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Two-step bioprocess employing whole cell and enzyme for economical biodiesel production

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Abstract−A preliminary research for feasible and economical bioprocess for biodiesel production was carried out. A microorganism producing lipase was isolated from grease-contaminated soil. After chemically mutating twice, the microorganism exhibited 2.5 times higher intracellular lipase activities than wild type one and was used as a whole cell biocatalyst for biodiesel production. 0.1% toluene treatment followed by freeze drying was the optimum preparation method for whole cell biocatalyst. 1 g whole cell biocatalyst corresponded to 0.15 g commercial enzyme under mild methanol inhibition condition, while corresponding to 0.7 g enzyme under severe methanol inhibition environment. A two-step process, biodiesel production by the whole cell and sequentially by commercial enzyme, was suggested. This novel process, combining the advantages of whole cell and enzyme, saved the usage of commercial enzyme and alleviated enzyme deactivation by methanol.

Key words: Biodiesel, Whole Cell, Preparation Method, Enzyme, Methanol Inhibition, Two-step Bioprocess

INTRODUCTION

Global warming and energy shortages have been imminent worldwide problems [1]. Fuels account for about 30% of total energy usage, and this urgently requires sustainable alternative fuels that emit less toxic exhaust and greenhouse gases than fossil fuels [2]. Of alternative energies, biodiesel is a renewable, biodegradable and nontoxic fuel that can be used in a diesel car without modifying existing engines [3]. Biodiesel, a fatty acid methyl ester, is usually produced through transesterification of vegetable oils or animal fats with alkali catalysts because they promote high conversion rate. However, the alkali process has several drawbacks including energy intensiveness, difficulty of glycerol recovery, removal of the alkaline catalyst from the product and treatment of the highly alkaline wastewater [4].

Biological biodiesel production using enzymes has recently attracted great attention because of its environmental friendly nature, easy recovery and mild operating condition in terms of temperature and pH [5]. In particular, glycerol, now being used as valuable intermediate in many industries [6], can be commercialized with a simple recovery process without washing. However, the enzymatic biodiesel production is thought to be still far from being commercialized because of the high price of enzyme and easy deactivation of the enzyme by acyl donor such as short-chain alcohol like methanol [7]. It was reported that the enzyme is easily deactivated by methanol when the reaction mixture contains more than 1.5 molar equivalents of methanol against the oil [8,9]. Stepwise methanol addition is the most widely used method to avoid methanol inhibition [8-11], pre-incubation of enzyme in methyl oleate [12] and introducing a solvent like *tert*-butanol [13]. Although these attempts have been successful in increasing enzyme stability, the inherent disadvantage of the enzyme process, high cost, cannot be easily overcome.

As an alternative to enzyme in the biological process, whole cell biocatalyst has been applied for economical biodiesel production [4,14,15]. *Rhizopus oryzae* has been studied extensively as the whole cell and glutaraldehyde or *tert*-butanol were used to improve its stability [16,17]. The whole cells are believed to be promising for the economical biodiesel production relative to enzyme. However, there are some other issues which should be discussed for the industrial application of whole cell. Low conversion rate can decrease the economical advantage of whole cell [4]. Whole cell of *Rhizopus oryzae* took 24 and 72 hours to achieve approximately 25 and 80% of conversion, respectively [5]. In the case of yeast whole cell, it took 165 hours to obtain 71% conversion [18]. Mass transfer resistance, which may be one of the reasons for a low conversion rate, also should be taken into consideration [19] because reactants (oil and methanol) and products (biodiesel and glycerol) should easily cross the cell wall while cell components remain inside the wall. So far, however, there have been few attempts to enhance cell permeability for enhancing biodiesel production.

In this study, a microorganism was isolated from grease-contaminated soil and chemically mutated for increasing its lipase production performance. An efficient preparation method of whole cell biocatalyst was suggested. Finally, a two-step process, combining the advantages of whole cell and enzyme, was suggested to save commercial enzyme usage and alleviate deactivation of enzyme by methanol.

