Biotechnology and Bioprocess Engineering 21: 274-282 (2016)
DOI 10.1007/s12257-016-0046-3

RESEARCH PAPER

Enhancing Biodiesel Production by Immobilized Whole Cells by Optimizing Reaction Conditions and Adding Glycerol and Water

Sung Ho Yeom

Received: 24 January 2016 / Revised: 24 March 2016 / Accepted: 7 April 2016

Abstract In this study, several methods were devised and evaluated to enhance biodiesel production by whole cells immobilized onto the polyurethane foam coated with activated carbon. Biodiesel conversion was increased to 76.4% with the increase in the number of polyurethane foam until it occupied 18.0 or 2.4% of reaction mixture based on apparent or actual volume of supports, respectively. Stepwise methanol addition to prevent methanol inhibition on the immobilized whole cells was optimized in terms of number of aliquot and feeding interval. When 4.5 molar ratio of methanol to soybean oil was divided into 4 equal aliquots and each aliquot was fed to the reaction mixture every 24 h, the highest final biodiesel conversion of 82.4% was achieved. Chemical treatment of the immobilized cells with 0.1% of chloroform for 2 h enhanced biodiesel conversion to 90.5%. The initial addition of 5% glycerol in the fresh reaction mixture increased biodiesel conversion to 90.3% while the removal of glycerol during biodiesel production barely increased biodiesel conversion. The biodiesel conversion was increased with the increase of initial water content in the fresh reaction mixture and the highest value was 92.7% at 3.0% of water content, but decreased thereafter. The effects of co-addition of glycerol and water on biodiesel production were also investigated, and the co-addition of 3.125% of glycerol and 1.875% of water relative to soybean oil substantially increased biodiesel conversion to 95.0%. By these optimization of reaction conditions and co-adding glycerol and water, initial biodiesel production rate and final biodiesel conversion were remarkably enhanced by 26.8 and 24.1%, respectively.

Sung Ho Yeom*
Department of Biochemical Engineering, Gangneung-Wonju National University, Gangneung 25457, Korea
Tel: +82-33-640-2406; Fax: +82-33-641-2410
E-mail: shyeom@gwnu.ac.kr

Keywords: biodiesel, whole cells, immobilization, optimal conditions, enhancement, biodiesel conversion

1. Introduction

Biodiesel is a renewable, biodegradable, and nontoxic fuel that can be used in diesel cars without requiring modification of existing engines [1-3]. Biodiesel, a fatty acid methyl ester, is commercially produced through transesterification of vegetable oils or animal fats with alkali catalysts, as these promote a high conversion rate [4-6]. However, use of alkalis has several drawbacks, including its energy intensiveness, difficulty of glycerol recovery, and the necessity to remove the alkaline catalyst from the product and to treat the highly alkaline wastewater [4,5].

Biological biodiesel production using enzymes has attracted great attention because of its environmentally friendly nature, easy recovery of products, and mild operating conditions in terms of temperature and pH [5,7]. However, enzymatic biodiesel production is still considered to be far from commercialization because of the high price of enzymes and the easy deactivation of the enzyme by methanol [4,5,7,9]. It has been reported that the enzyme is easily deactivated by methanol when the reaction mixture contains more than 1.5 molar equivalents of methanol to the oil [9,12]. Stepwise methanol addition is the most widely used method to avoid methanol inhibition [7,9,12]. Pre-incubation of the enzyme with methyl oleate [13] and introduction of a solvent like *tert*-butanol [14] could be solutions to this problem. This alcohol is considered to be an ideal medium that enhances miscibility of methanol with vegetable oils, as well as being a regenerating agent for lipase [8,10,15]. We have previously suggested the use of a pseudo-two phase partitioning bioreactor (P-TPPB), composed of a hydrophobic first phase

