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Process for biodiesel production from *Cryptococcus curvatus*

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ABSTRACT

The objective of the current report is process optimization for economical production of lipids by the well known oleaginous yeast *Cryptococcus curvatus* and conversion of the lipids to biodiesel. A high cell density fed-batch cultivation on low cost substrate viz. crude glycerol resulted in a dry biomass and oil yield of up to 69 g/L and 48% (w/w), respectively. The process was scaled up easily to 26 L. The oil extraction process was also optimized using environmentally safe solvents. The oil profile indicated a high oleic acid content followed by palmitic acid, stearic acid and linoleic acid. The oil was trans-esterified to biodiesel and thoroughly characterized. This is the first end to end report on production of biodiesel from the *C. curvatus* oil.

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1. Introduction

To qualify as an alternative fuel, the fuel should be environmentally safe, economical, scalable, sustainable based on life cycle analysis, and should provide a net energy gain over the sources used to produce it (Meng et al., 2009).

Biodiesel is an alternative energy form derived from renewable biomass by trans-esterification of triacyglycerols, yielding fatty acid methyl or ethyl esters. It contributes to the reduction of carbon dioxide and sulfur emissions to the atmosphere and has potential of being an environmentally benign solution for global warming, the energy crisis and depleted fossil fuel supplies (Meng et al., 2009). Biodiesel is compatible with current commercial diesel engines. Various sources of lipids including vegetable oils (soybean, palm, rapeseed), animal fats, used cooking oils have been used for production of biodiesel. However, one of the major drawbacks is the extremely high cost of the vegetable oil contributing to about 70–90% of the cost of biodiesel (Vicente et al., 2009). Besides, traditional oil-rich crops are limited by land availability, influenced by the climate and are in constant debate due to the food versus fuel issues. Therefore, there has been extensive research in exploring newer sources of oil.

One of the alternative ways to produce biodiesel in a green and sustainable manner, without competing with food crops, is to use microbes. Microbial oils, also called single cell oils, are produced by some oleaginous microorganisms, such as yeast, fungi, bacteria, and microalgae (Ma, 2006). Some of these microbes have the inherent ability to accumulate or store oil/lipid up to 60% of their dry weight, when grown under nitrogen-limited conditions. These lipids usually consist of 80–90% triacylglycerols with a fatty acid composition similar to many plant seed oils (Ratledge and Evans, 1984; Ykema et al., 1988).

It has been demonstrated that such microbial oils can be used as feedstock for biodiesel production (Ma, 2006). In comparison to other vegetable oils and animal fats, the production of microbial oil has many advantages: microbes have a short life cycle as compared to plants so the time to harvest is shorter, less labor is required, microbial oil production is less affected by venue, season and climate, and scale-up is easier (Li and Wang, 1997). Therefore, microbial oil has a tremendous potential to become one of the major oil feedstocks for biodiesel production in the future. Although not a new concept, work in this area has been very limited (Li et al., 2008).

Among the oleaginous yeasts, *Yarrowia, Candida, Rhodotorula, Rhodosporidium, Cryptococcus, Trichosporon* and *Lipomyces* have been studied for their microbial oil properties. *Cryptococcus curvatus*, one of the oleaginous yeasts, can accumulate large amounts of oil, up to 60% of the cell's dry weight (Ratledge, 1991), utilizing cheap carbon sources like whey permeate (Ykema, 1989) and other carbohydrate-rich agricultural or food processing wastes. The yeast oil produced by *C. curvatus* resembles plant seed oils like palm oil (Davies, 1988). The oil accumulated by the yeasts are in the form of triacylglycerols that are predominantly oleic (18:1), linoleic (18:2), stearic (18:0) palmitic (16:0) or palmitoleic acids (C16:1) acids (Meng et al., 2009). Recently,

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there has been intensive research on high cell density cultivation and improving lipid production in *Cryptococcus* and *Rhodosporidium* species (Li et al., 2007; Wu et al., 2011).

Currently, the production of yeast oil is more expensive than the production of vegetable oils. Therefore, single-cell oil fermentations will be economically feasible only when the oil can be produced with high added value. Accordingly, various process engineering approaches need to be used to yield a higher lipid production rate, a higher cellular lipid content, and higher biomass production, all geared to make the process more economically feasible. Different cultivation modes, including fed-batch and continuous fermentations, have been reported to increase the cell density of oleaginous microbes in culture. Hassan et al. (1993) optimized the growth of *Apiotrichum curvatum* in a continuous culture system using glucose and reached a lipid production rate of 0.42 g/L/h and lipid content of 31.9% (w/w). Pan et al. (1986) obtained a cell density of 185 g/L in an 84 h fed-batch culture of *Rhodotorula glutinis* aerated with oxygen-enriched air. However, there are no reports yet on a holistic approach towards optimization of the complete process starting from oil production to extraction to biodiesel conversion.

The current study is an attempt to address the entire process parameters from oil accumulation in yeast to biodiesel production to arrive at an economically feasible process.

