Bioresource Technology 192 (2015) 718–725

Contents lists available at ScienceDirect

Bioresource Technology

journal homepage: www.elsevier.com/locate/biortech

Strategies of preserving lipids in microorganism after fermentation

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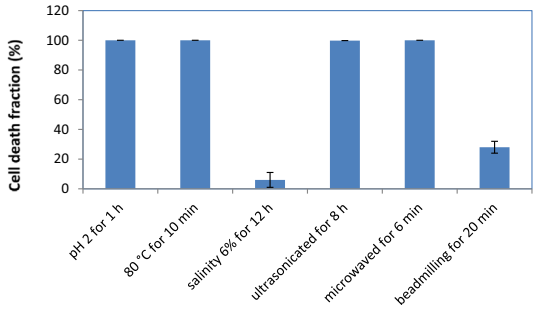
HIGHLIGHTS

- Treatments used to inactivate *Trichosporon oleaginosus* cells.
- 100% cells were inactivated after 1 h at pH 1 and 10 min at 80 °C.
- 100% cells were inactivated after 8 min ultrasonication and 6 min microwave.
- Treatments had no impact on lipid profile.

GRAPHICAL ABSTRACT



ARTICLE INFO

Article history:
Received 24 March 2015
Received in revised form 10 June 2015
Accepted 11 June 2015
Available online 17 June 2015

Keywords:
Microorganism
Lipid preservation
Cell death
Treatments
Biodiesel

ABSTRACT

Microbial oil is accumulated by microorganisms as stored energy during cultivation, and will be degraded to generate energy when they are not able to obtain external energy source. As the lipid is the desired production, the *in situ* degradation of oil by microorganisms after fermentation or during downstream processing has to be prevented. This study investigated the effect of pH, thermal, salinity, bead milling, ultrasonication, and microwave treatment on the viability of *Trichosporon oleaginosus* when it was used for lipid production. The cells in broth were completely inactivated with the treatment of pH 1 and 2 for 1 h, temperature 80 °C for 10 min, ultrasonication for 8 min, and microwave for 6 min, respectively. It was observed that these treatments had no impact on final product (biodiesel) composition and were considered as safe and efficient methods to preserve lipid in cells.

1. Introduction

Biodiesel is produced by transesterification of vegetable oil and animal fat. The process becomes more and more unaffordable due to the increasing price of these oil and fat. Microbial lipid as replacement has grabbed significant attention due to several advantages for biodiesel production: rapid growth rate of oleaginous microorganisms; no arable land requirement for growth; high lipid accumulation capacity (up to 70% w/w dry cells); and controllable cultivation conditions (Arumugam and Ponnusami, 2014; Ryu et al., 2013; Sung et al., 2014). There are mainly four steps in conversion of microbial lipid to biodiesel, which are microbe cultivation, harvesting, lipid extraction, and transesterification. In microbe cultivation step, the goal is to provide an optimal condition for the microbes to accumulate the highest lipid amount in their body. After microbe cultivation is over, the biomass is concentrated before sending for lipid extraction. In lab scale studies, the fermented broth can be immediately centrifuged and then directly subjected to lipid extraction. However, at industrial scale, there would be a time gap between the end of fermentation and lipid extraction due to the large volume cultivation broth and limitation of concentrating and extraction devices. In order to well utilize and minimize the purchasing cost of devices, normally the quantities of the devices were calculated based on their capacities and the time required to process the materials (Ríos et al., 2013; Richardson et al., 2012; Slade and Bauen, 2013). For example, when the volume of the broth to be centrifuged is 10,000 m^3 and the time

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http://dx.doi.org/10.1016/j.biortech.2015.06.053
0960-8524/

given for centrifugation is 48 h, the number of centrifuges would be 5 with each capacity of 50 m^3/h. In batch cultivation of oleaginous microbes, the time required to reach the highest lipid accumulation is around 48–96 h, which means that the next batch of fermentation broth would be ready only after 48–96 h (Ryu et al., 2013; Leiva-Candia et al., 2014; Tanimura et al., 2014). It suggested that 48–96 h would be considered as the time that harvesting equipment had to process each batch broth, which indicated that some fermentation broth would be standing for around 48–96 h before to be processed. However, the practise is normally to employ sufficient number of fermenters of specific sizes to operate in a staggered manner so that at any time, the fermented broth need not be held for 48 or 96 h. The holding time could be still up to several hours depending upon the downstream equipment availability in practice.

The microorganisms do not die right away after the cultivation is stopped. They would remain alive until there is no more energy source and oxygen available. In fact, lipid droplet is stored in microorganism cells as energy reserves. It indicates that there is a possibility of the microorganism would consume the lipid droplet when external food is exhausted or cannot be reached (microbes are too weak to transport food inside the cells). During cultivation, all the efforts have been made to maximize the lipid accumulation. If the lipid was finally decomposed to supply energy to cells, it would reduce the output of the final product (biodiesel).

This study was aimed to investigate if the lipid accumulated in the cells would be consumed (during harvesting the cells and extraction of lipids) and the consumption rate. The strategies to stop the lipid consumption were introduced. The impact of the strategies on the quality of the final product biodiesel was also studied.