(soybean oil) and a hydrophilic second phase, as an attempt to avoid methanol inhibition as well as to extend the usage of TPPB and have applied it to the production of biodiesel [4]. *n*-Pentanol was found to be the optimal for the second phase, since P-TPPB containing *n*-pentanol showed the greatest total biodiesel conversion and highest fatty acid methyl ester content. The enzyme was repeatedly used to produce biodiesel in P-TPPB, while maintaining its activity at over 95% relative to that of the intact enzyme. Although these attempts have been successful in increasing enzyme stability, the inherent disadvantage of the enzyme process, *i.e.*, high cost, cannot be easily overcome. As an alternative to use of an enzyme in the biological process, whole cell biocatalysts have been applied for economical biodiesel production [16-20]. In this context, use of whole cell *Rhizopus oryzae* has been studied extensively, and glutaraldehyde or *tert*-butanol have been used to improve its stability [21-23]. In particular, after treated with glutaraldehyde, *R. oryzae* IFO 4697 (presently NBRC 4697) could maintain high activity even after being reused for 35 batches [23]. Using the same microorganism, it was previously found that vacuum-drying of the immobilized cells on polyurethane foam coated with activated carbon was more efficient than natural or freeze-drying processes [5]. While the immobilized cells were severely inhibited by a molar ratio of methanol to soybean oil in excess of 2.0, stepwise methanol addition (3 aliquots at 24 h feeding intervals) significantly prevented methanol inhibition. In addition, we constructed packed-bed bioreactor containing the immobilized whole cells and investigated the performance of the bioreactor under various operating condition. Overall, whole cells were believed to be promising for more economical production of biodiesel, as compared to enzyme-based production.

However, there are some other issues that should be discussed for industrial application of whole cells. A low conversion rate can mar the economic advantage of whole cells. Using Jatropha oil, *R. oryzae* IFO 4697, immobilized onto a polyurethane foam-based support, and required 60 h to attain a biodiesel conversion of 80% [19]. Yeast whole cells required 165 h to obtain a 71% conversion [24]. *R. oryzae* IFO 4697 treated with glutaraldehyde required about 70 h for 60 ~ 80% biodiesel conversion during each batch operation, although it maintained stability for 35 batches as stated above [23]. In the previous study, it took 144 h to achieve the highest biodiesel conversion of 86.5 [5]. Mass transfer resistance, which may be one of the reasons for a low conversion rate, should also be taken into consideration [25] because reactants (oil and methanol) and products (biodiesel and glycerol) need to easily cross the cell wall, while cell components including enzymes remain inside the wall. In addition, methanol inhibition on intracellular lipase was reported to be one of the reasons for a low conversion rate [26,27].

In this study, various methods were devised and evaluated to increase biodiesel production by the immobilized whole cells. By comparing biodiesel conversions before and after methods employed, simple but feasible methods for substantially enhanced biodiesel production were suggested.

2. Materials and Methods

2.1. Microorganisms and chemicals

Rhizopus oryzae NBRC 4697 (formerly, IFO 4697) [5] was purchased from NBRC in Japan. This microorganism has been widely used as biocatalyst for the production of biodiesel [17,18]. The medium composition was 70 g/L polypeptone, 1.0 g/L $NaNO_3$, 1.0 g/L KH_2PO_4, 0.5 g/L $MgSO_4 \cdot 7H_2O$ according to the suggestion by culture collection institutes. Soybean oil was purchased from a domestic supplier (CJ, Korea); 99% of the 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 and smaller percentages of other acids. Methanol (Showa, Japan) was used as the acyl donor. The commercial lipase enzyme used in this study was Novozym 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.

