete hydrolysis of xylan to D-xylose is performed outside the yeast cells as denoted by β-xylosidase activity mainly detected in the extracellular space and as cell-wall activity (>90%).

A fed-batch approach revealed to be potentially advantageous for xylan bioconversion into MEL, with a first stage for the production of biomass and xylanolytic enzymes and a second stage dedicated to MEL production. The direct conversion of xylan into MEL potentiates the utilization of lignocellulosic materials for the production of this biosurfactant. The complete utilization of the polysaccharides present in lignocellulosic materials for MEL production will require the supplementation with external cellulolytic enzymes [30] to complement the role of xylanolytic enzymes and achieve the hydrolysis of both the cellulosic and the hemicellulosic fractions. Other approach would be to engineer *P. antarctica* to produce heterologous cellulolytic enzymes in order to confer this yeast with the ability to also utilize cellulose, as a complete CBP system. Conversely, the xylanolytic performance of *Pseudozyma* spp. can be exploited toward a broad use of their extracellular crude extracts in lignocellulose conversion processes, either as cellulase-free xylanases or as supplementation of commercial cellulolytic cocktails for the production of biofuels and other bio-based products.

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