MATERIALS AND METHODS

1. Microorganism and Mutation

A microorganism producing lipase was isolated from grease-contaminated soil and identified as *Serratia marcescens* (Seoul Medical Science Institute, Korea). The screening was carried out according

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to another's procedure [20]. Transparent halos appeared around colonies on agar plate containing LB medium (10 g/L tryptone, 5 g/L yeast extract, 10 g/L NaCl) supplemented with 1% tributyrin, and 300 colonies were picked up by the ratio of halo to colony. Each colony was incubated in a 100 mL flask containing basal medium (1.0 g/L yeast extract, 2.0 g/L NaCl, 0.4 g/L $MgSO_4$, 0.5 g/L $(NH_4)_2SO_4$, 0.3 g/L K_2HPO_4 and 0.3 g/L KH_2PO_4) supplemented with 5.0 mL/L olive oil as a sole carbon source [21]. After 24 hours of incubation, the microorganism showing highest lipase activity was chosen for chemical mutation. After the candidate cells were harvested during exponential growth phase in the flask, they were centrifuged at 12,000 rpm for 5 minutes (Combi-514R, Hanil Industrial, Korea) and washed with distilled water several times to remove culture medium. After being mixed with 40 mL distilled water, 10 mL of each aliquot was added with 0.1, 0.3, 0.5 or 1.0% EMS (ethyl methanesulfonate, Fluka). The mixture was incubated at 30 °C for 1 hour (JeioTech SI-600R, Korea). After EMS was removed with distilled water, mixtures were spread on the agar plates containing LB medium supplement with 1% tributyrin. The following procedure for choosing the cells showing highest lipase activity was identical to that described above. The cells showing highest lipase activity after first mutation were mutated again to enhance lipase activity more. It was found that the cells from the third and fourth mutation did not improve lipase activity compared to the cells from the second mutation. The final microorganism from chemical mutation was named as *Serratia marcescens* JYM110 (abbreviated as JYM110).

2. Chemicals

Soybean oil was purchased from a domestic supplier (CJ, Korea) and 99% of this oil was triglycerides composed of 51.8-56.0% linoleic acid, 22.0-27.1% oleic acid, 9.6-11.5% palmitic acid, 6.2-11.1% linolenic acid *etc*. Methanol (Showa, Japan) was used as an acyl donor. Commercial lipase enzyme used in this study was Novozyme 435 (Novo Nordisk, Denmark). Palmitic acid methyl ester, oleic acid methyl ester, linoleic acid methyl ester and stearic acid methyl ester were purchased from Sigma-Aldrich (USA) to identify and measure the components of biodiesel. The other chemicals were of analytical grade.

3. Analysis

To prepare a calibration curve for the components of biodiesel, each methyl ester mentioned previously was dissolved in chloroform (Wako, Japan) to be 100-1,000 mg/L. 1 μl of the dissolved sample was injected into a GC (HP 5890 II, USA) equipped with an FID detector and HP-5 column (30 m×0.32 mm×0.25 μm film thickness). The temperature of both injector and detector was 250 °C and that of column was elevated from 150 to 250 °C at 5 °C/min after the oven temperature was initially maintained for 2 minutes. Helium was used as a carrier gas. Methyl heptadecanoate (Fluka, Japan) was used as an internal standard for GC analysis.

4. Enzyme Activity Measurement

Enzyme activity was measured according to the p-NPP method [22] in which p-NP (p-nitrophenol) and palmitic acid were produced from p-NPP (p-nitrophenylpalmitate) by lipase and p-NP was quantified for the determination of enzyme activity. One unit was defined as the amount of lipase to release 1 μmol of p-NP in 1 minute. The p-NPP was dissolved in acetronitrile to be 10 mM and 1 mL of this solution was mixed with 4 mL of ethanol. Thereafter, 95 mL of 50 mM Tris-HCl (pH 8.0) buffer was gently added to the solution and this was stored in a refrigerator at 4 °C. 3 mL of the reagent solution was mixed with 0.1 mL or 0.1 g of experimental sample and placed at 28 °C for 30 minutes in a shaking incubator. After centrifugation, the absorbance of the supernatant was measured with a UV spectrophotometer (Jasco V-550, Japan) at 405 nm.

5. Biodiesel Production

Biodiesel was produced in a 50 mL flask in which 8.724 g soybean oil and 0.24 g methanol were initially placed and stirred for 10 minutes before biocatalyst was added. The same amount of methanol was added every 3 hours until the total amount of methanol fed to the flask became 1.44 g, which means the total molar ratio of methanol to soybean oil was 4.5 [23]. The reaction was carried out at 30 °C for 24 hours.

