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The pH-based fed-batch for lipid production from *Trichosporon oleaginosus* with crude glycerol

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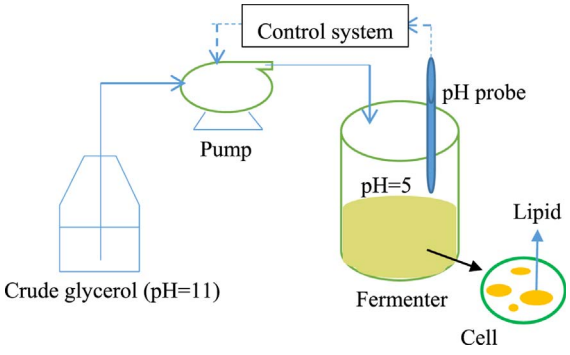
GRAPHICAL ABSTRACT



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ABSTRACT

In this study, it was found that the optimal pH for the growth of *Trichosporon oleaginosus* was related to the fermentation medium. A neutral or weak acid pH condition was optimal for the growth of *Trichosporon oleaginosus* in the extract-peptone-dextrose and wastewater sludge medium. Significant inhibition was observed at neutral pH in the wastewater sludge + crude glycerol medium due to the high soap content of the crude glycerol. By converting the soap to free fatty acid (FFA) at pH 5, the soap inhibition could be prevented. Fed-batch fermentation was employed to produce lipid from *Trichosporon oleaginosus* at pH 5 controlled by feeding crude glycerol. A remarkably high biomass (65.63 g/L) and lipid (35.79 g/L) concentration were achieved from the pH-based fed-batch fermentation in this study.

1. Introduction

For nearly a century, researchers have been exploring the opportunities to produce oil using microorganisms. The microorganisms, which contain more than 20% oil of the total dry biomass weight, are defined as oleaginous microorganisms (Liang and Jiang, 2013).

Oleaginous yeasts, such as *Trichosporon oleaginosus* (previously named as *Cryptococcus curvatus*), *Lipomyces starkeyi and Yarrowia lipolytica*, are widely studied to produce microbial oil (lipid), due to their capacity to accumulate high lipid content (Sitepu et al., 2014; Zhang et al., 2014a). Nowadays, the lipid production cost is high which is mainly from the utilization of the high-grade fermentation substrate (Cho and Park, 2018). In order to reduce the lipid production cost, organic wastes (low or free of cost), such as crude glycerol and wastewater sludge, are investigated to replace high-cost substrate for oleaginous yeast fermentation (Zhang et al., 2014a; Cortes and de

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Carvalho, 2015). Even though utilization of organic wastes as raw materials can reduce the lipid production cost, inhibition of impurities on the growth of oleaginous yeast and lipid production was observed (Xu et al., 2015; Yen and Chang, 2015; Yen et al., 2015). To enhance the cell growth and lipid production of oleaginous yeast from organic wastes, the optimization of fermentation parameters was generally performed (Sitepu et al., 2013; Tanimura et al., 2014; Chang et al., 2015).

The fermentation parameters, which affect the cell growth and lipid production, are C/N ratio, temperature, and pH (Zhang and Jahng, 2012; Nagano et al., 2013; Espinosa-Gonzalez et al., 2014). Among all, pH was normally controlled in an optimal range during the fermentation because either lower or higher pH caused a remarkable decrease in lipid production (Cappai et al., 2014; Nguyen et al., 2014; Alfè et al., 2015; Fumasoli et al., 2015). However, it is found that the optimal pH for a lipid-producing strain (such as oleaginous yeast *Trichosporon Oleaginosus*) varied with the change of the fermentation medium (Zhu et al., 2008; Cui et al., 2012; Yu et al., 2014).

For high lipid production, fed-batch fermentation is proved to be superior to the batch fermentation. So far, the highest biomass concentration of 185 (g/L), the highest lipid content of 76% (w/w) and the highest lipid productivity of 1 (g/L/h) were obtained in fed-batch fermentations (Koutinas et al., 2014). Fed-batch fermentation is a biotechnological process that substrates are fed to the reactor through multiple steps. To achieve a good performance of fed-batch fermentation, the feeding strategy is essential and important. The feeding strategy can be based on time, pH, DO (dissolved oxygen) or limiting substrate's dilution rate (Zhang et al., 2011; Chen et al., 2017; Farinha et al., 2017). Our previous research shows that fed-batch based on time could highly increase the lipid production (Chen et al., 2017). However, the operation of the time-based fed-batch fermentation was complicating and the substrate for the feed was not accurate (Chen et al., 2017). Therefore, an easier but more reliable feeding strategy should be investigated.

In this study, the optimal pH for oleaginous yeast *T. oleaginosus* was investigated and discussed with respect to different media. A pH-based fed-batch fermentation was designed and operated to enhance lipid production.

2. Materials and methods

2.1. Materials

2.1.1. The strain

Trichosporon oleaginosus (ATCC 20905) was employed as the lipid producing strain in this study.

2.1.2. The pre-culture and mediums

2.1.2.1. The pre-culture and YPD medium. The pre-culture medium was prepared from the yeast extract-peptone-dextrose (YPD) (50 g/L). The optimal pH was investigated with YPD medium as well.