2. Methods

2.1. Sludge collection

Municipal secondary wastewater sludge obtained from Communauté Urbain de Québec (CUQ), Québec, Canada, was concentrated by gravity settling at 4 °C for 24 h. The resulting solution had a suspended solids concentration (SS) around 23 g/L. The fermentation required a final SS concentration of 30 g/L. The final fermenter content contains 8.5 L sludge, 0.5 L crude glycerol solution and 1 L pre-culture grown in sludge medium (SS 30 g/L). It was found that in order to obtain 30 g/L SS in the fermenter the sludge solution (8.5 L) should contain a SS concentration of 32 g/L. To achieve the 32 g/L SS concentration, the sludge was centrifuged at 5000 rpm for 15 min.

2.2. Microorganism

Oleaginous yeast *Trichosporon oleaginosus* (ATCC 20509) was used in the study. It was maintained in malt extract agar plates at 4 °C.

2.3. Production of pre-culture (inoculum)

One loop full of *T. oleaginosus* was inoculated to a 4 L shake flask with 1 L sterilized sludge medium with SS concentration of 30 g/L (before sterilization) and 30 g/L yeast extract-peptone-dextrose. The fermentation was conducted in an incubator at 28 °C and 170 rpm.

2.4. Fermentation (lipid production)

The fermentation was performed in a 15 L fermenter equipped with accessories and programmable logic control (PLC) system for controlling dissolved oxygen (DO), pH, anti-foam, impeller speed, aeration rate, and temperature. The software (iFix 3.5, Intellution, USA) allowed automatic set-point control and integration of all parameters via PLC.

The fermentation volume was 10 L. 8.5 L sludge solution with SS 32 g/L was adjusted to pH 12 with 4 M NaOH and then transferred to the fermenter for sterilization. Before sterilization cycle, the polarographic pH-electrode (Mettler Toledo, USA) was calibrated using buffers of pH 4 and 7 (VWR, Canada). The oxygen probe was calibrated to zero (using sodium carbonate water) and 100% (air saturated water). Polypropylene glycol (PPG, Sigma-Canada) as an anti-foam agent was added to the sludge medium with concentration of 0.1% v/v (0.1 mL PPG in 99.9 mL medium). The fermenter with the medium was then sterilized *in situ* at 121 °C for 20 min. After the fermenter cooled down to 28 °C, DO probe was recalibrated to zero and 100% saturation by sparging N_2 gas and air, respectively, at agitation rate of 500 rpm. The pH of the fermenter solution was adjusted to 6.5 with 2 M H_2SO_4. Thereafter, 0.5 L crude glycerol (80% w/v) was transferred to the fermenter as carbon source under aseptic condition. Agitation was provided to mix the solution, after mixing, 1 L pre-culture of *T. oleaginosus* was added to the fermenter.

During fermentation, air flow rate was kept constant 1 L/min. Agitation rate were varied during fermentation in order to keep the DO above 30% saturation. The temperature was maintained at 28 °C by circulating water through the fermenter jacket. Fermentation pH was controlled automatically at 6.5 ± 0.1 through computer-controlled peristaltic pumps by the addition of pH control agents: 4 M NaOH and 4 M H_2SO_4. 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 samples were taken every 12 h. The fermentation was stopped at 48 h, which was determined based on previous study (the maximum lipid accumulation was observed at 48 h fermentation) (Zhang et al., 2014a). Thereafter, 6 L of the fermentation broth was immediately taken out of the fermenter to perform other experiments. The rest of the broth was kept in the fermenter without agitation and samples were taken every 1 h (the broth was mixed with agitator for 5 min before each sample was drawn). The samples were analysed to determine SS, cell concentration, and lipid content of the biomass.

To determine SS concentration, 10 mL of the broth was centrifuged and then washed two times with distilled water. The resulting solid was then transferred to an aluminum cup and dried at 105 °C to a constant weight. Then the SS was calculated by measuring the weight of the dried biomass. Cell concentration was estimated by counting colony-forming units (CFU). To measure the lipid content of the biomass, 30 mL of the broth was centrifuged and then washed with distilled water twice to remove the materials attached to the cell surface. Resulting solid was transferred to the bead milling container, which was filled with 6 mL Zirconia beads with 1 mm diameter and 30 mL of the mixture of chloroform and methanol (2:1 v/v). The solution was subjected to beads milling for 3 min followed by filtration. The filtered solids and beads were then transferred to the bead milling container and filled with 20 mL of the mixture of chloroform and methanol (1:1 v/v). The bead milling was performed again for 2 min. The solution was filtered and the filtrate (lipids in solvents) was mixed with the filtrate from the first filtration. Then the solvents were transferred to a pre-weighed glass tube and subjected to nitrogen gas evaporation until a constant weight was obtained. The lipid content was calculated as:

$$\text{Lipid content} = (\text{Wa} - \text{Wb})/(\text{SS} \times V)$$

where Wa is the weight of the glass tube after evaporation (g); Wb is the weight of the empty glass tube (g); SS is the suspended solids