2. Methods

2.1. Organism and growth medium

C. curvatus ATCC 20508 was maintained on MGYP medium (glucose, 10 g; peptone, 5 g; yeast extract, 5 g; malt extract, 3 g in 1 L of water, pH 5.5) at 28 °C. For seed inoculum, the yeast was grown in MGYP for 24 h and typically, 20% (v/v) was added to the production medium.

2.2. Analytical procedures

The yeast growth was monitored by measuring optical density (O.D.) at 600 nm and determining the dry weight at regular intervals. Residual glycerol was estimated by HPLC on an organic acid (Rezex) column with a refractive index detector and 0.005 mol/L H_2SO_4 as mobile phase at 0.5 mL/min flow rate (Hiremath et al., 2011). Total fatty acid composition was determined by GC (Agilent) on a SGE Forte column (Capillary Column BPX70, Length 60 m, id 0.25 mm, film 0.25 μm) using a FID detector (Li et al., 2007).

2.3. Fed-batch fermentation

Fed-batch fermentation was carried out in a 6 L bioreactor (Sartorius B-plus, Germany) at a working volume of 4.0 L. To grow in high cell density, *C. curvatus* was grown in medium containing crude glycerol (obtained from Kakinada biodiesel plant, Reliance Industries Ltd., India), 10 g/L; corn steep liquor (CSL), 20 g/L; baker's yeast autolysate, 5 g/L (Local bakery) and malt extract (HiMedia Labs, India), 2 g/L. The fermentor was inoculated with 20% (v/v) inoculum and maintained at 700 rpm agitation, 1 vvm aeration, pH 5.5 and 28 °C. Residual glycerol was monitored periodically and feed of crude glycerol was added when the glycerol was either consumed totally or was below 3 g/L. Each feed consisted of adding crude glycerol from an 80 g/L stock to a final concentration of 10 g/L. The process was scaled up to 26 L in a 30 L Bioflow-5000 reactor (New Brunswick). All other fermentation parameters were as described above except that the agitation was carried out at 380 rpm. Optimization experiments were carried out where baker's yeast autolysate was replaced by the deoiled yeast cake from previous fermentation batches and in another experiment, malt extract was eliminated completely. The effect of keeping the same composition for seed and production medium was also tested. All experiments were carried out at least thrice to ensure reproducibility.

2.4. Homogenization of cells

Direct homogenization of fermented broth was performed, thereby eliminating the step of centrifugation to collect cell mass. After harvesting, the fermented broth was subjected to homogenization at 4 °C under 800 bar pressure in a high pressure homogenizer (GEA, Italy) to break the cells. Five passages were taken and reduction in optical density was measured at various passages. Typically, the initial optical density of 190 was reduced to 71 after five passages.

2.5. Oil extraction

The oil extraction from yeast cells was carried out in 50 L SS agitator tank using a modified method described by Markham et al. (2006). Homogenized yeast cells and *n*-hexane were used at a ratio of 1:2. The homogenized cells (5 L) were mixed with 10 L *n*-hexane and stirred for 1 h at 950 rpm. The mixture was allowed to stabilize for 10–12 h at ambient temperature. The bottom aqueous layer was removed; isopropyl alcohol (IPA) (2.5 L) was added to separate the organic layer which was subsequently collected and concentrated to recover oil.

Jatropha oil was obtained by extracting crushed *Jatropha curcas* seeds with petroleum ether in a Soxhlet apparatus.

2.6. Refining of oil and transesterification

For biodiesel production, the microbial oil was subjected to free fatty acid measurement before the transesterification reaction. Free fatty acid levels are measured by titration with 0.1 mol/L KOH. To remove the impurities and free fatty acids; microbial oil was subjected to neutralization process (Bhattacharyya and Bhattacharyya, 1987). The neutralization process included degumming, free fatty acid removal and bleaching. Degumming is a process in which microbial oil was heated at 60 °C in presence of 0.1% of H_3PO_4 and 1% hot water (at a temperature of >90 °C). After heating for 1 h, the solution was allowed to settle at ambient temperature for 2 h and the gum was drained away. For free fatty acid removal the oil was heated to 70–75 °C in presence of NaOH with slow stirring. The solution was allowed to settle for 2 h at ambient temperature. The soap formed by the reaction was then drained away. Two to three hot water (at a temperature of >90 °C) washes were then performed to remove additional soap. The free fatty acid content, moisture and volatile matters were measured at each step of neutralization process. The moisture and volatile matter content is determined by taking 10 g (W) of oil sample in a petri dish, measuring the weight (W1) and heating to 105 ± 1 °C for 1 h in a hot air oven. The weight of the dish with the oil is again measured (W2) and moisture content is calculated by the formula $100 \times (W1 - W2)/W$. After free fatty acid removal as described above, the oil was dried under vacuum at 105 ± 1 °C for 1 h. For bleaching, oil after neutralization was treated with 1% SiO_2 at 100 °C under vacuum for 1 h. After this, the mixture was filtered to remove impurities and SiO_2 and refined microbial oil was collected. Trans-esterification was carried out by treating the refined microbial oil with 30% sodium methylate at 60–65 °C for 2 h. The lower glycerol layer was removed and crude biodiesel was subjected to hot water washes to remove soap and impurities. Characterization of microbial oil, refined microbial oil and microbial-based biodiesel was done by GC analysis.

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