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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

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