RESULTS AND DISCUSSION

1. Lipase Production by *Serratia marcescens* JYM110

Serratia marcescens JYM110 was cultured in a flask containing basal medium supplemented with olive oil of which composition was described above. Its lipase production was compared with other well-known lipase producers (*Burkholderia cepacia* ATCC 25416, 29424, 39356, 17759) as shown in Fig. 1 [24,25]. Except ATCC 29424 showing approximately 9 times higher extracellular lipase activity per culture volume, the other lipase producers showed lower extracellular lipase activity than JYM110. On the other hand, the intracellular lipase activity of JYM110, 4.2×10^{-3} U/mL, was 30% higher than that of ATCC 29424 (3.1×10^{-3} U/mL). Since the purpose of this study includes whole cell biocatalyst development, related to intracellular lipase rather than extracellular one, JYM110 can be considered as a good microorganism for whole cell biocatalyst.

To investigate lipase production by JYM110 in detail, cell mass, extracellular and intracellular lipase activities were monitored during cell culture in a 5 L fermentor (KF-5L(D), KoBioTec, Korea) with 3 L working volume. As shown in Fig. 2, both extracellular and intracellular lipase activities steadily increased for the first 6 hours of operation and the extracellular activity stood at $0.5\text{-}1.1\times10^{-3}$ U/mL throughout the operation. In the case of intracellular lipase activity, it was sharply increased to 5.6×10^{-3} U/mL at 12 hours of opera-

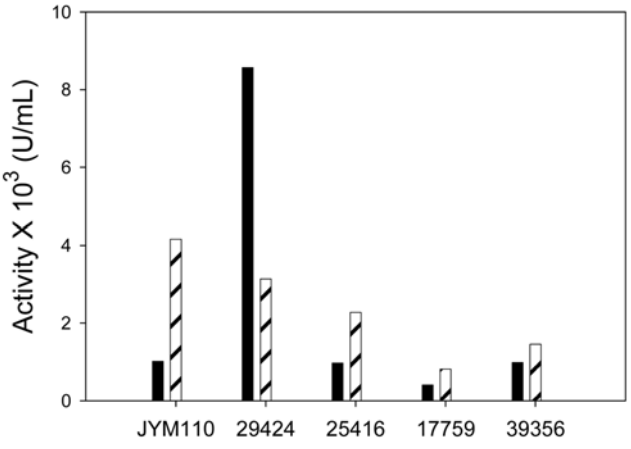

Fig. 1. Comparison of lipase production between *Serratia marcescens* JYM110 and ATCC lipase producer. ■: extracellular lipase, ▨: intracellular lipase.

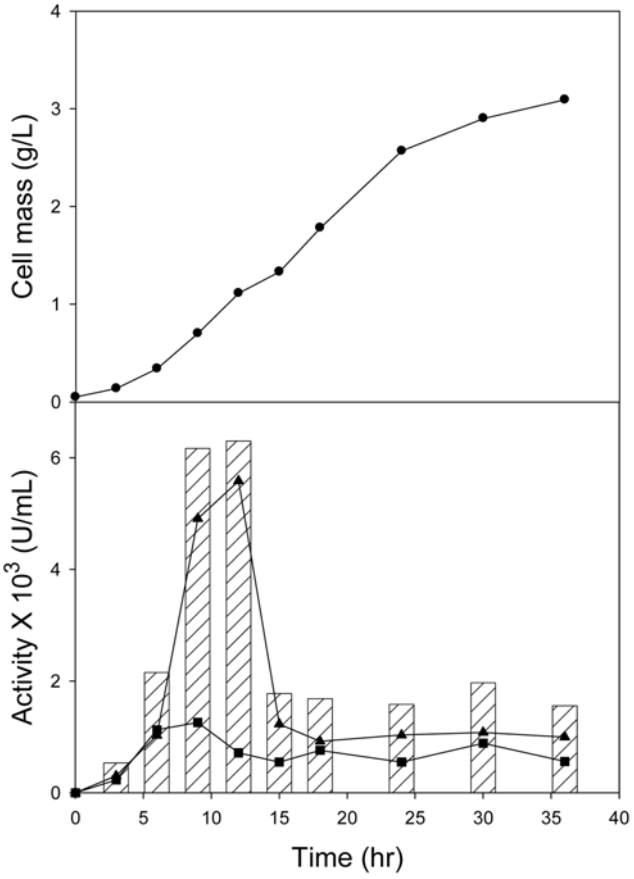

Fig. 2. Lipase production by *Serratia marcescens* JYM110 in a fermentor. ●: cell mass, ■: intracellular lipase activity, ▲: extracellular lipase activity, ▨: total lipase activity.

