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Solid state fermentation by cellulolytic oleaginous fungi for direct conversion of lignocellulosic biomass into lipids: Fed-batch and repeated-batch fermentations

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ABSTRACT

Lignocellulosic wastes from palm oil mill are one of attractive feedstocks for microbial lipid production by oleaginous microorganisms because of their low cost, renewable nature and abundance. In this study, four filamentous fungi with cellulolytic activity were screened as potential oleaginous microorganisms for direct conversion of these lignocellulosic wastes into lipid. Among them, *Aspergillus tubingensis* TSIP9 accumulated lipid at the highest amount of 39.5 ± 2.2 mg per gram dry substrate (gds), and simultaneously produced high activities of cellulase (2.35 ± 0.22 U/gds) and xylanase (11.83 ± 0.18 U/gds) through solid state fermentation (SSF) of palm empty fruit bunches (EFB). The use of EFB mixed with palm kernel cake (PK) promoted lipid production by the fungi up to 79.9 ± 3.5 mg/gds. When the enzymes were extracted from the first batch and reused in the next batch, *A. tubingensis* TSIP9 produced much higher amount of enzymes and accumulated lipid faster. Fed-batch SSF with intermittent adding of EFB could be applied for lipid production but with a decrease in the enzyme activity. When repeated-batch SSF with 90% replacement with new substrate was applied, both lipid and enzymes were efficiently produced for long period of fermentation. This new strategy for solid state fermentation may contribute greatly to the commercialized enzyme and lipid productions from abundant lignocellulosic biomass.

1. Introduction

Biomass-based biofuel production is now concerned as an approach to face the challenges from high energy prices and potential depletion of crude oils reservoirs, to reduce greenhouse gas emissions, and to enhance a sustainable economy. Biodiesel produced from plant oil is an attractive alternative biomass-based biofuel because it is biodegradable, nontoxic, clean and having similar properties to the conventional diesel fuels. However, the use of pure plant oil as a raw material for biodiesel production would compete with its use as edible oil; thus, leading to an increase in its food price. Therefore, new sources of biodiesel feedstocks have been intensively searched. Microbial lipids produced by oleaginous microorganisms, are considered to be promising potential biodiesel feedstock due to their plant-like oil composition (Zhu et al., 2008). Among oleaginous microorganisms, oleaginous fungi including both molds and yeasts, are increasingly been reported as good lipid producers.

Lignocellulosic biomass (agricultural waste and forest biomass), which contains 55–65% carbohydrate, is widely recognized as a promising feedstock for bio-fuel production (Chundawat et al., 2011). It is also an attractive feedstock for microbial lipid production. However, the lignocellulosic biomass needs to be hydrolyzed into fermentable sugars by a pool of cellulolytic enzymes including cellulase, xylanase, and other accessory enzymes. Filamentous fungi are considered to be the most suitable source for cellulolytic enzymes due to their high production yields and the ability to utilize a wide range of inexpensive agro-residues (Dhillon et al., 2011; Ncube et al., 2012; Liang et al., 2012). It was thought that if oleaginous fungi with cellulolytic activity were screened, a direct pathway for conversion of lignocellulosic biomass to sugars and consequently lipid could be developed.

Solid-state fermentation (SSF) has been widely used for cultivation of filamentous fungi (Kunamneni et al., 2005; Dharani and Kumaran, 2012; Pensupa et al., 2013; Saleem and Ebrahim, 2014). This is because a solid medium simulates better natural habitat of the fungi (Robinson et al., 2001). SSF has also experienced particular

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interest due to its many advantages in comparison to submerged fermentation, e.g., smaller bioreactor volume, reduced downstream processing costs, higher productivity, simpler technique, reduced energy requirement, and low wastewater output (Mitchell et al., 2006). The hyphal development of the fungi also allows them to effectively colonize and penetrate the solid substrate. Furthermore, they can utilize the bound water of their substrates; and thus, grow in the absence of free water (Krull et al., 2010).

To date research work that has been associated with the direct conversion of lignocellulosic biomass into microbial lipid has been limited. One research group has focused on the isolation of oleaginous fungi with cellulase activity (Peng and Chen, 2007). However, the cellulase activities of their strains were very low at only 0.31–0.69 filter paper units (FPU) and the lipid yield were 19–42 mg/g of the substrate, dry wheat straw. Therefore, there was a need to add exogenous cellulases to boost the lipid yield to as much as 74 mg/g dry wheat straw (Peng and Chen, 2008). Recently, the direct conversion of wheat and rice straws into lipid by cellulase producing fungi was attempted (Lin et al., 2010; Dey et al., 2011). However, there was no available information on xylanase production by their fungi and its relationship to lipid production.

Currently, the palm oil industry has considerably expanded its production in Thailand and this has produced much lignocellulosic wastes including 13.5% palm pressed fiber (PPF), 22% palm empty fruit bunches (EFB) and 5.5% palm kernel cake (PK) (Pua et al., 2013). These wastes, therefore could be suitable lignocellulosic raw materials for microbial lipid production. The aim of this study was to directly convert such lignocellulosic wastes from palm oil mill into lipid by cellulolytic oleaginous fungi. Firstly, oleaginous fungi with high cellulolytic activity were isolated from soils and wastes associated with palm oil mill. The production of lipid and enzymes by these isolated oleaginous fungi was then tested through solid state fermentation (SSF) of palm empty fruit bunches (EFB) added with palm kernel cake (PK) as an alternative nitrogen source. The process development for solid state fermentation including reuse of crude enzymes, fed-batch strategy with intermittent adding of a carbon source and repeated-batch strategy with various percent replacements, was attempted for sustainable production of lipid and enzymes from lignocellolusic biomass.