2.2. Analysis

To prepare a calibration curve for the components of biodiesel, each of the previously mentioned methyl esters was dissolved in chloroform (Wako, Japan) to concentrations of 100 ~ 1,000 mg/L. After shaking, 1 μL of the dissolved sample was injected into a GC (HP 5890, USA) equipped with a FID detector and HP-5 column (30 m × 0.32 mm × 0.25 μm film thickness). The temperature of both the injector and detector was 250°C and that of the column was increased from 150 to 250°C at 5°C/min after the oven temperature was initially maintained for 2 min. Helium was used as a carrier gas. Methyl heptadecanoate (Fluka, Japan) was used as an internal standard for GC analysis. After samples were centrifuged at 12,000 rpm (Combi-514R Hanil, Korea) for 15 min, the upper layer (oil layer) was separated and 0.01 g of the layer was mixed with 10 mL of chloroform. After shaking vigorously, 1 μL of the solution was injected into the GC. Since the internal standard was used for GC analysis, errors during the analysis were thought to be insignificant. Nevertheless, after the reliability of GC

data was confirmed by verifying negligible deviation of three GC data from a sample, each sample was analyzed only once by the GC.

2.3. Preparation of immobilized whole cell biocatalyst

Since *R. oryzae* NBRC 4697 is a filamentous fungus, attachment is a more favorable immobilization method than entrapment or cross-linking. Polyurethane foams coated with activated carbon, with 50 pores per square inches and 5-mm thickness (Woongjin, Korea), was cut to 6 mm × 6 mm squares and used for cell immobilization. While the cells were cultivated in a 500-mL flask containing 200 mL of culture medium at 30°C and 250 rpm for 90 h, they attached to the polyurethane pieces by covering them with filaments and inserting filaments into the polyurethane foams. The immobilized cells were harvested and washed with distilled water and dried *via* vacuum-drying (Isotemp 280A, USA), which was found to be better than natural or freeze dryings in the previous study [5]. The SEM and photograph images were presented in the previous study [5].

2.4. Chemical treatment

Chemical treatment of immobilized cells was performed in two ways. After 90 h of culture, 0.1% (v/v) organic solvent relative to culture volume was added to the culture broth and incubated for 2 h. The supports with immobilized cells were then harvested, washed, and vacuum-dried [5]. Alternatively, the supports were harvested, vacuum-dried and then treated in 0.1% (v/v) organic solvent solution for 2 h. Thereafter, the supports were vacuum-dried again before being used. Seven organic solvents were evaluated, *i.e.*, toluene, benzene, methanol, chloroform, ether, glutaraldehyde and dimetyl sulfoxide (DMSO).

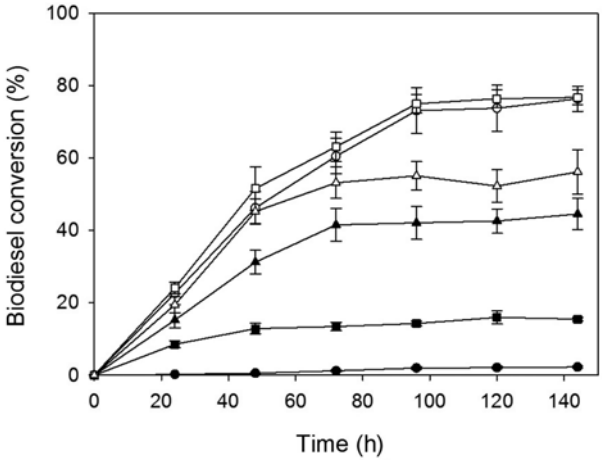

Fig. 1. Effect of number of supports onto which whole cells were immobilized on biodiesel production. ●: 25, ■: 50, ▲: 75, ○: 100, □: 150, △: 200.

2.5. Biodiesel production

Biodiesel production was performed in a 250 mL flask in a shaking incubator at 250 rpm. The amounts of soybean oil and methanol used in the biodiesel production were 0.1 M (87.24 g) and 0.45 M (14.42 g), respectively, and their total volume was approximately 120 mL. The methanol was divided into aliquots of equal volume. Supports onto which whole cells were immobilized and soybean oil were placed in the flask and an aliquot of methanol was fed to the flask to trigger biodiesel production. Each aliquot of methanol was fed by regular interval. All the experiments were carried out in triplicate and average values with standard deviation were presented in this study.