tion but decreased to about 0.9×10^{-3} U/mL after 18 hours of operation. Considering the cell mass was 0.34, 1.1 and 1.8 g/L for 6, 12 and 18 hours, respectively, the specific intracellular lipase activities were 3.0, 5.1 and 0.5 U/mg-cell for 6, 12 and 18 hours, respectively. Therefore, the cells were harvested at 12 hours of operation from the fermentor and used for whole cell development.

2. Solvent Treatment for Whole Cell Preparation

The harvested cells from the fermentor were freeze dried and used as whole cell biocatalyst for biodiesel production. When 10% (w/w) of the freeze dried cells relative to soybean oil was placed in the reaction mixture, the conversion was as low as 2.1% after 24 hours. If the cell content in the reaction is increased, higher conversion will be obtained. However, high cell mass in the reaction could cause difficulty in agitation and separation of biodiesel and glycerol. If whole cells are immobilized, conversion is expected to be enhanced without causing such operational problems [5]. Biodiesel production using immobilized whole cell will be investigated in the next study. In this study, in order to increase biodiesel conversion, whole cells were treated with various chemicals which are known to increase cell permeability and they were toluene, ether, DMSO (dimethyl sulfoxide), benzene, methanol and chloroform. Since Flores et al. [26] and Ban et al. [5] reported that glutaraldehyde improved whole cells of *Kluvernomyces lactis* with β-galactosidas and *Rhizopus oryzae* with lipase, respectively, it was also considered. After the cells were

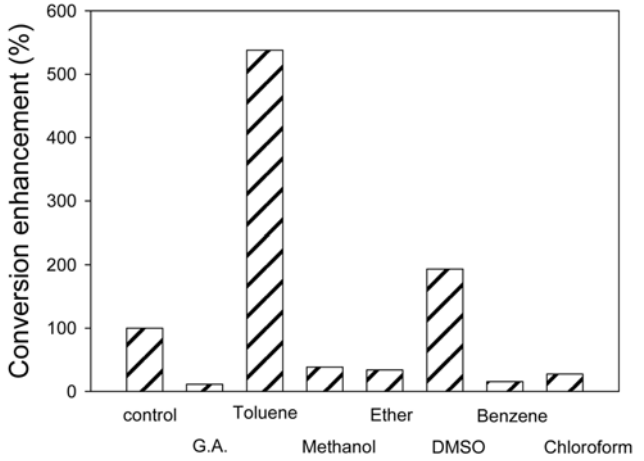

Fig. 3. Effect of chemical treatment for *Serratia marcescens* JYM110 on the biodiesel conversion.

treated with 0.1% of each chemical for 1 hour, they were washed with distilled water three times and dried at 60 °C or freeze dried. When the cells were dried at 60 °C, biodiesel conversion became much lower than that by non-treated freeze dried cells by approximately 90% irrespective of chemical (data not shown). When the cells were freeze dried after being treated with toluene, they showed the highest conversion enhancement, 538%, relative to non-treated cells, as shown in Fig. 3. Benzene treatment enhanced biodiesel conversion by 193% while other chemicals caused reverse effect on the biodiesel conversion, for example, glutaraldehyde decreased biodiesel conversion by 89%. Various toluene concentrations ranging 0.01-1.0% were tested for the chemical treatment. The toluene concentration of 0.01-0.05 % enhanced the biodiesel conversion by approximately 250% relative to the control as shown in Fig. 4. The 0.1% of toluene turned out to be the optimum for biodiesel conversion while 0.5 and 1% of toluene showed 383 and 163% enhancement, respectively. The results may imply that toluene concentration less than 0.1% is low to obtain enough cell permeability, while high toluene concentrations cause inhibition on enzyme inside the cell wall.

