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Implementation of a repeated fed-batch process for the production of chitin-glucan complex by *Komagataella pastoris*

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ABSTRACT

The yeast *Komagataella pastoris* was cultivated under different fed-batch strategies for the production of chitin-glucan complex (CGC), a co-polymer of chitin and β-glucan. The tested fed-batch strategies included DO-stat mode, predefined feeding profile and repeated fed-batch operation. Although high cell dry mass and high CGC production were obtained under the tested DO-stat strategy in a 94 h cultivation (159 and 29 g/L, respectively), the overall biomass and CGC productivities were low (41 and 7.4 g/L day, respectively). Cultivation with a predefined profile significantly improved both biomass and CGC volumetric productivity (87 and 10.8 g/L day, respectively). Hence, this strategy was used to implement a repeated fed-batch process comprising 7 consecutive cycles. A daily production of 119–126 g/L of biomass with a CGC content of 11–16 wt% was obtained, thus proving this cultivation strategy is adequate to reach a high CGC productivity that ranged between 11 and 18 g/L day. The process was stable and reproducible in terms of CGC productivity and polymer composition, making it a promising strategy for further process development.

1. Introduction

Chitin-glucan complex (CGC) is a cell wall component of most yeasts and fungi, contributing to the stability and protection of the cell [1,2]. Composed of chitin and β-glucans, this nontoxic and biologically active copolymer is a valuable biomaterial for use in pharmaceutical, cosmetic and food applications [3–6]. CGC has also the advantage of being a non-animal chitin source [7].

Komagataella pastoris, formerly known as *Pichia pastoris* [8], is a methylotrophic yeast that was recently proposed as a source of CGC in a patented bioprocess [5,9] based on the use of glycerol [7,10]. *K. pastoris* is widely used for the production of recombinant proteins due to its capacity in achieving high cell densities and high product productivities [11]. Most of *K. pastoris* production processes are performed under fed-batch mode, using generally glucose/glycerol and methanol as carbon sources, for the growth and heterologous protein induction phases, respectively [12–14]. The fed-batch mode is usually preferred over batch operation, since it allows for higher cell densities, while easily ensuring the culture's stability during the extended cultivation periods [14,15].

Different feeding strategies have been tested for the fed-batch cultivation of *K. pastoris*, including the use of exponential feeding profiles [7,16,17], the DO-stat mode [18–21], sequential pulse feeding [21,22], constant feeding flow [23,24] and repeated fed-batch operation [16,18,22,25]. Most of these substrate feeding strategies were developed to maximize the production of heterologous proteins by different *P. pastoris* strains [26]. Since different target products were envisaged in each of those processes, it is difficult to compare the performance of the tested fed-batch strategies. Moreover, many of them were based on monitoring and controlling methanol concentration to avoid its accumulation to inhibitory values in the broth [26].

Among the proposed fed-batch strategies, the repeated fed-batch presents several advantages. This type of fermentation process consists in a cyclic fed-batch operation, where, at the end of each cycle, a defined volume of the fermentation broth is purged from the reactor and replaced by fresh medium. The broth left inside the reactor serves as inoculum for the following cycle [25,27]. Operation under a repeated fed-batch mode joins in the same fermentation process the advantages of the conventional fed-batch with those of continuous fermentation. It allows for achieving higher product productivities by keeping the culture's viability for longer operation periods, so that high cell density and high product production is maintained along this multi-cycle operation [14,27]. Moreover, it avoids the need for reactor cleaning

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and sterilization steps between fed-batch cycles [25]. This cultivation strategy was successfully used for the production of Lipase A [22] and rGuamerin [18] by *P. pastoris* strains X33 and GS115, respectively, wherein high cell densities were achieved (120–160 g/L) during 10–11 consecutive fed-batch cycles.

In previous work, *K. pastoris* was cultivated under a fed-batch mode with and exponential feeding profile for the production of CGC [7]. In this work, alternative fed-batch strategies were tested for the first time aiming to improve the process in terms of CGC production and productivity. The tested cultivation strategies included the DO-stat mode, feeding with a predefined profile and the repeated fed-batch operation. The produced copolymers were extracted and characterized to evaluate the impact of each cultivation strategy on CGC content in the biomass and its composition.

2. Materials and methods

2.1. Yeast strain and culture medium

In all experiments, *Komagataella pastoris* strain DSM 70877 was cultivated in standard Basal Salts Medium (BSM) (Pichia Fermentation Process Guidelines, Invitrogen), as described by Roca et al. [7]. BSM was supplemented with glycerol (86–88 wt%) to give a concentration of ≈50 g/L. Ammonium hydroxide (25%, v/v) was used to set the medium's pH to 5.0, serving also as the nitrogen source.

