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Maximizing biodiesel production from *Yarrowia lipolytica* Po1g biomass using subcritical water pretreatment

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ABSTRACT

The yeast *Yarrowia lipolytica* Po1g is one of the oleaginous microorganisms with a potential for biodiesel production. Sub-critical water (SCW) treatment has been known as an effective method for increasing the amount of extractable lipids in microorganisms. In this work, the amount of neutral lipids and fatty acid profiles in neutral lipids extracted from *Y. lipolytica* Po1g with and without SCW pre-treatment were investigated. The effects of temperature (125, 150 or 175 °C), amount of water (20, 30 or 40 mL/g biomass) and time (10, 20 or 30 min) showed that maximum neutral lipid (42.69%, w/w) could be achieved at 175 °C using 20 mL water for 20 min. The maximum neutral lipid from unpretreated samples was 23.21%. No difference in fatty acid profiles was observed, but long chain fatty acids were observed in higher amount in SCW pretreated samples. SCW pretreatment increased biodiesel yield twofold.

1. Introduction

Microbial lipid has already been developed as sources of high-valued oils including cocoa butter equivalent (CBE) and polyunsaturated fatty acids (Papanikolaou and Aggelis, 2010). Recently, it has also been suggested as a potential feedstock for biodiesel industry (Adamczak et al., 2009; Easterling et al., 2009; Tsigie et al., 2011), because microbial lipid can be produced using various cheap feedstocks, especially renewable biomaterials such as lignocelluloses.

Biodiesel is an attractive replacement for petroleum diesel because it is domestically available, biodegradable, compatible with existing diesel engines, and reduces tailpipe emissions of most criteria air pollutants (Janaun and Ellis, 2010). Biodiesel, produced from vegetable oils and animal fats, is rather an attractive alternative for its biodegradable, non-toxic and clean renewable characteristics as well as the similar properties to the conventional diesel fuels. Although biodiesel has presently been used in many countries, the high cost of biodiesel has become one of the major obstacles for its further development and wide application. Besides, the use of vegetable oils as raw material for biodiesel production would compete with edible oils, thus leading to the soar of food price. Using recovered animal fats and used frying oils as feedstock can efficiently reduce the price of biodiesel, however, the amount of waste oils is limited and cannot meet the increasing needs for clean renewable fuels (Zhu et al., 2008). Chemically, biodiesel is a fatty acid alkyl ester, commonly known as fatty acid methyl ester (FAME) that is produced via esterification and/or transesterification of various lipid sources in the presence of a base, acid, enzyme or solid catalyst (Knothe, 2005).

Microbial oils, otherwise referred to as single cell oils (SCO) produced by various microorganisms, are now believed as a potential feedstock for biodiesel production due to their specific characteristics such as: they are not affected either by seasons or by climates, they own high lipid content, can be produced from a wide variety of sources with short period of production, especially from the residues with abundant nutrition, and so on (Papanikolaou et al., 2004; Xue et al., 2006).

The oleaginous yeast *Yarrowia lipolytica* can accumulate large amount of lipid and through its efficient mechanism, it can break down hydrophobic substrates. The complete sequencing of its genome and application of methods of genetic manipulation have enabled researchers to use this yeast for biotechnological applications as reviewed by Beopoulos et al. (2009). This oleaginous yeast can also be developed into a versatile and high-throughput microbial factory that, by using specific enzymatic pathways from hydrocarbonclastic bacteria, efficiently mobilizes lipids by directing its versatile lipid metabolism towards the production of industrially

Abbreviations: SCW, sub-critical water; FAME, fatty acid methyl ester; FFA, free fatty acids; MAG, monoacylglycerides; DAG, diacylglycerides; TAG, triacylglycerides; TLC, thin layer chromatography; GC, gas chromatography.

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valuable lipid-derived compounds such as wax esters (WE), isoprenoid-derived compounds (carotenoids, polyenic carotenoid ester), polyhydroxyalkanoates (PHAs) and free hydroxylated fatty acids (HFAs) (Sabirova et al., 2011).

Subcritical water (SCW) treatment is an environmentally friendly technique with a wide range of applications, such as extraction, hydrolysis, and wet oxidation of organic compounds (Holliday et al., 1998; Kruse and Dinjus, 2007). SCW is defined as hot water at temperatures ranging between 100 and 374 °C under high pressure to maintain water in the liquid state. Dielectric constant, which can be changed by temperature, is the most important factor when using water as an extraction solvent; it decreases from 80 (at room temperature) to 27 (at 250 °C) which is almost equal to that of ethanol at ambient temperature (Herrero et al., 2006). Thus, SCW can be used for extraction of organic compounds instead of using organic solvents which are environmentally unacceptable. On the other hand, SCW has been widely used for hydrolysis of organic compounds. Recently growing attention has led to extensive research activities using SCW for hydrolysis and conversion of biomass and carbohydrates to useful compounds (Bicker et al., 2005; Kruse and Gawlik, 2002; Salak Asghari and Yoshida, 2006; Sasaki et al., 1998; Yoshida et al., 1999).