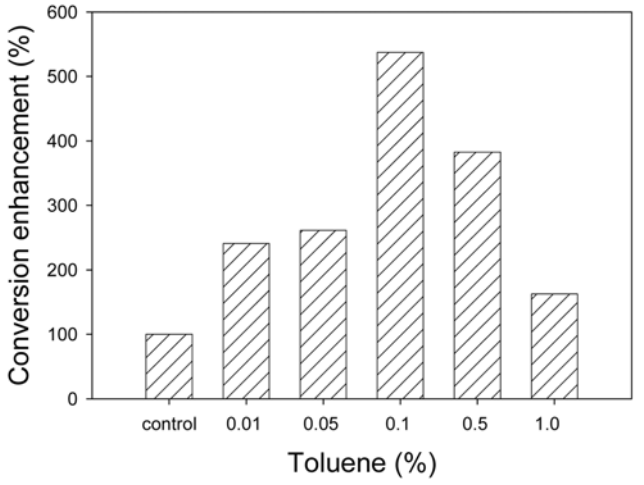

Fig. 4. Effect of toluene concentration in the chemical treatment on the biodiesel conversion.

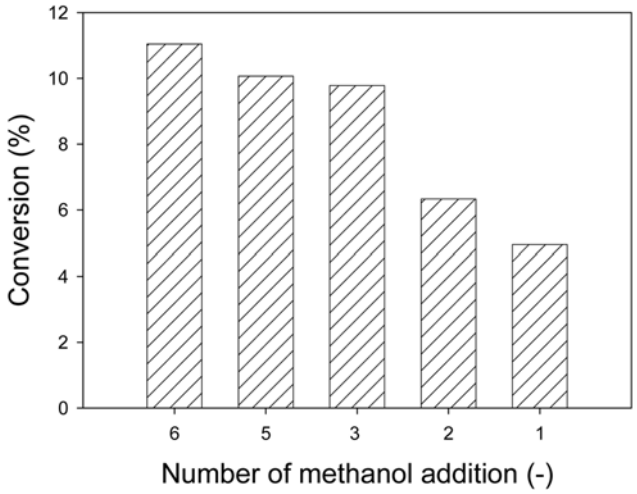

Fig. 5. Methanol inhibition on the biodiesel conversion by whole cell.

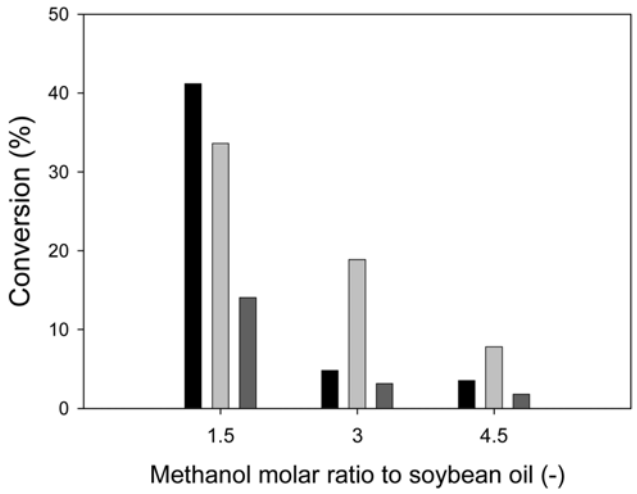

Fig. 6. Comparison of biodiesel conversions in single enzyme bioprocess and two-step bioprocess. ■: 3% enzyme, ▨: 10% whole cell 10%+1% enzyme, □: 1% enzyme.

3. Methanol Inhibition on Whole Cell

Serratia marcescens JYM110, which had been treated with 0.1% toluene followed by freeze drying, was used for biodiesel production. Reaction was carried out at 30 °C and the whole cell was 10% (w/w) to soybean oil. Methanol was fed to the flask containing soybean oil and whole cell in 5 different modes: 6 times of 0.75 molar ratio of methanol to soybean oil every 3 hours, 5 times of 0.9 molar ratio every 3 hours, 3 times of 1.5 molar ratio every 3 hours, 2 times of 2.25 molar ratio every 3 hours or 4.5 molar ratio once, but the total molar ratio of methanol to soybean oil was 4.5 irrespective of feeding mode. When 4.5 molar ratio of methanol to soybean oil was fed to the flask at start, biodiesel conversion was 5.0% as shown in Fig. 5 while no biodiesel was produced by the commercial enzyme, Novozyme 435, with this methanol feeding mode [27]. With 6 stepwise methanol feedings, the optimum feeding mode for the commercial enzyme [27], the biodiesel conversion was 11.0%. Therefore, although the biodiesel conversion with 1 methanol feeding was only 5.0%, it is 45% of the likely maximum biodiesel conversion using whole cells. These results imply that whole cell biocatalyst is much less inhibited by methanol than the commercial enzyme.

