Bioresource Technology 129 (2013) 650–654

Contents lists available at SciVerse ScienceDirect

Bioresource Technology

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

Short Communication

Microbial oil produced from biodiesel by-products could enhance overall production

Esra Uçkun Kiran [a,b], Antoine Trzcinski [a], Colin Webb [a,*]

[a] School of Chemical Engineering and Analytical Science, University of Manchester, UK
[b] Middle East Technical University, Biotechnology Department, 06531 Ankara, Turkey

HIGHLIGHTS

- Microbial lipids were produced from biodiesel derived glycerol and rapeseed meal.
- Rapeseed meal biomedium is better than yeast extract for lipid accumulation.
- Fatty acid profiles of the lipids are excellent for biodiesel production.

ARTICLE INFO

Article history:
Received 18 May 2012
Received in revised form 17 November 2012
Accepted 20 November 2012
Available online 17 December 2012

Keywords:
Biodiesel
Microbial bio-oil
Rapeseed meal
Crude glycerol
Solid state fermentation

ABSTRACT

Glycerol and rapeseed meal, two major by-products of biodiesel production, have been tested for possible use as low-cost raw materials for the production of microbial bio-oil using the oleaginous yeast *Rhodosporidium toruloides*. Using fed-batch fermentation with crude glycerol and a novel nitrogen rich nutrient source derived from rapeseed meal as feed, it was shown that 13 g/L lipids could be produced, compared with 9.4 g/L when crude glycerol was used with yeast extract. When 100 g/L pure glycerol was used, the final lipid concentration was 19.7 g/L with the novel biomedium compared to 16.2 g/L for yeast extract. The novel biomedium also resulted in higher lipid yields (0.19 g lipid/g glycerol consumed compared to 0.12 g/L) suggesting it provides a better carbon to nitrogen balance for accumulating lipids. FAMEs produced from the microbial lipids indicated a high degree of unsaturation confirming that the fatty acids produced from the novel biomedium have potential for biodiesel production.

1. Introduction

Global production and use of biodiesel has increased dramatically in recent years and the fuel represents a promising alternative for use in compression-ignition (diesel) engines. It is generally produced by transesterification of vegetable oils such rapeseed oil. In the case of rapeseed, following extraction of the oil, a protein rich solid by-product, rapeseed meal, is generated, which is usually sold as organic fertilizer or animal feed. Global rapeseed production was 47 million tons in 2010 (FAO, 2010), resulting in the production of more than 25 million tons of rapeseed meal. However, the utilization of rapeseed meal as an animal feed is limited because it contains some anti-nutritional constituents such as phytic acid, erucic acid and fibre and precursors of toxic compounds such as glucosinolates and phenol (Koutinas et al., 2007). As a consequence, the continued expansion of the biodiesel industry is likely to result in the production of greater quantities of rapeseed meal than the current demand can justify. Alternative uses for this by-product would therefore be desirable.

Another by-product of the biodiesel industry is crude glycerol, a mixture of the glycerol produced during the transesterification process along with residual methanol and salts of the reaction catalyst (sodium or potassium hydroxide). This can be purified but refining crude glycerol to high purities is expensive and energy-intensive and, again, the market is limited. The utilization of crude glycerol directly, without refining, could help to make biodiesel production more profitable and sustainable. There are many reports of the biological conversion of crude glycerol to value-added products such as animal feeds (Nitayavardhana and Khanal, 2011), 1,3-propanediol (Chatzifragkou et al., 2011), succinic acid (Vlysidis et al., 2011), single cell oil (Papanikolaou and Aggelis, 2009; Saenge et al., 2011) and citric acid (Papanikolaou and Aggelis, 2009; Rymowicz et al., 2010). However, these studies have generally relied on the use of yeast extract or peptone as the nitrogen source to accompany the glycerol carbon source. Such materials are too expensive for use in a large biorefinery. In a sustainable, integrated biorefinery, it would make sense to utilise the available nitrogen source (rapeseed meal) alongside the crude glycerol for such

* Corresponding author. Tel.: +44 1613064379.
E-mail addresses: esrauckun@yahoo.com (E. Uçkun Kiran), antoinetrzcinski@hotmail.com (A. Trzcinski), colin.webb@manchester.ac.uk (C. Webb).

0960-8524/$ - see front matter © 2012 Elsevier Ltd. All rights reserved.
http://dx.doi.org/10.1016/j.biortech.2012.11.152

processes. Furthermore, it would be very attractive to be able to convert these into additional oil using an oleaginous microorganism, such as *Rhodosporidium toruloides*. The objective of this study was, therefore, to investigate the production of additional lipids using biorefinery derived rapeseed meal and glycerol.

2. Methods

2.1. Rapeseed meal

Rapeseed meal was kindly supplied by the Oilseeds Processing Division of Cargill Plc (Liverpool, UK). Its composition was determined and reported in a previous publication (Wang et al., 2010). The rapeseed meal was kept in an air-tight plastic container and stored at room temperature. Rapeseed meal proteins are not easily accessible for microorganisms and a pre-treatment is therefore required to obtain a suitable N source for the subsequent microbial oil fermentation (Wang et al., 2010). *Aspergillus oryzae,* an excellent protease producer was used to break down proteins present in the rapeseed meal into peptides and amino acids. Firstly, rapeseed meal was moistened with the required amount of tap water to obtain a 65% moisture content in a 1 L bottle then sterilized at 121 °C for 15 min. The meal was allowed to cool to room temperature before inoculating with approximately 10^6 spores of *A. oryzae*·g^{-1} meal. The content was mixed by stirring with a sterile aluminium rod and vigorous shaking. After mixing, approximately 10–13 g of content was distributed into each 9-cm Petri dish and incubated at 30 °C for 3 days. Following the fermentation, autolysis of the filamentous fungus was initiated by mixing the required amount of distilled water with the fermented solids to obtain approximately 55–60 g L^{-1} solid concentration. The content was homogenised using a kitchen blender then incubated at 55 °C for 3 days in a tightly capped bottle. Afterwards, the autolysate was centrifuged at 12,000*g* for 10 min and the supernatant was filtered through a 0.2 μm filter and kept at −20 °C until required.

