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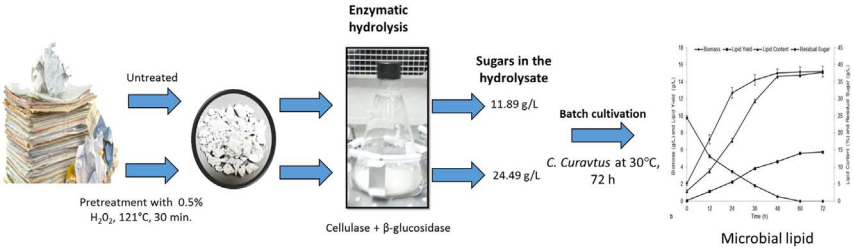
Full Length Article

Enhanced production of microbial lipids from waste office paper by the oleaginous yeast *Cryptococcus curvatus*

Neelamegam Annamalai[a,b], Nallusamy Sivakumar[a,*], Piotr Oleskowicz-Popiel[c]

[a] Department of Biology, College of Science, Sultan Qaboos University, PO Box 36, Muscat – 123, Oman
[b] Hawaii Natural Energy Institute, University of Hawaii at Manoa, 1680, East-West Road, Honolulu – 96822, HI, USA
[c] Institute of Environmental Engineering, Faculty of Civil and Environmental Engineering, Poznan University of Technology, Berdychowo 4, 60-965 Poznan, Poland

GRAPHICAL ABSTRACT



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ABSTRACT

Waste paper has a potential to serve as renewable feedstock for the biorefineries of fuels, chemicals and materials due to rich in cellulose and its abundance at low cost. In the present study, pretreated waste office paper (WOP) was enzymatically hydrolysed and used for lipid production by *Cryptococcus curvatus*. The results suggested that the WOP hydrolysate supplemented with ammonium sulphate (2 g/L) and yeast extract (0.5 g/L) as nitrogen source at a C/N ratio of 80 were the most suitable for high yield of lipids. The biomass, lipid yield, lipid content and lipid coefficient achieved from batch cultivation of *C. curvatus* using untreated and pretreated WOP hydrolysates were 6.32 and 15.20 g/L, 1.39 and 5.75 g/L, 22 and 37.8%, and 99.9 and 234.6 mg/g sugar with the productivity of 0.02 and 0.08 g/L/h, respectively. The fatty acid profile of the lipids indicated that the oleic acid was the major fatty acid followed by palmitic acid, stearic acid and linoleic acid which is quite similar to plant/vegetable oils. Thus, the results suggested that the waste office paper could be an alternative feedstock for production of microbial lipids for biodiesel.

1. Introduction

In recent years, there has been an increased production of biomass-derived biofuels to overcome negative environmental impact of fossil fuels and their future shortage [1]. Of which, biodiesel (fatty acid methyl esters, FAME) has received much attention due to its production from renewable resources and several environmental benefits [2–4]. Biodiesel derived from plant/vegetable oils can effectively decrease the emission of sulfur, carbon monoxide, polyaromatics, smoke during combustion process and no addition to the atmospheric carbon dioxide [5]. However, the high cost of these food crop feedstocks is the major problem for commercial production of biodiesel as it affects the continuous food supply chain and also leads to rise of food prices [6,7].

Oleaginous microorganisms that can accumulate 20–80% of their dry weight in the form of lipids (triacylglycerols) when grown under nitrogen-limited conditions are considered as an alternative raw

* Corresponding author.
E-mail addresses: annabact@gmail.com (N. Annamalai), apnsiva@squ.edu.om (N. Sivakumar).

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material for biodiesel production [8–10]. The microbial lipids or single cell oils have a similar fatty acid profile to that of oils from agricultural oil crops, which makes them generally suitable as a substitute for biodiesel [11,12]. However, the costs of microbial oil production are currently higher than those of vegetable oil due to the carbon sources which is estimated to be about 80% of the total medium cost, but there are many methods to drastically improve the techno-economics of microbial oil production processes [13,14].

Lignocellulosic biomass (agricultural, municipal and industrial wastes), which contains 55–65% carbohydrate is widely considered as a promising feedstock for biofuels due to its sustainability and abundance [13,15,16]. Microbial lipid production from lignocellulosic biomass is considered as a potential alternative feedstock for large-scale production of biodiesel at low cost and also to prevent shortage of feedstock [4,17,18]. Waste paper is considered as one of the major components of municipal and industrial solid wastes which account more than 35% of total lignocellulosic wastes [19]. Annually, more than 400 million tons of waste paper is generated and only about 50–65% is being recycled due to shortening of fibers during recycling which decreases the quality of paper [20]. For better recycling of waste paper, it would be beneficial to develop various techniques to convert waste paper to value added products such as ethanol, methane and biodiesel which reduces the feedstock related costs [21].

The yeast, *Cryptococcus curvatus* is a well-known and promising candidate to produce lipids, the precursors of hydrocarbons with high energy density, from cellulose [10,22]. The oleaginous yeast, *C. curvatus* has been selected for this study due to its broad substrate spectrum and also several studies have been reported on lipid production using various cheaper substrates such as glycerol, N-acetylglucosamine, wheat straw, sorghum bagasse and corn stover [4,18,23,24]. Hence, the present study was aimed to explore the possibility of microbial lipid production by *C. curvatus* using waste office paper as a feedstock. This work focuses on pretreatment of waste office paper, enzymatic hydrolysis and subsequent production of microbial lipids for biodiesel.

