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Morphology and rheological behaviour of *Yarrowia lipolytica*: Impact of dissolved oxygen level on cell growth and lipid composition

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ABSTRACT

Yarrowia lipolytica was investigated as potential ascomycetes yeast for higher lipid productivity. Moreover, lipogenesis seems to be very sensitive to oxygen variation and to achieve efficient microbial conversion of crude glycerol into energetic molecules (single cell oils, SCO) is a real challenge in the bioprocess. Dissolved oxygen (DO) was selected as a key parameter for translating results from shake flask to large scale for a successful bioprocess. Lipid production under different oxygenation rates was carried out to identify the ideal scenario required for higher accumulation rate. A combination of metabolomic, morphological and rheological profiling was investigated in the presence of crude glycerol as a carbon substrate. Results showed that cells require a dissolved oxygen concentration of 30% to achieve the highest lipid production of 44.8 ± 1.2% (w/w). The increase or decrease of oxygen level might affect the lipid content. However, cells exhibited the same rheological behaviour and presented unimodal distribution despite the oxygen variation. Furthermore, broth rheology was ultimately dependent on nutrient limitation and aeration. The variation of oxygen level (i.e. volumetric oxygen transfer coefficient (K_La) and oxygen uptake rate (OUR)) affects the biomass concentration, lipid accumulation and composition. These parameters played a crucial role in determining bioprocess performance.

1. Introduction

The increasing emphasis on sustainable and renewable energy and global warming has encouraged scientists to seek for other lipid-derived biofuels as potential substitutes of fossils fuels. In this regard, microbial oils have received a high interest owing to their advantages that include the high efficiency and the low land consumption [1,2]. *Yarrowia lipolytica,* a "non-conventional" oleaginous ascomycetous yeast, was reported to be an attractive cell factory for the production of single cell oils (SCO) because of its native lipid accumulation and easy engineering. In this regard, Blazeck et al. [3] have reported genotypic and phenotypic optimization in order to create *Yarrowia lipolytica* producer with significant lipogenesis capability (90% lipid content and titres exceeding 25 g/L lipids, which represents a 60-fold improvement over parental strain and conditions). The high titres and carbon-source independent nature of this lipogenesis in *Y. lipolytica* highlight the potential of this organism as a platform for efficient oleochemical production. Moreover, its robust growth on diverse variety of industrial by-products, such as raw glycerol and saturated fatty acids to achieve higher lipid productivity [4,5] and to produce other secondary metabolites [6,7], make *Y. lipolytica* a potential candidate for biotechnological process. Many factors were reported to have a major effect on lipid accumulation, such as nitrogen source, phosphorous, pH, temperature, as well as carbon to nitrogen ratio [8]. However, significant knowledge gaps regarding the key biological processes involved in relation to oxygen saturation are observed. The study of dissolved oxygen effect on lipid accumulation is not a new concept. Thus, Aiba et al. [9] have reported that oxygen limitation may affect the growth and biomass production [9]. Besides, Kamzolova et al. [10] have reported that oxygen requirements for growth and citric acid (CA) synthesis were depended from the iron concentration in the medium. Even at relatively low oxygen pressure (pO2 = 20%) and a high iron concentration (3.5 mg/L), CA consisted of 120 g/L [10]. Authors suggested that the application of iron-enriched media makes it possible to cultivate citrate producers at relatively low oxygen concentrations. This finding is of special importance, since oxygen supply is generally a limiting factor in industrial aerobic processes [10]. On the other side, an increase in concentration of pO_2 induced CA production in *Aspergillus niger* and *Candida lipolytica* Y1095, respectively [11,12]. *C. lipolytica*, anamorph (i.e. asexual state) of *Y. lipolytica* is reported to be among oleaginous microorganisms of the genera Candida, accumulating high titers of lipids as compared to Cryptococcus, Rhizopus, and Trichosporon [2]. Moreover, not only CA production, but also lipid productivity and profiles were reported to be highly affected by oxygen transfer

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(aeration and agitation). For instance, low level of dissolved oxygen (DO) was required for lipid accumulation in oleaginous species [13,14]. Besides, Yen et al. [15] observed that low level of DO enhances lipid accumulation, but inhibits cell growth whereas a higher level of DO enhances biomass accumulation rather than lipid accumulation. These observations confirmed the choice of two stage DO strategy to control biomass and lipid production [15,16].

As discussed below, the effect of DO on lipid accumulation of *Y. lipolytica* has not been conclusive so far and only few studies dealt with the effects of oxygen on lipid accumulation while using crude glycerol concentration. Besides, some changes of physical state of cells during cell culturing in bioreactor can occur. For example, physical parameters (aeration, mixing, heat and mass transfer) and microorganism physiology and activity are known to closely interact and evolve in a complex system [17]. So, understanding the physical mechanism involved in lipogenesis will guide the easy transition from downstream process to scale up and increase the lipid accumulation task. Besides, the study of the morphogenesis and the rheological behaviour of culture will provide useful information about the dynamic biological processes during the transition from biomass production to lipid accumulation. Taken together, understanding these parameters is determinant to drive cell culture up to a defined goal (biomass production, extra or intra cellular metabolite production, substrate biodegradation, etc.) and to optimize bioprocess [18,19]. The present investigation is a part of a work aiming to study biological response (growth rate, substrate assimilation, lipid accumulation) and physical properties (rheology) of *Y. lipolytica* under controlled operating conditions using crude glycerol as carbon source. A two-stage DO controlled strategy was also conducted to improve the cell growth during exponential growth, later, the variation of oxygen concentration and its effect on lipid production and rheology of substrate was evaluated. Another aspect investigated was the impact of DO on the scaling up of process during the lipid accumulation.