2.2. Fed-batch bioreactor cultivation

For the bioreactor experiments, inocula were prepared by inoculating 2 mL of the cryopreserved culture in 300 mL BSM supplemented with 50 g/L glycerol and incubating during 40 h, at 30 °C and 200 rpm. The broth thus obtained was used to inoculate a 5 L bioreactor (BioStat B-plus, Sartorius) with an initial working volume of 3 L. All experiments were performed with controlled temperature at 30 °C. The pH was controlled at 5.0 by the automatic addition of a 25% (v/v) ammonium hydroxide solution. The air flow rate was kept constant at 3 SLPM (standard liters per minute). The dissolved oxygen (DO) concentration was controlled at 50% of the air saturation by an automatic cascade comprising the variation of the stirring rate (300–2000 rpm) and supplementation of the air stream with pure oxygen that was triggered when the maximum stirring rate was not enough to maintain the DO concentration at the set point.

All experiments comprised an initial 24 h batch phase, followed by the fed-batch phase wherein the feeding solution was supplied to the culture. The feeding solution was composed of glycerol (86–88 wt%) supplemented with PTMs (*Pichia* trace mineral solution) (24 mL per liter of glycerol). Online data acquisition was performed using BioCTR software developed by Eusébio [28]. Samples (≈25 mL) were periodically withdrawn from the bioreactor for determination of the dry cell weight (DCW), glycerol and ammonium concentration, CGC content in the biomass and polymer composition.

In experiment A (DO-stat mode), the feeding flow rate was controlled as a function of DO concentration (under a constant stirring of 1300 rpm), i.e., when the DO concentration rose above 50%, the substrate was automatically fed to the bioreactor until the DO reached the set point again. In experiment B (predefined feeding profile), the feeding profile was set at the Biostat B-plus control unit, which automatically fed the reactor with substrate at a rate that gradually increased from 9.3 to 24 g/L h (considering the reactor's starting volume) over the 22.5 h of the fed-batch phase, giving an overall glycerol feeding of 1178 g.

2.3. Implementation of CGC production under repeated fed-batch mode

The repeated fed-batch cultivation of *K. pastoris* for CGC production comprised 7 cycles. The experiment was initiated by inoculating the bioreactor as described above for experiments A and B. The first cycle comprised a 24 h batch phase and a 23 h fed-batch phase wherein the feeding rate increased from 8 to 12 g/L h to give an overall glycerol feeding of 722 g. The subsequent 6 cycles took 23 h, with a 6 h batch phase and a 17 h fed-batch phase, with the feeding rate increasing from 14 to 16 g/L h (overall glycerol feed of 764–799 g). At the end of each cycle, 90% of the culture broth (≈3.5 L) were withdrawn from the reactor under aseptic conditions. The remaining broth (≈380 mL) was kept in the reactor, serving as inoculum for the following cycle. The new cycle was initiated by filling the bioreactor with 2.6 L of fresh BSM medium.

In all cycles, the reactor was operated as described above for experiments A and B in terms of temperature (30 °C), pH (5.0) and DO concentration (50%) control. Online data was acquired by the BioCTR software [28]. Samples (≈25 mL) were periodically withdrawn from the bioreactor for determination of the DCW, glycerol and ammonium concentration, CGC content in the biomass and polymer composition.

2.4. CGC extraction

For extraction of CGC from *K. pastoris*, dried biomass samples (≈100 mg) were treated with NaOH 5M (30 mL), at 65 °C, for 2 h. After cooling, the suspension was centrifuged (8000*g*, 10 min) and the alkaline insoluble material (AIM) was resuspended in deionized water (30 mL), neutralized with HCl 1M and centrifuged as described above. The AIM was further washed twice with deionized water (30 mL, for each wash) and, finally, the CGC samples were freeze dried. All extractions were performed in duplicate.

2.5. Analytical techniques

For the determination of the DCW, 5 mL culture broth samples were centrifuged at 8000*g* for 10 min. The cell-free supernatant was used for glycerol quantification, while the pellet was used for the gravimetric quantification of biomass concentration. The pellet was washed twice (resuspension in 5 mL deionized water and centrifuged at 8000*g* for 10 min) and freeze-dried. Three replicas were used for the quantification of DCW.

For glycerol quantification, the cell-free supernatant was analyzed by High Performance Liquid Chromatography (HPLC), in a Dionex ICS 3000 equipment with a Shodex RI-101 detector. The analysis was performed using an Aminex 87H Biorad column, at 30 °C with H_2SO_4 10 mN as eluent, at a flow rate of 0.6 mL/min. Glycerol (Scharlau) was used as standard, at concentrations of 0.005–1.0 g/L. The samples were diluted in order to have their concentration in glycerol below 1.0 g/L. Ammonia was quantified in the cell-free supernatant by segmented flow analysis (Skalar san++, Skalar Analytical, The Netherlands).

For the compositional analysis, dried CGC samples were subjected to two acid hydrolysis, as described by Farinha et al. [29]. Trifluoroacetic acid (TFA) was used to hydrolyze the β-glucan fraction into the constituent glucose monomers, while hydrochloric acid (HCl) was used to hydrolyze the chitin fraction of the copolymer that was quantified as glucosamine. Both hydrolysates were analyzed by HPLC, as described by Chagas et al. [10], using glucose and glucosamine (Sigma) as standards, at concentrations between 0.01 and 0.1 g/L. The standards were subjected to the same hydrolysis procedures as the samples.

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