2.2. Synthetic crude glycerol

In addition to real crude glycerol, obtained from a local biodiesel plant, a synthetic crude glycerol was also prepared based on the composition 65% glycerol, 27% water, 4% methanol and 4% salts (Gonzalez-Pajueloa et al., 2005). Chemicals were supplied by Sigma.

2.3. Microbial-oil production

The oleaginous yeast *R. toruloides* Y4, which has previously been shown by Li et al. (2007) to be capable of high cell density culture was kindly provided by Professor Zongbao (Kent) Zhao of the Dalian Institute of Chemical Physics in China and was used throughout this study. It was kept at 4 °C on Petri dishes containing 3 g/L malt extract, 10 g/L yeast extract, 10 g/L peptone, 10 g/L NaCl, 50 g/L glycerol and 15 g/L agar. Microbial-oil production was carried out using the solid state fermentation autolysate (SSFA) as the sole N source and pure or crude glycerol as the C source. In control experiments, yeast extract and pure glycerol were used as N and C sources, respectively.

The effect of initial glycerol concentration on yeast growth was investigated in flasks. For the inoculum preparation, *R toruloides* Y4 was grown for 3 days in 100 mL liquid medium composed of: 3 g/L malt extract (Sigma), 10 g/L yeast extract (Fisher), 10 g/L peptone (Oxoid), 10 g/L NaCl (Sigma) and 50 g/L glycerol (Sigma). For experiments in 500 mL Erlenmeyer flasks, the fermentation medium (100 mL) was prepared using 90 mL water containing different concentrations of pure or crude glycerol and the components above. The flasks were autoclaved at 121 °C for 20 min, and then inoculated aseptically with 10 mL of inoculum. A control without glycerol was also carried out in parallel. Fermentations were carried out in triplicate for 72 h at 30 °C on a 200 rpm rotary shaker.

For bioreactor (1 L, Electrolab) experiments, the fermentation medium was prepared using 900 mL filtered SSFA as nitrogen source and diluted to a Free Amino Nitrogen (FAN) concentration of 300 mg L^{-1}. The medium was supplemented with 0.215 g/L $(NH_4)_2SO_4$, 0.4 g/L KH_2PO_4, 1.5 g/L $MgSO_4.7H_2O$. Glycerol was autoclaved separately, and added at the beginning of the fermentation to obtain concentrations of 100 g/L and 50 g/L for pure and crude glycerol, respectively. The inoculum (100 mL) was transferred aseptically and the fermentation was performed at pH 6 and 30 °C. Samples were taken at regular intervals to follow the yeast growth, glycerol and FAN consumption. For batch fermentations the bioreactor was operated for 72 h, after which, for fed-batch mode, glycerol was added every 24 h in order to maintain a C/N ratio, in the range 70–90, for lipids accumulation.

2.4. Analytical methods

FAN concentration was analysed by the ninhydrin colorimetric method (Lie, 1973). Glycerol concentration was measured in triplicate using an Analox GL6 analyser (Analox, England). Fungal spores and yeast cells were quantified microscopically using a haemacytometer (Improved Neubauer, Weber England, Depth 0.1 mm, 1/400mm^2). For dry cell biomass determination, 5 mL fermentation broth was filtered through a 0.2 μm filter and dried at 60 °C overnight. Oil content of dried cells was determined by chloroform:methanol (1:1 v/v) extraction in a Soxtec-HT6 system (Höganäs, Sweden). The extraction time was 2 h at 140 °C, followed by 20 min of rinsing. Oil content was determined in triplicate.

Fatty acid analysis of lipids was conducted by gas chromatography (GC). For this, 1 mg of lipid was subjected for 10 h to methanolysis at 50 °C in the presence of 4 g/L NaOH with 1:20 biomass:methanol ratio. The resulting fatty acid methyl esters were analysed by GC on a Varian CP-3800 equipped with a DB-23 capillary column (60 m by 0.25 mm; film thickness of 150 nm) and a flame ionization detector (Agilent Technologies). A 2 μL portion of the organic phase was analysed after split injection (1:50); helium (constant flow of 0.2 ml min^{-1}) was used as a carrier gas. The temperatures of the injector and detector were 250 °C. The following temperature program was applied: 50 °C for 1 min, increase of 25 °C min^{-1} to 175 °C, increase of 4 °C min^{-1} to 230 °C, and 230 °C for 5 min. Substances were identified by comparison of their retention times with those of standard fatty acid methyl esters.

3. Results and discussion

Pure and crude glycerols were used, in separate sets of flask experiments, as the carbon source in order to determine the effect of glycerol concentration on *Rhodosporidium toruloides* Y4 growth. It can be seen from Fig. 1 that the optimum concentration for pure glycerol was 70 g/L while for growth on crude glycerol, it was 50 g/L. Beyond these concentrations growth started to decrease and above 100 g/L growth was markedly affected.

In 1 L bioreactor studies, the effect of agitation was investigated using an initial glycerol concentration of 100 g/L. Since *R. toruloides* Y4 is an obligate aerobe, it grows better in oxygen rich media. Hence, cell growth should be enhanced by using a high mixing speed. It was indeed observed that greater specific growth rates (up to 0.085 h^{-1}) were obtained when fermentations were carried out at high agitation speeds of up to 1200 rpm. The maximum