Fuel 194 (2017) 180–187

Contents lists available at ScienceDirect

Fuel

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

Full Length Article

# Improved microbial oil production from oil palm empty fruit bunch by *Mucor plumbeus*

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ARTICLE INFO

*Article history:*
Received 1 June 2016
Received in revised form 16 November 2016
Accepted 5 January 2017
Available online 10 January 2017

*Keywords:*
Biodiesel
Empty fruit bunch
Lignocellulose
Lipid
Microbial oil
Palm oil

ABSTRACT

This study investigated the effect of cultivation parameters on microbial oil production from hydrolysate of oil palm empty fruit bunch (EFB) using fungus *Mucor plumbeus*. The parameters selected for evaluation were sugar concentration (30–100 g/L), yeast extract concentration (0–13.3%, g yeast extract/g sugar), pH (5–7) and spore concentration (4.3–6.3, log spore number/mL medium). Response surface methodology was used to optimise the cultivation conditions which were based on the oil concentration and oil yield. Sugar concentration was the most influential parameter that affected oil concentration. However, the cultivation at high sugar concentration (~100 g/L) also resulted in ethanol accumulation. The optimum condition for oil yield was found at 30 g/L sugar, 0 g/L yeast extract and pH 5.0. Cultivation in 1 L bioreactor under the optimum conditions resulted in ~1.8-fold increase in oil yield compared to the shake-flask cultivation. Microbial oil produced from EFB hydrolysate has the potential to be used as the feedstock for biodiesel production from non-food feedstock, with cheaper cost of biodiesel production in comparison to glucose-derived microbial oil.



## 1. Introduction

Lignocellulosic biomass is an attractive feedstock for microbial oil production due to its high availability and low price. Microbial oil or lipid can be produced through biochemical conversion of lignocellulosic biomass, which typically involves pretreatment and enzymatic hydrolysis of lignocellulosic biomass for breaking down polysaccharides to hexoses and pentoses, that can be utilised by oleaginous microorganisms for oil production. Oils in the form of triacylglycerides (TAG) can be used as feedstocks for the production of the second generation biodiesel through transesterification process. In comparison to the first generation biodiesel derived from plant oils, the production of biodiesel from microbial oil has several advantages, including low requirement of land-use and labour, as well as higher oil productivity [1,2].

Oleaginous filamentous fungi are promising candidates for microbial oil production from lignocellulosic biomass due to their capacity to grow on a broad range of carbon substrates (*e.g.*, glucose, xylose, glycerol, etc.) [3,4], and their ability to tolerate low concentrations of growth inhibitors (e.g., furfural and 5-hydroxymethylfurfural (HMF)) resulting from chemical pretreatment of lignocellulosic biomass [5]. The morphology of filamentous fungi, either in pellet or filamentous hyphal form, allows simple filtration technique for down-steam processing [6,7].

Cultivation conditions play important roles in oil production. Carbon-to-nitrogen (C/N) ratio is possibly the most important factor as oleaginous microorganisms accumulate oil under limiting-nitrogen conditions [8,9]. Other cultivation conditions that have influence on microbial growth and oil production are cultivation pH [10], and inoculum concentration. pH of the cultivation medium may affect microbial cells' membrane permeability [11]. Spore inoculum concentration of fungi was reported to have an effect on fungal morphology and metabolic activity [12], which subsequently could influence oil accumulation. Despite the effect of a variety of cultivation conditions on oil production, studies with a systematic approach to process optimisation of oil production from lignocellulosic biomass by filamentous fungi are limited.

Oil palm empty fruit bunch (EFB), is the lignocellulosic by-product of palm oil processing and makes up the highest percentage of wastes generated in palm oil mills. In this study, the cultivation of the filamentous fungus, *Mucor plumbeus*, on EFB

*Abbreviations:* HMF, 5-hydroxymethylfurfural; C/N, carbon-to-nitrogen ratio; DO, dissolved oxygen; EFB, empty fruit bunch; EH, enzymatic hydrolysate; RMC, raw material cost; RSM, Response surface methodology.

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http://dx.doi.org/10.1016/j.fuel.2017.01.013
0016-2361/

hydrolysates for microbial oil production was optimised. *M. plumbeus* was shown to be the best candidate for oil production from EFB [13]. EFB hydrolysate was prepared through dilute acid pretreatment and enzymatic hydrolysis. Response surface methodology (RSM) was used to optimise the oil production by assessing the impact of the parameters of cultivation (sugar concentration, yeast extract concentration, spores concentration, and pH) on the oil concentration (g/L) and oil yield (mg oil per g sugars consumed). Oil yield is an important response parameter for the optimisation as it measures the efficiency of converting the carbon substrates (*i.e.*, an operating cost) to product (*i.e.*, a revenue) [3]. Subsequently, microbial oil production was scaled up into a bioreactor system to investigate the effect of reactor operation on microbial biomass and oil production. Studies of cultivation in the bioreactor systems are important for assessing the feasibility and the economics of progressing to industrial scales (*e.g.*, >1000 L). However, there are limited studies on microbial oil production from lignocellulosic hydrolysates in the bioreactor systems. Microbial oil from EFB could be a promising source for biodiesel production. The use of cheap feedstock like EFB could potentially improve the economics of microbial oil production, which is one of the challenges for commercialising biodiesel production.