2. Material and methods

2.1. Microorganism and medium

Y. lipolytica SM7, having gene bank accession (KF908251) has been newly isolated and was used in the present study for its capacity to produce lipids in crude glycerol based media [7,16]. The strain was grown on yeast extract peptone dextrose agar (YPDA) medium containing (g/L) (20 bacto peptone, 10 yeast extract, 20 dextrose, 20 agar) at 28 °C for 2 days, maintained at 4 °C and sub-cultured every three months. The pre-culture was obtained by inoculating a separate colony of *Y. lipolytica* SM7 in yeast extract peptone dextrose (YPD) medium containing (g/L): Glucose 20, peptone 20 and yeast extract 10 and incubating it at 28 °C in a rotary shaker incubator, under agitation of 180 rpm for 24 h prior to cultivation.

2.2. Batch operation in fermenter with controlled dissolved oxygen

About 150 mL of seed medium was transferred into a 5-L stirred desk-top fermenter of 3 L working volume with 100 g/L of crude glycerol, 1 g/L yeast extract, 1.5 g/L $(NH_4)_2SO_4$, 3 g/L K_2HPO_4, 3 g/L NaH_2PO_4 H_2O, 0.5 g/L $MgSO_4$ $7H_2O$, 0.040 g/L $ZnSO_4$ $7H_2O$, 0.016 g/L $FeSO_4$ $7H_2O$, 0.25 µg/L biotin). pH was re-adjusted in all solutions by using NaOH and H_2SO_4 (4N). Crude glycerol was provided by Rothsay, Ontario, Canada, resulting from the transesterification of yellow grease and rendered animal fats. Its high composition of glycerol and low quantities of impurities such soap and salts makes it a potential source for lipid production. Its characterization was given below (g % (w/w): glycerol 83.5 + 1.5%, methanol 1.5 + 0.5%, moisture 8.83 + 1.5%).

The fermenter was operated at 28 °C with dissolved oxygen (DO) concentration controlled at 15–20%, 30–35%, and 40–60% of saturation, respectively. The fermenter used for lipid production has 5-liter capacity, with single-phase induction motor for running the agitator. The agitator has two blades and it rotates at a speed of 100–1500 rpm. Dissolved oxygen and pH were continuously monitored by means of a polarographic dissolved oxygen probe and of a pH sensor (Mettler-Toledo, USA), respectively. The agitation during the process was maintained above 35% saturation by adjusting agitation rate (300–500 rpm) and airflow rate (0.2–2 L/min). Fermentation pH was controlled automatically at 6.5 ± 0.1 through computer-controlled peristaltic pumps by the addition of pH control agents. Operating conditions were chosen according to previous results [16]. In the batch process, with DO controlled at 40–60% of saturation, supplemental pure oxygen in the inlet gas was provided to prevent the potential cell damage from the high shear force. In the batch operation with two-stage controlled DO, the DO was set at 60% of saturation for the first 24 h (in the exponential phase) and was adjusted after according to the experiments. The reason of providing higher DO at the beginning was to enhance the cell growth rate and accelerate biomass production.

2.3. Analytical procedures

2.3.1. Glycerol analysis and biomass measurement

For the measurement of yeast growth, dry biomass concentration was determined gravimetrically. For the measurement of glycerol and others organics acids in the broth, LC/MS/MS technique was employed. A Thermo TSQ Quantum model, equipped with an Electrospray Ionization (ESI) in negative ion mode; Zorbax Carbohydrate (4.6 mm × 150 mm; 5 mm, Agilent) analytical column; 75% acetonitrile; 0.1% NH_4OH; 25% water and 0.1% NH_4OH mobile phase and 10 mL injection volume, was used. Glycerol, CA and organics acids (all from Sigma) were used as the internal standards.

2.3.2. Lipid extraction and analysis

Extraction of lipids from lyophilized biomass was modified from the procedure of Bligh and Dyer's method [20]. 0.05 g of biomass was blended with 5 mL chloroform/methanol (2:1) and the mixture was agitated for 20 min in an orbital shaker at room temperature. The same protocol of extraction was repeated with methanol/chloroform (1:1, v/v). Resulted organic phases were, collected, mixed and washed twice with 0.88% (w/v) KCl solution for 10 min. Finally, lipids were recovered as dry material after the evaporation of the solvent at 40 ± 1 °C, until a constant weight. The fatty acids profile of the lipid was determined by methylation for conversion of fatty acids to fatty acid methyl esters (FAMEs) according to [21]. The FAMEs were analyzed using a Gas Chromatography linked to Mass Spectroscopy (GC–MS) (Perkin Elmer, Clarus 500). The dimensions of the column used were 30 m × 0.25 mm, with a phase thickness of 0.25 µm. The calibration curve was prepared with a mixture comprising 37 FAMEs (47885-U, 37 Component FAME Mix; Supelco, Bellefonte, PA, USA). 1,3-Dichlorobenzene was also used as an internal standard.

2.3.3. Volumetric oxygen transfer coefficient (KLa) oxygen uptake rate (OUR), and (OTR)

The dynamic gassing-out method was used for K_La measurements [9]. The DO control was momentarily stopped during the dynamic gassing-out method to facilitate correct K_La measurements. Oxygen uptake rates were obtained by measuring the slope of DO decrease during air-off of the dynamic gassing-out protocol.

2.3.4. Rheological measurements

Rheological analyses were carried out for fresh samples by using a rotational viscometer (DVII+, Brookfield) equipped with small sample adapter spindle (SC4 34, Brookfield). The viscosity data were examined by using the software Rheocalc V2.6 (Brookfield Engineering Labs 1999). The shear stress versus shear rate data was analyzed as per the