2. Materials and methods

2.1. Yeast strain, cultivation and materials

The oleaginous yeast strain, *C. curvatus* DSM 70022 was obtained from DMSZ (Germany) and propagated every two weeks on yeast peptone dextrose (YPD) agar slants (yeast extract 10; peptone 10; glucose 20; agar 15 (g/L), pH 6.0). Yeast inoculum was prepared in YPD liquid medium (pH 6.0). For production of lipids, the yeast *C. curvatus* was grown in fermentation medium [Yeast extract – 0.5; KH_2PO_4 – 0.8; K_2HPO_4 – 0.2; $(NH_4)_2SO_4$ – 2; $MgSO_4$ – 0.5 (g/L), (pH – 6.0)].

The chemicals and materials used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) or as indicated. Cellulase from *Trichoderma reesei* ATCC 26921 (C2730) and β-glucosidase from *Aspergillus niger* (49291) were purchased from Sigma-Aldrich (MO, USA). The activity of cellulase and β-glucosidase were 185 FPU/mL and 500 CBU/mL, respectively. The cellulase activity was determined by standard filter paper assay [25]. One unit of enzyme activity (FPU) is defined as the amount of enzyme required to liberate 1 μmol of glucose from filter paper in 60 min at 50 °C and pH 4.8. The β-glucosidase activity was determined by measuring the amount of *p*-nitrophenol released from *p*-nitrophenyl – β–D-glucopyranoside (*p*NPG) [26]. One unit of enzyme activity (CBU) is defined as the amount of enzyme required to produce 1 μmol of *p*-nitrophenol from *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG) per minute at 50 °C and pH 5.0.

2.2. Preparation and pretreatment of waste office paper

Waste office paper (WOP) collected from the local market was shredded into small pieces (2 × 6 mm) using a mechanical shredder (Atlas, China). The shredded papers were soaked in deionized water (5%, w/v), milled and dried at 60 °C for 24 h. The dry WOP was then milled again to homogenize the cellulose structure and used for further analysis.

For pretreatment, the WOP (5%, w/v) was mixed with 0.5% hydrogen peroxide and then autoclaved at 121 °C for 30 min. The solid residue was collected by centrifugation at 5000 × *g* for 10 min, washed repeatedly with deionized water till obtain neutral pH and then dried at 60 °C for 24 h. The dried materials were milled again and used for further enzymatic hydrolysis studies.

2.3. Enzymatic hydrolysis of WOP

The enzymatic hydrolysis was carried out in 500 mL hydrolysis flasks containing 3% (w/v) of WOP in 50 mM citrate buffer (pH 4.8), incubated at 50 °C with 150 rpm for 120 h with the enzyme loading of cellulase (37 FPU/g solid) and β-glucosidase (25 CBU/g solid). Samples were withdrawn at regular intervals of 24 h, centrifuged at 10,000 × *g* for 10 min and the supernatant was subjected to sugar analysis. The percentage of hydrolysis (%) was calculated based on the amount of glucan (cellulose) and xylan (xylose) in initial substrates and the sugars released from the hydrolysis.

2.4. Effect of nitrogen and C/N ratio on cell biomass and lipid production

To study the effect of various nitrogen sources on cell biomass and lipid production, the yeast *C. curvatus* was grown in 50 mL of fermentation medium (KH_2PO_4-0.8; K_2HPO_4-0.2 and $MgSO_4$-0.5 (g/L) (pH – 6.0) containing WOP hydrolysate supplemented with various organic and inorganic nitrogen sources (2 g/L) [yeast extract (10% N, w/w), peptone (14% N, w/w), $(NH_4)_2SO_4$ and NH_4Cl] and incubated at 30 °C with 160 rpm for 72 h. The effect of combined addition of organic (yeast extract and peptone) and inorganic ($(NH_4)_2SO_4$ and NH_4Cl) nitrogen was investigated at two different levels (0.5 and 2 g/L). The effect of different C/N ratio (40, 60, 80, 100 and 120) on cell mass production and lipid accumulation was investigated. The C/N ratio was maintained by adjusting the nitrogen source according to the total sugars in the hydrolysate.

2.5. Lipid production by batch cultivation using WOP hydrolysate

For lipid production, the experiment was carried out in 500 mL conical flasks with 100 mL fermentation medium [Yeast extract-0.2; KH_2PO_4-0.8; K_2HPO_4-0.2; $(NH_4)_2SO_4$-0.5 and $MgSO_4$-0.5 (g/L), (pH 6.0)] prepared with WOP hydrolysate (C/N:80). After filter sterilization, the flasks were inoculated with 1% preculture of *C. curvatus* grown in YPD liquid medium for 24 h at 30 °C (OD – 1.0) and incubated in a shaker incubator at 30 °C for 72 h at 160 rpm. The cell growth was monitored by measuring the optical density at 600 nm using spectrophotometer from the aliquots withdrawn at regular intervals (24 h). The cell mass was estimated gravimetrically by centrifuging the culture broth (5 mL) at 5000 × *g* for 10 min at 4 °C, washed with deionized water and dried at 105 °C for 24 h and expressed as cell dry weight (DCW, g/L). Residual sugar content in the medium was analyzed by HPLC-RID.

2.6. Analytical methods

The structural carbohydrates, acid soluble and insoluble lignin were determined by NREL/TP-510-42618 method [27]. The sugars (glucose and xylose), hydroxymethylfurfural (HMF) and furfural were analyzed by HPLC (Shimadzu; LC10AD) equipped with aminex HPX – 87H (Bio-Rad) column at 65 °C using 5 mM sulfuric acid as mobile phase (0.6 mL/min) with refractive index detector (Shimadzu; RID10A). The total phenolic content was determined by Folin – Ciocalteu method [28] with gallic acid as standard. All the experiments were performed in triplicate and the results are presented as mean ± standard deviation.