## 2. Material and methods

### 2.1. Materials

Oil palm EFB was provided by KKS East Mill, Sime Darby Plantation Sdn. Bhd, Malaysia. Air-dried EFB consisted of 34.0% glucan, 17.2% xylan, 29.6% lignin, 7.5% moisture, 6.5% ash, 14.2% water extractive and 6.3% ethanol extractive based on compositional analysis procedure developed by National Renewable Energy Laboratory [14,15].

*Mucor plumbeus* (FRR no.: 2412) strain was purchased from FRR Culture Collection (Australia). The spores of fungal strain were maintained on potato dextrose agar (PDA) at 4 °C [2].

### 2.2. EFB hydrolysate preparation

EFB was pretreated at 170 °C with 0.8 wt% sulfuric acid and a solid/liquid ratio of 1:6 in 7.5 L Parr reactor (Model 4554, Parr Instrument Company, USA). The stirring speed was 100 rpm and the reaction time was 15 min. Following pretreatment, the liquid fraction and the solid residue were separated by filtration using Whatman filter paper (Grade 1, Whatman, England). The solid residue was washed twice with tap water.

Enzymatic hydrolysis of the washed EFB solid residue was performed at a glucan loading of 7% (w/w) with a cellulase dosage of 20 FPU/g glucan (Accelerase™ 1500, Batch no: 4901298419). The pH of the mixture was adjusted to 5.0 and the mixture was then placed in a shaking incubator (OM15, Ratek, Australia) for 72 h at 50 °C and 150 rpm. At the end of enzymatic hydrolysis, the liquid fraction of enzymatic hydrolysis was separated by centrifugation. The supernatant was labelled as EFB enzymatic hydrolysate (EH). EH was concentrated using a rotary evaporator (Rotavapor, BUCHI, UK) at 60 °C. The concentrated EH consisted of 118.52 g/L glucose, 9.55 g/L xylose and 1.00 g/L arabinose.

### 2.3. Optimisation in shake flasks

A response surface methodology (RSM) with face-centred central composite design (CCD) (Supplementary Data A.1) was applied for the cultivation of *M. plumbeus* on EFB enzymatic hydrolysate (EH) by varying the parameters of cultivation (independent variables) which were:

1. Sugar concentration of EH (g/L) (X1)
2. Relative concentration of yeast extract to sugars in EH (%, g yeast extract/g sugar in EH) (X2)
3. Spore concentration (log spores number/mL medium) (X3)
4. Initial pH (X4)

The response factors (dependant variables) for optimisation were oil concentration (g/L) ($Y_1$) and oil yield (mg/g) ($Y_2$). The coded and actual values of each variable and its levels (−1 for low value and 1 for high value) for this experimental design are shown in Table 1. The levels were determined based on the preliminary study (unpublished data), where the selection of the pH level was on the basis that *M. plumbeus* showed no capacity to grow at pH 4 and poor growth above pH 8.

For this optimisation study, a total of twenty-six experimental runs were conducted in random, which consisted of the combination of sixteen factorial points, six axial points and a centre point with five replicates. Design of experiments, mathematical modelling and optimisation of process parameters were performed using the Design Expert software (Stat-Ease Inc., USA). Three dimensional surface plots were drawn using MATLAB R2009a (The MathWorks, Inc., USA). Each response variable was fitted to a quadratic model to correlate the response variable to the independent variables. Analysis of variance (ANOVA) was evaluated through statistical analysis of the model. The statistical significance of the model terms was assessed using the *p*-value approach.

For cultivation experiments, sugar concentrations, yeast extract and spore inoculum concentration as well as pH were used according to Table 1. The cultivation media were prepared by supplementing EH with the same nutrients compositions used in previous study (0.4 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $KH_2PO_4$, 3 mg/L $MnSO_4{\cdot}H_2O$ and 0.1 mg/L $CuSO_4{\cdot}5H_2O$) [13]. The cultivation was performed with 30 mL working volume in 250 mL Erlenmeyer flasks at 28 °C and 200 rpm on an OM15 orbital shaking incubator (Ratek, Australia) for seven days. Fungal biomasses were harvested by vacuum filtration and washing, followed by biomass freeze-drying to constant weight [3].

A validation experiment was performed using the experimental conditions of the optimised parameters cultivation. The cultivation of control was performed using the media preparation based on repeatedly used cultivation methods [2,16]. The nutrients supplementation used in the control was the same as the present study. Yeast extract concentration for the control cultivation was 1.5 g/L and the initial pH was 5.5.

### 2.4. Cultivation in bioreactor

The cultivation of *M. plumbeus* on EH was scaled up and performed in a 1 L bioreactor (New Brunswick™ BioFlo®/CelliGen® 115 Fermentor and Bioreactor, Eppendorf AG, Germany). The cultivation was carried out at 28 °C with an initial agitation speed of 200 rpm and aeration rate of 1 vvm (volume air per working volume per minute). A cascade control for dissolved oxygen (DO) regulation was applied to keep the DO level higher than 20% by automated increment of agitation speed and aeration rate. The cultivation in the bioreactor was carried out using EH at the same concentrations of sugar (30 g/L) and yeast extract (0 g/L), and initial pH (pH 5.0) of the optimised conditions, and supplemented with the same nutrients formulation of 0.4 g/L $MgSO_4{\cdot}7H_2O$, 2 g/L $KH_2PO_4$, 3 mg/L $MnSO_4{\cdot}H_2O$ and 0.1 mg/L $CuSO_4{\cdot}5H_2O$ [13]. Samples from the cultivation medium were observed under an Olympus BX41TF microscope (Japan) with an Olympus DP11 Microscope Digital Camera system (Japan).