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An integrative process model of enzymatic biodiesel production through ethanol fermentation of brown rice followed by lipase-catalyzed ethanolysis in a water-containing system

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ABSTRACT

We attempted to integrate lipase-catalyzed ethanolysis into fermentative bioethanol production. To produce bioethanol, ethanol fermentation from brown rice was conducted using a tetraploid *Saccharomyces cerevisiae* expressing α-amylase and glucoamylase. The resultant ethanol was distilled and separated into three fractions with different concentrations of water and fusel alcohols. In ethanolysis using the first fraction with 89.3% ethanol, a recombinant *Aspergillus oryzae* whole-cell biocatalyst expressing *Fusarium heterosporum* lipase (r-FHL) afforded the highest ethyl ester content of 94.0% after 96 h. Owing to a high concentration of water in the bioethanol solutions, r-FHL, which works best in the presence of water when processing ethanolysis, was found to be more suitable for the integrative process than a commercial immobilized *Candida antarctica* lipase. In addition, r-FHL was used for repeated-batch ethanolysis, resulting in an ethyl ester content of more than 80% even after the fifth batch. Fusel alcohols such as 1-butanol and isobutyl alcohol are thought to decrease the lipase activity of r-FHL. Using this process, a high ethyl ester content was obtained by simply mixing bioethanol, plant oil, and lipase with an appropriate adjustment of water concentration. The developed process model, therefore, would contribute to biodiesel production from only biomass-derived feedstocks.

1. Introduction

Biodiesel fuel, fatty acid alkyl esters produced by alcoholysis of plant oils or animal fats, is expected to serve as an alternative to fossil fuel [1]. Although an alkaline-catalyzed method has traditionally been used for biodiesel production, increases in environmental concerns have led to a growing interest in alternatives such as a lipase-catalyzed method that avoids conventional difficulties in the recovery of glycerol and catalysts such as potassium and/or sodium salt [2–4].

Methanol is most frequently used as an acyl accepter in plant oil transesterification because of its low cost and high reactivity [5,6]. However, methanol has some drawbacks such as toxicity to biocatalysts and petroleum-derived alcohols. Ethanol and 1-butanol would be an alternative to methanol for biodiesel production because they are less toxic to biocatalysts and can be produced through fermentation of sugars from starchy or lignocellulosic biomass using several microorganisms such as yeast, solventogenic clostridia and *Escherichia coli* [6–8]. Although ethanolysis and butanolysis have been widely studied, biodiesel production using bioethanol or biobutanol obtained by fermentation of biomass has not been reported. Here, we report the production of biodiesel from biomass-derived alcohol and oil. To produce a high concentration of alcohol from biomass, we selected a starchy biomass, brown rice, which leads to a theoretical ethanol yield during fermentation using a tetraploid, *Saccharomyces cerevisiae*, that expresses α-amylase and glucoamylase (MNIV/δGS) [9,10]. In the present study, a simple distillation tower was used to distill the fermentation broth. According to the equilibrium curve of ethanol/water, an ethanol solution containing more than 95% ethanol cannot be obtained solely by the distillation of bioethanol because of azeotropy; therefore, another purification step that is associated with high cost is necessary to further increase the ethanol concentration. Given the cost increase, we attempted the direct use of a distilled ethanol solution for enzymatic biodiesel production.

To produce biodiesel from a distilled bioethanol solution, a recombinant *A. oryzae* expressing *Fusarium heterosporum* lipase (r-FHL) was employed. In a previous study, r-FHL catalyzed

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ethanolysis efficiently, resulting in an ethyl ester (EE) content of more than 90% [11]. Moreover, because r-FHL tolerates a high water concentration in reaction media [11,12], it has promise as a biocatalyst for ethanolysis when using a bioethanol solution with a significant amount of water.

The aim of the present study was to develop an integrative process model of plant oil ethanolysis catalyzed by r-FHL using a distilled bioethanol solution from brown rice (see *Graphical Abstract*). The effects of the composition of a bioethanol solution and impurities on ethanolysis were investigated for efficient biodiesel production.