As stated above, the biodiesel conversion can be enhanced by increasing the mass of whole cell in batch operation or circulating reactants in a packed-bed bioreactor containing immobilized cells, which will be investigated in the next study.

4. Two-step Process for Biodiesel Production

As shown above, the whole cell biocatalyst is less inhibited by methanol than enzyme. However, it has much lower activity per mass than commercial enzyme in terms of biodiesel conversion. Accordingly, a substantial amount of whole cell is required to achieve high biodiesel conversion. In addition, the conversion rate is lower than that by commercial enzyme. These results imply that the replacement of whole cell for commercial enzyme may be limited. Therefore, it is necessary to combine the advantages of whole cell and commercial enzyme. In this study, a two-step process for biodiesel production is suggested, biodiesel production by whole cell and subsequently by enzyme. Fig. 5 shows biodiesel conversion by 3% commercial enzyme, 10% whole cell followed by 1% commercial enzyme and 1% commercial enzyme for various ratios of methanol to soybean oil. In the previous study, 3% enzyme was found to be optimum usage for biodiesel production in terms of biodiesel conversion and productivity [27]. The total reaction time was 24 hours. In the case of 10% whole cell and 1% commercial enzyme, biodiesel was initially produced by 10% whole cell for 12 hours and cells were removed for further biodiesel production by 1% enzyme for another 12 hours.

When methanol molar ratio was 1.5, the biodiesel conversions were 41.2, 33.6 and 14.0% for 3% enzyme, 10% whole cell and 1% enzyme, and 1% enzyme, respectively. 1% whole cells was estimated to yield 2.0% of biodiesel conversion while 1% enzyme yielded average 13.6% of biodiesel conversion. Accordingly, 1 g whole cell corresponded to 0.15 g commercial enzyme in terms of biodiesel conversion. On the basis of these estimations, 20 g of whole cell is required to obtain the same biodiesel conversion as 3 g commercial enzyme. In the case of 3.0 molar ratio of methanol to soybean oil which causes severe inhibition on the enzyme, biodiesel conversions by 3% and 1% enzyme were only 4.8 and 3.1%, respectively, while that by 10% whole cell and 1% enzyme was 18.9%. In this case, 1 g whole cell corresponded to 0.8 g enzyme. In the same manner, 1 g whole cell corresponded to 0.7 g enzyme for the 4.5 molar ratio of methanol to soybean oil. These results imply that whole cell replaces commercial enzyme sufficiently and that it can save much more commercial enzyme under methanol inhibition environment.

In this two-step process, biodiesel was first produced by whole cell which is more resistant to methanol than enzyme. The mixture of biodiesel, glycerol, methanol and soybean oil was then transferred from whole cell to enzyme for further conversion. Since methanol is soluble in biodiesel and glycerol, the methanol inhibition on enzyme can be alleviated. As stated above, a substantial amount of whole cell is required to obtain high biodiesel conversion. In this process, however, since biodiesel is produced by whole cell and subsequently by enzyme, the requirement of whole cell can be controlled by the amount of enzyme. Clearly, the enzyme usage is greatly reduced by whole cell. Accordingly, the two parts for biodiesel pro-

duction are tightly related and it is necessary to optimize the share of biodiesel production between two parts to minimize operating cost and maximize performance of the two-step process, which is another issue for future study.

CONCLUSIONS

Microorganism producing lipase was isolated and chemically mutated consecutively to enhance intracellular lipase activity by 250%. Treatment of the cells with 0.1% toluene followed by freeze drying increased the performance of the whole cell by 538%. Two-step process employing whole cell and subsequently commercial enzyme was suggested in this study. In the mild methanol inhibition condition, 1 g whole cell can replace with 0.25 g enzyme while the same amount of whole cell does 0.7 g of enzyme under severe methanol inhibition condition. Also, the whole cell showed much higher tolerance to methanol compared with commercial enzyme. Although whole cell can save expensive commercial enzyme, the conversion rate by whole cell is lower than that by enzyme. Since whole cell can save enzyme and vice versa, it is necessary to optimize the share of biodiesel production between them.

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