3. Results and Discussions

3.1. Effect of volume occupation of supports in the reaction mixture

Since whole cells serve as a biocatalyst in biodiesel production, the rate of the biodiesel production and the final biodiesel conversion (FBC) can be enhanced by increasing the amount of the whole cells. In the previous study, we immobilized the whole cells onto the polyurethane foam by attachment method because the microorganism used was a fungus with many filaments [5]. Both the types of microorganisms and supports substantially influence cell immobilization [5,26]. The amount of whole cells immobilized was 10.2 mg/piece in the previous study, which is approximate twice and three-fold higher compared with that of wild and recombinant microorganisms, respectively in others' report [5]. Since it is hardly possible to control the amount of cells attached to the support (here, polyurethane foam), I controlled the amount of whole cells involved in the biodiesel production by changing the number of supports onto which whole cells were immobilized. The number of supports was 25, 50, 75, 100, 150, and 200. As done in the previous study using whole cells [5], the total molar ratio of methanol to soybean oil (TMRMSO), the number of aliquot and feeding interval were set 4.5, 3, and 24 h, respectively. As shown in Fig. 1, the number of supports significantly influenced the initial biodiesel conversion rates (IBCRs) for the first 24 h and FBCs. IBCRs and FBCs with respect to the number of supports were presented in Fig. 2. The IBCRs were 0.01, 0.36, 0.63, 0.94, 1.00, and 0.81%/h for 25, 50, 75, 100, 150, and 200 of supports, respectively, that is, IBCRs consistently increased until 100 supports but it barely increased or decreased thereafter. For FBCs, when the number of supports increased from 25 to 50, 75 and 100, FBCs were also increased from 2.3% to 15.5, 44.5, and 76.4%. However, when the number of support increased further to 150 and 200, the FBCs were

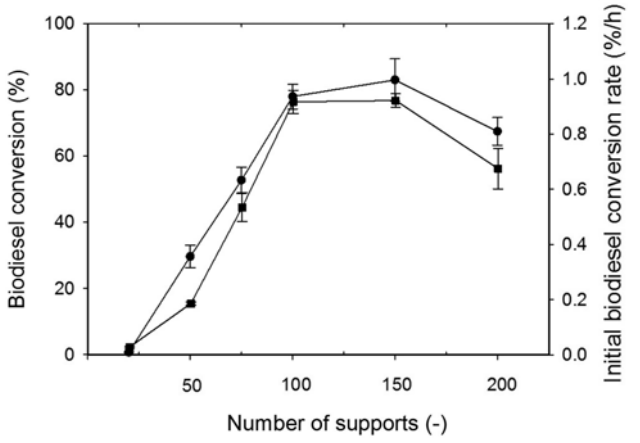

Fig. 2. Effect of number of support on initial biodiesel conversion rate and final biodiesel conversion. ●: initial biodiesel conversion rate (IBCR), ■: final biodiesel conversion (FBC).

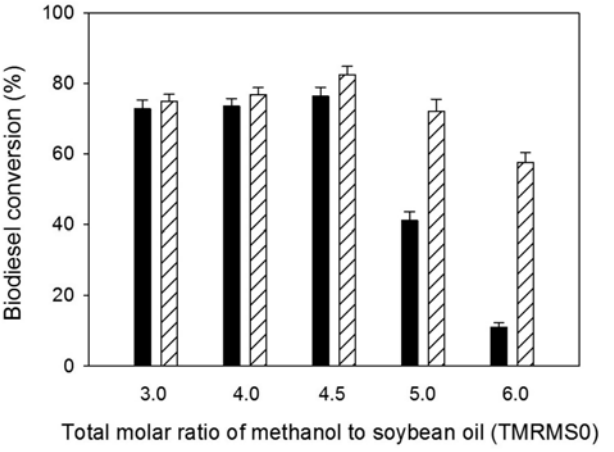

Fig. 3. Effect of total molar ratio of methanol to soybean oil (TMRMSO) and number of aliquot on biodiesel production by immobilized cells. ■: 3 aliquots, ▨: 4 aliquots

marginally increased (76.8% for 150) or noticeably decreased (56.2% for 200). From the economical viewpoint, it was concluded that the optimal number of support is 100.