2. Materials and methods

2.1. Strains, media and chemicals

A recombinant tetraploid *S. cerevisiae* strain expressing α-amylase and glucoamylase obtained by δ-integration and cell fusion (MNIV/δGS) [10] was used for ethanol fermentation. The yeast was aerobically cultivated in SD medium (20 g/L D-glucose and 6.7 g/L Yeast nitrogen Base w/o amino acids) for 24 h and then in YPS medium (10 g/L Yeast extract, 20 g/L Bacto-Peptone and 50 g/L Soluble starch) for 72 h. Ethanol fermentation was conducted using a 2 L jar fermenter in the medium containing 200 g/L brown rice in flour form as the only carbon source.

Recombinant *A. oryzae* which expresses a lipase from *F. heterosporum* [12] was grown in dextrin-peptone medium consisting of 20 g/L glucose, 20 g/L polypeptone, 5 g/L KH_2PO_4, 1 g/L $NaNO_3$, and 0.5 g/L $MgSO_4 \cdot 7H_2O$.

Rapeseed oil used in biodiesel production was purchased from Wako Pure Chemical Industries (Osaka, Japan).

2.2. Preparation of BSP-immobilized cells

Sakaguchi flasks (500 ml) containing 100 ml DP medium, fungal spores, and 300 Biomass support particles (BSPs) were incubated at 30 °C for 96 h on a reciprocal shaker (150 opm). The BSPs used for cell immobilization were 6 mm × 6 mm × 3 mm cuboids of reticulated polyurethane foam (Bridgestone Co. Ltd., Osaka, Japan) with a particle voidage of more than 97% and a pore size of 50 pores per linear inch (ppi). Fungal cells were spontaneously immobilized within BSPs as a natural consequence of their growth during shake-flask cultivation.

2.3. Effect of water content on alcoholysis

Three kinds of alcoholysis (methanolysis, ethanolysis and butanolysis) were carried out at 30 °C on a thermo block rotator (35 rpm). At the beginning of each form of alcoholysis, one molar equivalent of each alcohol was added to the reaction mixture. The compositions of reaction mixtures were varied—methanolysis used rapeseed oil at 9.65 g and methanol at 0.35 g; ethanolysis used rapeseed oil at 9.5 g and ethanol at 0.5 g; butanolysis used rapeseed oil at 9.23 g and 1-butanol at 0.77 g—with 100 pieces of BSPs and various amounts of distilled water (0, 0.5, 1.5 or 3.0 g). To fully convert oil to its corresponding alkyl esters, each one molar equivalent of alcohol was added stepwise at scheduled times (0, 24, 48, and 72 h).

2.4. Preparation of distilled bioethanol solutions for biodiesel production

To prepare bioethanol for ethanolysis, ethanol fermentation was performed in a 2 L jar fermenter, as reported previously [9]. After fermentation for 96-h, the reaction mixture was centrifuged at 5000 rpm for 10 min to remove the yeast and residues, and then the supernatant containing about 8% ethanol was subjected to distillation. In the distillation step, a separable flask (3 L) and a glass column (length; 800 mm, external diameter; 25 mm) with McMahon packing were used as a reboiler and as a distillation tower, respectively. The distillate was separated into three fractions with different ethanol concentrations. GC was used to analyze the ethanol concentration of each fraction, as described in Section 2.6.

2.5. Ethanolysis of oil using a distilled bioethanol solution

Either r-FHL or Novozym 435 was used to catalyze the ethanolysis with each of the distilled bioethanol solutions. The reaction mixture contained 9.5 g of rapeseed oil, 0.56 g of the first fraction or 0.737 g of the third fraction corresponding to 0.5 g of ethanol, and 100 particles of r-FHL or 0.4 g of Novozym 435 with or without the addition of 0.5 g of water. Bioethanol solution containing one molar equivalent of ethanol to rapeseed oil was added to the reaction mixture at scheduled times (0, 24, 48, and 72 h).

For the repeated use of r-FHL during the ethanolysis with bioethanol, r-FHL was recovered from the reaction mixture after one cycle, washed with distilled water, dried at room temperature, and then added to a fresh reaction mixture for the next cycle.