2.1.2.2. Wastewater sludge medium. Studies have revealed that municipal secondary wastewater sludge was better for *T. oleaginosus* cultivation compared to the primary and mixed sludge (Zhang et al., 2014a). Hence, secondary wastewater sludge collected from a municipal wastewater treatment plant Communauté Urbain de Québec (CUQ), Québec, Canada was utilized in this study. The characteristics of the secondary wastewater sludge were presented in the previous study accomplished in our lab (Zhang et al., 2014b). After collection, the sludge was allowed to settle at 4 °C for 24 h. The supernatant was withdrawn. The suspended solids (SS) concentration of the resulting sludge solution was around 20 g/L. Previous studies in our lab found that SS concentration of 30 g/L was optimal for *T. oleaginosus* cultivation (Zhang et al., 2014a). To obtain SS concentration of 30 g/L, part of the resulting sludge was centrifuged to concentrate the sludge. The concentrated sludge was then mixed with the resulting sludge to obtain the SS concentration of 33 g/L. The sludge (SS 30 g/L) after sterilization at 121 °C for 30 min was cooled down to the room temperature and inoculated with 10% of pre-culture to make the final SS of 30 g/L.

2.1.2.3. Wastewater sludge and crude glycerol medium. The crude glycerol solution used in this study was obtained from a biodiesel production industry in Quebec, Canada. The characteristic of the crude glycerol was done following the methods presented in our previous study (Chen et al., 2017). The crude glycerol solution contained 31.14 ± 1.22 (% w/v) of methanol, 26.80 ± 1.35 (% w/w) of soap, 15.05 ± 0.39 (% w/w) of glycerol and other minor components.

The 200 mL pre-concentrated sludge with SS concentration of 37.5 g/L (same method as above) was mixed with 25 mL methanol free crude glycerol solution (methanol evaporated) to prepare 225 mL sludge and crude glycerol medium. After sterilization and cooling down to the room temperature, 25 mL pre-culture was inoculated into the sludge and crude glycerol medium. After inoculation, the concentration of SS, glycerol and soap of the medium was 30 g/L, 20.50 g/L and 36.50 g/L, respectively.

2.1.2.4. Crude glycerol synthetic medium. To prepare one-liter synthetic medium, the chemicals of 5.4 g KH_2PO_4, 1.9 g Na_2HPO_4, 3.232 g NH_4Cl, 0.1 g EDTA, 0.2 g $MgSO_4{\cdot}7H_2O$, 0.8 g peptone, 0.04 g $CaCl_2{\cdot}2H_2O$, 0.0055 g $FeSO_4{\cdot}7H_2O$, 0.001 g $ZnSO_4{\cdot}7H_2O$, and 0.00076 g $MnSO_4{\cdot}H_2O$ were used. The amount of crude glycerol solution varied according to the required carbon concentration. The detailed information was described below.

2.2. Methods

2.2.1. The primary screening of the optimal pH of T. oleaginosus growth

Nine shake flasks (each volume of 1 L) were filled with 250 mL YPD medium. After sterilizing and cooling down, the pH was adjusted to 3.5, 4.5, 5.5, 6.0, 6.5, 7.0, 7.5, 8.0 and 9.0 with 4 M H_2SO_4 solution or 4 M NaOH solution, respectively, in the laminar hood under aseptic condition. After pH adjustment, they were inoculated with a loopful of *T. oleaginosus* culture and then incubated at 30 °C and 180 rpm for 24 h. The Colony-Forming Units (CFU) of *T. oleaginosus* was determined after the incubation.

2.2.2. Verification of the optimal pH range for the cell growth of T. oleaginosus in wastewater sludge medium

Five shake flasks (each volume of 1 L) were filled with 225 mL of sludge with SS of 33 g/L. They were sterilized at 121 °C for 15 min. After cooling down, the pH of the sludge mediums was adjusted to 5.0, 5.5, 6.0, 6.5 and 7.0 with 4 M H_2SO_4 solution or 4 M NaOH solution, respectively, in the laminar hood under aseptic condition. The 25 mL of *T. oleaginosus* pre-culture with the corresponding pH was inoculated to the pH adjusted sludge medium and then incubated at 30 °C and 180 rpm, for instance. The pre-culture cultivated at pH 5.0 was inoculated to the sludge medium with pH 5. Samples were taken at every 4 h, and the CFU of *T. oleaginosus* was determined.

2.2.3. Verification of the optimal pH range for the cell growth of T. oleaginosus in the medium of wastewater sludge fortified with crude glycerol

The wastewater sludge was pre-treated using the method described in Section 2.2.2 to obtain the required SS concentration of 37.5 g/L. Three flasks with the capacity of 1 L were filled with 200 mL sludge (SS = 37.5 g/L) and 25 mL methanol free crude glycerol solution (glycerol concentration = 20.5 g/L, soap concentration = 36.5 g/L). After being sterilized and cooling, the pH of the sludge and crude glycerol media was adjusted to 5.0, 6.0, and 7.0 in a laminar hood under aseptic condition. The other operation steps in the wastewater sludge and crude glycerol media were similar to those in the sludge media (Section

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