Despite of larger amount of whole cells or larger number of supports, the negligible increase or unexpected decrease is thought to be related with poor mass transfer [28]. When supports are over-loaded in the reaction mixture, mixing is interrupted and this leads to poor mass transfer. It was observed that the movement of 200 supports in the flask became notably sluggish. The apparent volume of a support was 1.80×10^{-1} cm^3 (6 mm × 6 mm × 5 mm) but the actual volume of a support with 86.8% of porosity was determined to be 2.37×10^{-2} cm^3. Accordingly, 100 supports occupied 15.0% based on apparent volume and 2.0% based on actual volume of supports, and these occupations of supports in the reaction mixture is thought to be optimal for biodiesel production in this study.

3.2. Optimal stepwise methanol addition

Stepwise methanol addition is a proper method to prevent methanol inhibition on whole cells as well as enzyme during biodiesel production [7,9,11,12]. We have reported that the number of aliquot and feeding interval significantly affected biodiesel production using commercial enzyme in the previous study [11].The use of six feedings of methanol with an equivalent molar ratio of 0.75 to soybean oil at 3 h intervals was found to be the optimal mode for preventing methanol inhibition; approximately 95% of biodiesel conversion could be achieved within 20 h by using this method [11]. In this stepwise methanol addition, TMRMSO, aliquot number and feeding interval are important factors, and their effects on the biodiesel production using whole cells was investigated. The number of polyurethane piece was 100 as determined in the above section. I investigated firstly the effects of TMRMSO and aliquots number on biodiesel production at a fixed feeding interval (24 h), and subsequently determined optimal feeding intervals. TMRMSO was 3.0, 4.0, 4.5, 5.0 and 6.0 and each was divided into 3 or 4 aliquots. The first aliquot was fed to the mixture of soybean oil and polyurethane pieces onto which the cells were immobilized to initiate biodiesel production and the remaining aliquots were fed every 24 h thereafter. As shown in Fig. 3, when TMRMSO was 3.0 or 4.0, FBCs were about 75%, irrespective of aliquot number. When TMRMSO was 4.5, FBCs were 76.4% for 3 aliquots and 82.4% for 4 aliquots. When TMRMSO was 5.0, FBCs were 41.1% for 3 aliquots and 72.1% for 4 aliquots. When TMRMSO was 6.0, FBCs were 11.0% for 3 aliquots and 57.6% for 4 aliquots. Overall, FBCs were barely influenced by aliquots number at relatively low TMRMSO (3.0 and 4.0) while they were substantially influenced by aliquots number at relatively high TMRMSO (5.0 and 6.0). The FBC was highest at 4.5 of TMRMSO with 4 aliquots, but it was decreased at higher TMRMSO than 4.5, specifically, the decreases of FBCs were drastic at 3 aliquots of 5.0 or 6.0 of TMRMSO. These decreases may be due to the excess methanol feeding to the reaction mixture, which causes serious inhibition on the immobilized cells [5]. I further investigated the effect of feeding interval at the optimal TMRMSO (4.5) and aliquot number (4) as shown in Fig. 4. The feeding intervals were 6, 12, 18, 24 and 30 h, and the FBCs were 41.3, 63.5, 78.8, 82.4, and 75.2% at 144 h of reaction, respectively. Based on these results, I determined the optimal condition of stepwise methanol feeding was 4.5 TMRMSO, 4 aliquots and 24 h of feeding interval. This optimal condition was applied to the subsequent experiments.