2. Materials and methods

2.1. Lignocellulosic wastes from palm oil mill

EFB and PK were obtained from Thai Taro and Oils Co., Ltd., Surat Thani, Thailand and sun-dried for 2 days. The waste was cut into small pieces of about 1 cm in length. To remove the lignin, EFB were soaked with 10% NaOH at a ratio of solid to liquid of 10% and boiled at 100 °C for 15 min (Boonsawang et al., 2012). The pretreated solids were then washed with tap water to nearly a neutral pH. After pretreatment, the materials were dried to constant weight at 60 °C, stored in plastic bags and kept at room temperature before use. The hemicellulose, cellulose and lignin contents of EFB and PK were determined by standard method (A.O.A.C., 1999). The lipid content was determined by the method of Folch et al. (1957). The hemicellulose, cellulose, lignin and lipid contents of EFB used in this study were 18.2 ± 0.9% (w/w), 60.1 ± 0.3% (w/w), 14.3 ± 1.0% (w/w) and 2.13 ± 0.21% (w/w) or 21.3 ± 2.1 mg/g dry substrate, respectively. After pretreatment, EFB became softer and the cellulose content of EFB was increased up to 76.9 ± 0.8% (w/w), while the hemicellulose and lignin contents decreased to 3.3 ± 0.1% (w/w) and 11.6 ± 0.3% (w/w), respectively. The lipid content of pretreated EFB was 1.11 ± 0.49% (w/w) or 11.1 ± 4.9 mg/g dry substrate. The hemicellulose, cellulose and lignin contents of PK used in this study were 18.9 ± 1.0%, 40.5 ± 0.6% and 15.7 ± 1.2% (w/w), respectively. The lipid content of PK was 4.89 ± 0.2% (w/w) or 48.9 ± 2.0 mg/g dry substrate.

2.2. Media preparation

The enrichment medium for isolation consisted of a mineral salts solution (MS solution), 20 g/L palm fiber and 1.0 g/L yeast extract. The MS solution contained $(NH_4)_2SO_4$ 1.7 g, KH_2PO_4 2.0 g, $MgSO_4{\cdot}7H_2O$ 0.5 g, $CaCl_2{\cdot}2H_2O$ 0.2 g, $FeSO_4{\cdot}7H_2O$ 0.01 g, $ZnSO_4{\cdot}7H_2O$ 0.01 g, $MnSO_4{\cdot}4H_2O$ 0.001 g, $CuSO_4{\cdot}5H_2O$ 0.0005 g, 0.1% Tween-80 (w/v) and 1000 mL of deionized water, with the pH adjusted to 5.5. The carboxymethyl cellulose (CMC) agar medium consisted of 20 g/L CMC and the MS solution with the pH adjusted to 5.5. The Potato dextrose agar (PDA) used as a pre-culture medium contained 200 mL of potato soup (boiled potato 1 kg and water 1 L for 15 min), dextrose 20 g/L and agar 20 g/L with the pH adjusted to 5.5.

2.3. Isolation and screening of oleaginous fungi

The oleaginous fungi were isolated from soils and wastes of palm oil mill in southern region of Thailand. One gram of soils and wastes sample was enriched in 5 mL of enrichment medium. Then, 0.1 mL of a diluted culture was inoculated onto the carboxymethyl cellulose (CMC) agar medium containing 0.0001% chloramphenicol, using the spread-plate technique and incubated for 5 days at room temperature. Fungal strains were stained with the Sudan black B technique (Patnayak and Sree, 2005) to screen for fungi with high lipid content. Sudan Black B is a lysochrome (fat-soluble dye) diazo dye used for staining lipid. The stained fungi were observed with a phase contrast microscope using oil immersion to detect the presence of blue or grayish-colored lipid globules within the cells. The strains with large lipid globules were selected and purified on PDA medium.

The selected oleaginous fungi were identified based on their 18S rDNA sequences of their internal transcribed spacer regions (ITS) as described by White et al. (1990). Briefly, mycelia were harvested from liquid culture after 3 days of growth by filtration and were transferred to sterile mortar and liquid nitrogen was added. Mycelia were ground into a fine powder. After grinding with liquid nitrogen the genomic DNA was extracted by CTAB method. The ITS region (including ITS1, 5.8SrDNA and ITS2) was amplified using the universal primers ITS4 (5′-TCCTCCGCTTATTGATATGC-3′) and ITS5 (5′-GAAGTAAAAGTCGTAACAAGG-3′). PCR amplification was carried out under the following conditions: initial denaturation at 94 °C (1 min); followed by 30 cycles of 94 °C denaturation (1 min), 55 °C annealing (70 s), and 72 °C extension (1 min) and a final extension at 72 °C for 10 min. The obtained sequences were BLAST searched against the National Center for Biotechnology Information database.

2.4. Solid state fermentation (SSF)

One gram of dried lignocellulosic materials was added to 50 mL cotton plugged Erlenmeyer flasks and supplemented with 1 mL of MS solution. After sterilization, the media was cooled and the water content was then adjusted to 65% by adding 0.85 mL of spore suspension containing 10^7 spores/gds (Ismaili-Alaoui et al., 2003). The culture was incubated at 28 °C for 5 days. For time course studies, the whole flask replicates were collected as sample time points. In the fed-batch solid state fermentation, the SSF was first operated for 3 days, and 1.43 g of new substrate after water content adjusted to 65% (this contained 0.5 g dry substrate) was added every 3 days. Repeated-batch solid state fermentation was carried out by continuously repeating different cycles of batch fermentation. The fermented biomass was replaced with new substrate every 3 days.