SCW has also been known as a uniquely special medium or solvent for various chemical reactions and extraction of compounds. SCW extraction is able to selectively extract different classes of compounds at various temperatures since the polarity of water changes with temperature as long as the water is maintained at its sub-critical condition. Moreover, SCW can also act as an effective catalyst for a hydrolysis or biodegradation reaction. It has been demonstrated by Galkin and Lunin (2005) that SCW can completely remove a wide variety of organic pollutant from industrial waste within only a few minutes. SCW, therefore, has been becoming a promising extractant and catalyst (Rogalinski et al., 2005).

An experimental design is a fast, economic, and effective way to systematically investigate the effect of several variables simultaneously (multivariate data analysis). In the experimental study of M variables and N experiments, an $M \times N$ matrix constitutes a variable space (X). A response variable (Y) for each experiment is necessary for the analysis of the experimental data (Jalbani et al., 2006).

In the present work, the effect of SCW pretreatment of *Y. lipolytica* Po1g biomass on lipid and biodiesel production was investigated. A 2^3 two-level factorial design was applied to study three factors (temperature, amount of water added to the biomass and pretreatment time) which are believed to play important roles in the pretreatment of *Y. lipolytica* Po1g biomass for the extraction of lipid using organic solvents. The effect of temperature, pretreatment time and amount of water on the amount of extractable lipids and the fatty acid profile of subcritical water pretreated biomass was also studied. Finally, comparison of the results from untreated and SCW treated biomass samples was made.

2. Methods

2.1. Materials

All solvents and reagents were either gas chromatography (GC) or analytical reagent grade, obtained from commercial sources. For GC analysis, all the standards were purchased from Acros Organics (New Jersey, USA) and Sigma Aldrich (St. Louis, MO 63103, USA). Bacto peptone was supplied by Becton Drive (Sparks, MD 21152, USA). Thin layer chromatography (TLC) aluminum plates (20 cm × 20 cm × 250 cm) were purchased from Merck KGaA (Darmstadt, Germany). Qualitative filter paper (grade No. 2, 0.26 mm thickness, 80% collection efficiency and grade No. 5C) was obtained from Advantec (Tokyo, Japan).

2.2. Microorganism, media preparation, precultivation and cultivation

Y. lipolytica Po1g cells were obtained from YEASTERN Biotech Co. Ltd. (Taipei, Taiwan). The cells were maintained on YPDA medium at 4 °C. The preculture was performed on precultivation medium (g/L, D-glucose 20, peptone 10, yeast extract 10) at 26 °C and 160 rpm shaking for 24 h. Then, the preculture was inoculated to the culture medium with a ratio of 1:10 (v/v).

The culture medium in the flask consisted of detoxified sugar cane bagasse hydrolysate (SCBH, with a total sugar concentration of 20 g/L) and 5 g/L of peptone. Yeast extract (5 g/L), containing leucine (6.2%), was added to the medium. Cultivation was performed in 500 mL conical flasks each containing 250 mL culture media in an orbital shaker incubator model LM-570 (Chemist Scientific Co., Taiwan) at 26 °C and 160 rpm. The required amount of biomass was then collected for subsequent culturing experiments. The procedures in our previous work were followed (Tsigie et al., 2011).

2.3. Biomass pretreatment and sample preparation

To compare the amount of extractable lipid, neutral lipid and determine fatty acid profiles, biomass was prepared in two ways. The first type consisted of unpretreated freeze-dried biomass of *Y. lipolytica* Po1g. The second type of biomass was prepared by subcritical water pretreatment of freeze-dried biomass samples with subcritical water extractor. The effect of subcritical water pretreatment was studied by considering three variables: temperature (125–175 °C), amount of water added to 1 g biomass (10–30 mL), pretreatment time (10–30 min).

The equipment for subcritical water extraction used in this research was constructed by Ju-Shan Industrial Co. Ltd. in Taiwan. There are three main parts in this equipment, subcritical reactor, heater, and control devices. The reactor was made from stainless steel, and the total inner volume was about 90 mL. It is 25 mm thick and can withstand an estimated maximum operation pressure of 100 MPa. Ten M8 screws which can afford 12.8 tons of tensile force were used for tightening the reactor with its cap. Two layers of spacers were put between the cap and the reactor (Kasim et al., 2009). A thermocouple and a pressure gauge were connected to the reactor. The process was run under batch mode. For subcritical water extraction, nitrogen gas (99.9% purity) purchased from Dong-Xing Company (Taiwan) was used to maintain constant pressure (13 bar) inside the reactor.

Freeze-dried biomass (1 g) was dissolved in deionized water (10–30 mL) in the high pressure reactor. Temperature inside the reactor was measured by a thermocouple and controlled at either 125, 150 or 175 °C. The pressure inside the reactor was maintained at 13 bar by using nitrogen to assure that water remained as liquid. This condition was maintained for either 10, 20 or 30 min and then the system was allowed to cool to 30 °C. After that the pretreated biomass was collected, separated from the aqueous phase by vacuum filtration and then subjected to freeze drying (Freeze Zone – 2.5 l freeze dry system-Model 7670520, Labconco Corporation, Kansas city, USA). The freeze-dried, SCW pretreated biomass was then collected and stored 4 °C before use.

2.4. Experimental design

A 2^3 factorial design was used in this study. The optimization study was conducted with Design-Expert 8 software, by means of customized design of experiments. The experiments were done in random sequence. Upon completion of all the experimental