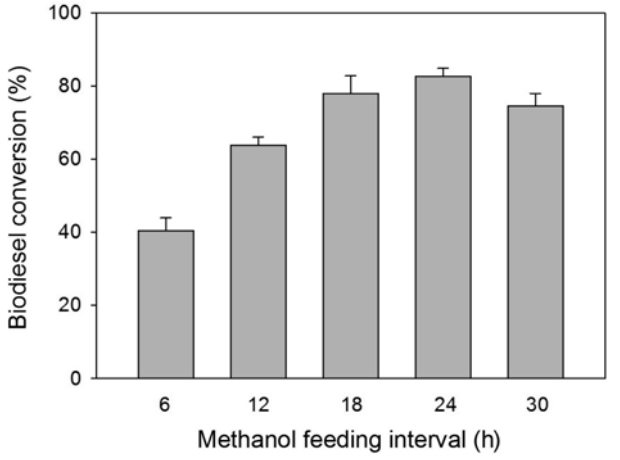

Fig. 4. Effect of feeding interval in stepwise methanol addition on the biodiesel production.

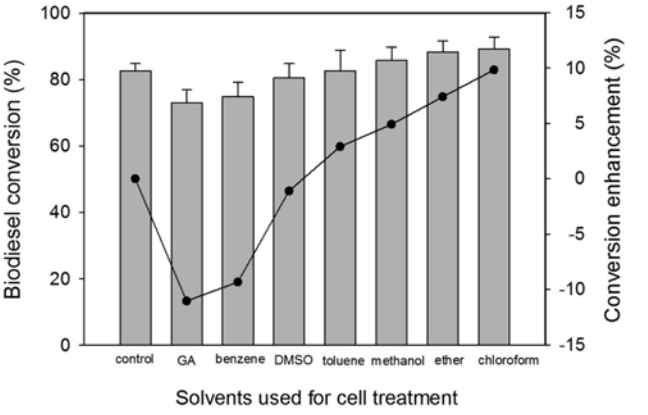

Fig. 5. Effect of chemical treatment of immobilized cells on biodiesel production. Whole cells immobilized on the supports were treated with each organic solvent of 0.1% for 2 h.

3.3. Chemical treatment

In the previous study using a bacterium, *S. marcescens* JYM110, biodiesel conversion was markedly increased when the bacterium was treated with toluene [1]. Chemical treatment of the bacterium with benzene also noticeably increased biodiesel conversion, while treatment with glutaraldehyde presented adverse influence [1]. However, after treated with glutaraldehyde, *R. oryzae* IFO 4697 could maintain high activity even after being reused for 35 batches of biodiesel production [23]. Therefore, the effect of chemical treatment on biodiesel production by whole cells should be investigated case by case. In this study, I used toluene, benzene, chloroform, ether, methanol, glutaraldehyde, and DMSO to treat the immobilized whole cells before or after vacuum-drying of the immobilized whole cells. In order to quantify the effect of chemical treatment, conversion enhancement was defined as the ratio of biodiesel conversion after chemical treatment to that without chemical treatment. The term of conversion enhancement was also used in the following sections to describe the addition effect of glycerol and water in the reaction mixture. As stated above, 0.1% (v/v) organic solvent relative to culture volume was added to the culture broth and incubated for 2 h. Chemical treatment of the whole cells with each organic solvent before vacuum-drying decreased biodiesel conversion compared to that of untreated supports (data not shown). As shown in Fig. 5, when the supports onto which whole cells were immobilized were treated with chloroform or ether after vacuum-drying, FBC was increased to 90.5 or 88.5% from 82.4%, respectively, corresponding to 9.8 or 7.4% of conversion enhancement, respectively. The other organic solvents barely influenced biodiesel production by the immobilized cells. I evaluated further the effect of chemical treatment with chloroform and ether at various concentrations (0.1, 0.5, 1.0, and 2.0%) and treatment durations (0.5, 1.0, 1.5, 2.0, and 3.0 h). However, no significant increase in biodiesel conversion was observed. Moreover, when the supports were treated with chloroform or ether at a concentration of more than 1.0% for 1.5, 2.0, or 3.0 h, biodiesel conversion became lower than those of untreated supports. The chemical treatment could affect the cell wall so that reactants, soybean oil and methanol, penetrate more easily into the cells, and so that products, biodiesel and glycerol, release readily out of the cells [11,26,27]. However, high concentration and long treatment duration could cause detrimental effects on the enzymes in the cells [26,27]. Despite of increase in the biodiesel conversion by chemical treatment, I did not consider further this treatment in the following experiments because its effect was fall short of expectation but requires an extra complex process.