2.6. Analytical methods

Samples of ethanolysis were obtained from the reaction mixture at intervals and centrifuged at 12,000 rpm for 5 min for phase separation. The upper oil layer was analyzed for EE content using a GC-2010 gas chromatograph connected to a ZB-5HT capillary column (0.25 mm × 15 m; Phenomenex, USA). All of the temperature conditions were unchanged from those described previously [13]. Chromatographic peaks were identified via comparison of their retention times with that of a standard solution. Tricaprylin served as the internal standard for the quantification of the alkyl esters in the reaction mixture. The detailed procedure for the determination of alkyl ester content was described in a previous paper [14].

The ethanol concentration of each fraction in a bioethanol solution was analyzed using a GC2010 gas chromatograph (Shimadzu, Kyoto, Japan) connected to a ZB-WAX plus capillary column (0.25 mm × 15 m; Phenomenex, USA). The temperature conditions of the injector and detector were set at 150 and 240 °C, respectively. The column temperature was set at 150 °C for 1 min, increased to 230 °C at 10 °C/min, and finally maintained at this temperature for 1 min. The concentrations of impurity alcohols in the bioethanol solution were quantified using a GC–MS (GCMSQP2010 Plus; Shimadzu) equipped with a DB-FFAP column (60 m, 0.25-mm internal diameter, 0.5-μm film thickness; Agilent Technologies, Tokyo, Japan). All of the analysis conditions were unchanged from those described previously [15].

Water content of each bioethanol fraction was measured by a moisture meter CA-21 (Mitsubishi Chemical Analytech Co., Ltd. Kanagawa, Japan).

2.7. Effect of the various alcohols in a bioethanol solution on lipase activity

To investigate the effect of the various alcohols in a bioethanol solution on lipase activity, a lipase activity assay of r-FHL using p-nitrophenyl butyrate (pNPB) as a chromogenic substrate was carried out after an incubation with a 10% ethanol solution (pH 7.0) containing 3% of each alcohol (1-propanol, 1-butanol, isobutyl alcohol or 3-methyl-1-butanol). Relative activity was calculated as the ratio of the hydrolytic activities of r-FHL incubated in an ethanol solution with each alcohol to those without each alcohol.

3. Results and discussion

3.1. Effect of water concentration on alcoholysis catalyzed by r-FHL

To investigate the effect of water on plant oil transesterification catalyzed by r-FHL, three kinds of alcoholysis—methanolysis, ethanolysis and butanolysis—were carried out with the addition of various amounts of water, 0–3.0 g (Fig. 1). In all cases, the alkyl ester contents with the addition of water were obviously higher than those without the addition of water. In a previous study of methanolysis, a small amount of water was effective for maintaining the lipase stability of immobilized *A. oryzae* [12,16]. In the present study, the addition of water also contributed to the high alkyl ester content in the reaction mixtures. Since a wide range of water content (5–30%) provided a sufficiently high EE content and high butyl ester content, we expected that bioethanol and biobutanol solutions with high water concentrations would be applicable to the enzymatic production of biodiesel. Therefore, bioethanol was employed for ethanolysis using r-FHL as described elsewhere in this paper.

3.2. Preparation and analysis of a distilled bioethanol solution

Ethanol fermentation of brown rice was conducted using the yeast strain (MNIV/δGS) and the fermented broth contained more than 80 g/L of ethanol. The ethanol solution was separated into three fractions by distillation. The first fraction contained 89.3% of ethanol and other peaks were not detected in GC–MS analysis (Supplementary data (Fig. S1)). The second fraction with 83.1% of ethanol contained 1-propanol, 1-butanol, isobutyl alcohol and 3-methyl-1-butanol at 0.78, 0.31, 1.56, and 1.96 g/L, respectively, whereas, in the third fraction with 67.8% of ethanol, the contents of these impurities increased to 3.21, 2.10, 12.1, and 37.6 g/L, respectively. The water contents of the first, second and third fraction were 8.08, 14.1 and 25.4%, respectively. The detected alcohols (1-propanol, 1-butanol, isobutyl alcohol, and 3-methyl-1-butanol),