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A two-stage process facilitating microbial lipid production from *N*-acetylglucosamine by *Cryptococcus curvatus* cultured under non-sterile conditions

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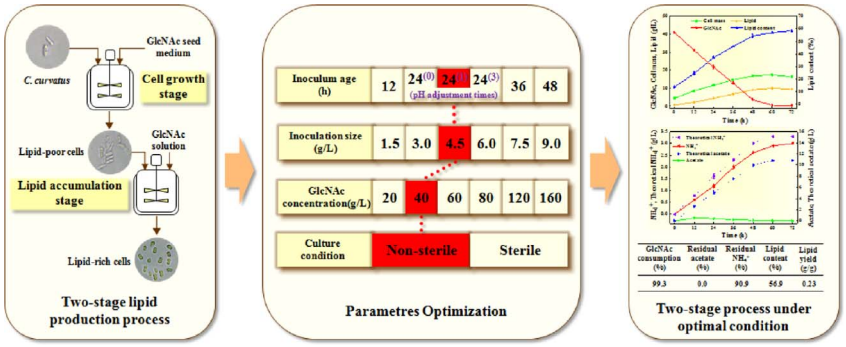
GRAPHICAL ABSTRACT



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ABSTRACT

N-acetylglucosamine (GlcNAc), the monomeric constituent of chitin, is rarely studied for lipid production by oleaginous species. This study demonstrated that *Cryptococcus curvatus* had a great capacity to convert GlcNAc into lipid with high yield using a two-stage production process. Optimal inoculum age and inoculation size strongly improved the two-stage lipid production efficiency. More interestingly, this process rendered superior lipid production under non-sterile condition. The acetate liberated from GlcNAc was consumed timely, while the NH_4^+ released was rarely assimilated. Lipid titre, lipid content and lipid yield reached 9.9 g/L, 56.9% and 0.23 g/g, respectively, which were significantly higher than those from the conventional process where cell growth and lipid accumulation were coupled. The resulting lipid samples had similar fatty acid compositional profiles to those of vegetable oil, suggesting their potential for biodiesel production. These findings strongly supported the two-stage process as an attractive strategy for better techno-economics of the chitin-to-biodiesel routes.

1. Introduction

Microbial lipids have been widely recognized as promising substitutes for high value-added exotic fats such as cocoa butter equivalents and polyunsaturated fatty acids (PUFA) and perfect precursor for biodiesel industries (Papanikolaou and Aggelis, 2011b). However, commercialization remains challenging due to the high lipid production costs. To reduce the cost, considerable efforts have been devoted to exploring low-cost substrates, e.g., lignocelluloses, biodiesel-derived glycerol, waste water, and volatile fatty acids (VFAs), for lipid

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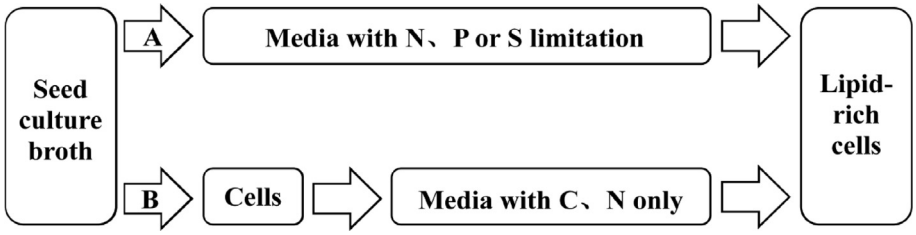

Fig. 1. Lipid production by oleaginous species based on conventional fermentation process (path A) and two-stage process (path B).

production (Qin et al., 2017).

Chitin, widely distributed in the biosphere as the integument of various crustaceans, is one of the most abundant renewable resources. *N*-acetylglucosamine (GlcNAc), the monomeric constituent of chitin, has been demonstrated as viable carbon and nitrogen sources for lipid production by several oleaginous yeasts including *Cryptococcus curvatus*, *Cryptococcus albidus*, *Trichosporon cutaneum*, and *Trichosporon fermentans* (Wu et al., 2008, 2010a; Zhang et al., 2011). However, the use of GlcNAc comes with some disadvantages such as relatively low lipid yield and productivity, difficulty of substrate metabolism at the late stage of culture, and discharge of wastewater with high concentration of ammonia (Wu et al., 2010a). The excess nitrogen from GlcNAc disfavors lipid accumulation, as lipid production is generally triggered by nitrogen starvation (Ratledge and Wynn, 2002). Enhancing the substrate consumption and improving lipid yield are critical for better techno-economics of microbial lipid technology.

Conventional lipid production by oleaginous species is generally considered as partially growth-coupled. That is, in such a fermentation process (Fig. 1, path A), nutrient composition is designed to support cell propagation and to deplete the essential nutrient such as nitrogen or phosphorus for lipid accumulation (Papanikolaou and Aggelis, 2011a; Wu et al., 2010b). When cell propagation and lipid accumulation processes are integrated, neither of them reaches the maximal capacity, reflecting the dilemma of different nutritional demand between them. Two-stage process (Fig. 1, path B), with cell growth and lipid accumulation process spatially separated, has been widely used to advance lipid production by oleaginous species. For example, the two-stage process has been described for lipid production using glucose, xylose or glycerol solution without auxiliary nutrients (Lin et al., 2011, 2014; Xu et al., 2017; Yang et al., 2014). Interestingly, this strategy has also been demonstrated for efficient conversion of lignocelluloses into lipids by *C. curvatus* (Gong et al., 2013). In the second stage of the process, high cell density and inhibited cell propagation from nutrients deficiency lead to enhanced lipid yield and productivity.

High lipid production entails both high cell density and high lipid content. Thus, it is crucial for balanced cell propagation and lipid biosynthesis. Two-stage lipid production process is uniquely suited for enhancing lipid production efficiency by separately optimizing the cell proliferation and lipid accumulation stage. Since cell growth can be easily achieved by using nutrient-rich media, efforts should be devoted to exploring the lipid accumulation behavior in the second stage. Inoculum age, inoculation size and initial GlcNAc concentration should play important roles on lipid production (Xu et al., 2017).

C. curvatus is a promising lipid producer with a broad spectrum of substrates including acetate and GlcNAc (Gong et al., 2015; Liu et al., 2017; Wu et al., 2010a,b). This study aims to propose a two-stage process to effectively convert GlcNAc into lipids by *C. curvatus* for the first time. Specifically, cells were firstly cultivated in a nutrient-rich medium to support cell proliferation. Then wet cells were collected, and inoculated at proper dosage for lipid production in GlcNAc solution under non-sterile condition for advanced lipid production. Important influential factors such as inoculum age, inoculation size and initial GlcNAc concentration were investigated. This two-stage process rendered significantly higher lipid content, yield and productivity than those obtained from the conventional process. Therefore, the two-stage process can potentially serve as an attractive strategy for lipid production from substrates rich in carbon and nitrogen.

2. Materials and methods

2.1. Materials, strain and media

C. curvatus ATCC 20509 was obtained from the American Type Culture Collection, stored at 4 °C and propagated every two weeks on yeast peptone dextrose (YPD) agar slants (yeast extract 10 g/L, peptone 10 g/L, glucose 20 g/L, agar 15 g/L, pH 6.0). GlcNAc was analytical grade (≥98%) and obtained from Shanghai Aladdin Reagent Co., Ltd. (Shanghai, China). Other reagents were analytical grade and purchased from local companies.

GlcNAc fermentation medium contained (g/L): GlcNAc 40, yeast extract 0.1, KH_2PO_4 2.7, $Na_2HPO_4{\cdot}12H_2O$ 2.4, $MgSO_4{\cdot}7H_2O$ 0.5, EDTA 0.1, and trace element solution 1% (v/v). Initial pH was adjusted to 5.5 with 4 M HCl. The composition of the trace element solution contained (g/L): $CaCl_2{\cdot}2H_2O$ 4.0, $FeSO_4{\cdot}7H_2O$ 0.55, citric acid·H_2O 0.52, $ZnSO_4{\cdot}7H_2O$ 0.10, $MnSO_4{\cdot}H_2O$ 0.076 and 100 μL of 18 M H_2SO_4 (Meesters et al., 1996). The media were sterilized by autoclaving at 121 °C for 20 min before use.

2.2. General procedure for conventional culture process

Pre-cultures were first grown in 50 mL GlcNAc seed medium (yeast extract 10 g/L, peptone 20 g/L, GlcNAc 16 g/L, pH 5.5) at 30 °C for 24 h unless otherwise stated. Cultures were initiated by adding 5 mL of preculture to 45 mL of the GlcNAc fermentation media in 250 mL unbaffled conical flasks, and incubated at a rotary rate of 200 rpm at 30 °C. Unless otherwise specified, the incubation and shaking procedures were carried out at a ZWY-2102 thermostatic culture oscillator purchased from Shanghai Zhicheng Analytical Instrument Manufacturing Co., Ltd. (Shanghai, China). Cultivation pH was adjusted to 5.5 with 4 M HCl in 12 h time intervals. Experiments were done in triplicates and data were presented as mean value ± standard deviation.

2.3. General procedures for two-stage culture process

For a typical two-stage culture process, *C. curvatus* cells were first incubated in 50 mL GlcNAc seed medium at 200 rpm, 30 °C for 24 h. Wet cells were harvested by centrifugation at 8000*g* for 5 min and washed twice with sterile water to remove residual nutrients. Then, 4.5 g/L of dry cell weight (DCW) equivalent wet yeast cells were resuspended in 50 mL of sterile GlcNAc solution (40 g/L) in 250 mL unbaffled conical flasks, and incubated at a rotary rate of 200 rpm at 30 °C. Cultivation pH was adjusted to 5.5 with 4 M HCl in 12 h time intervals. The water and the GlcNAc solution were sterilized by autoclaving at 121 °C for 20 min before use.

For the two-stage culture process under non-sterile conditions, GlcNAc solutions and water were not sterilized and all the operation procedures were performed in non-aseptic environment. All the experiments were done in triplicates and data were presented as mean value ± standard deviation.