3.4. Effect of glycerol removal or glycerol addition on biodiesel production

During biodiesel production *via* transesterification, glycerol is also generated and its amount is about 10% (w/w) relative to biodiesel produced. Since the transesterification with acid or alkali catalyst is reversible reaction, methanol is usually supplied in excess to promote forward reaction and to maximize equilibrium constant of the reaction [29]. Therefore, removal of glycerol may prevent reverse reaction, and facilitate biodiesel production. In addition, since glycerol is a viscous compound, its presence in the reaction mixture may hinder the mass transfer of compounds into and out of whole cells immobilized onto polyurethane foam. Accordingly, intermittent removal of glycerol during biodiesel production was expected to enhance biodiesel production. Biodiesel was produced under optimal conditions determined in above sections; 100 pieces of supports, 4.5 molar equivalent methanol to soybean oil, 4 equal aliquots and 24 h of feeding

interval. The first aliquot was fed to the flask containing soybean oil and 100 supports to trigger transesterification, and the remaining 3 aliquots were fed to the flask one by one every 24 h. Just before feeding 2nd, 3rd and 4th aliquot, reaction mixture was centrifuged, and glycerol was removed by a pipette. Contrary to expectations, the effect of glycerol removal on biodiesel production was negligible, that is, IBCRs and FBCs were very close to those from the experiments without glycerol removal (data not shown). Unlike the reversible transesterification by chemical catalysts, the transesterification by whole cells appeared to be apparently irreversible reaction. Moreover, since glycerol is a hydrophilic compound, some amount of methanol may have been removed along with glycerol. Therefore, glycerol removal does not have any advantages from economic viewpoint as well as from reaction kinetics.

In the previous study, we suggested pre-dissolution of methanol in biodiesel or water and this could prevent methanol inhibition on the enzyme [11], which evolved further to the development of pseudo-two phase partitioning bioreactor (P-TPPB) [4]. Since glycerol has strong affinity to methanol as stated above and it has minor inhibition on the commercial enzyme [11], I investigated the effect of glycerol addition on the biodiesel production using whole cells in this study. Glycerol was added to the mixture of soybean oil and cell-immobilized supports, and then an aliquot of methanol was fed to initiate biodiesel production. The biodiesel conversions with glycerol addition were compared with that without glycerol addition, and their ratios were defined as conversion enhancements. As presented in Fig. 6, when 5% (w/w) glycerol relative to soybean oil was initially added to the reaction mixture, FBC was increased to 90.3%, corresponding to 9.6% of enhancement. This increase in biodiesel was thought to be related with the mitigation of methanol inhibition on whole cells. However, when the amount of glycerol added initially was increased further to 20 or 25%, the biodiesel conversion became lower than that without glycerol addition. This decrease may be due to the inhibition on whole cells by excess glycerol, and the dilution of methanol by the glycerol, resulting in poor mass transfer of methanol to the interface with oil [30,31].

3.5. Effect of water on biodiesel production

In chemical esterification reaction in which an acid and an alcohol reacts to produce an ester and water, reverse reaction can occur in the presence of water. Accordingly, continuous removal of water can promote forward reaction, to produce the ester. On the other hand, water is essential for many enzymatic reactions, and some content of water was required to obtain high biodiesel conversion using enzymes or whole cells [26,32]. In addition, since methanol is a hydrophilic compound, it is readily dissolved in water and this could prevent methanol inhibition on whole cells. It has been demonstrated that pre-dissolution of methanol in water prevented methanol inhibition on the commercial enzyme [11]. Based on these backgrounds, I investigated the effect of water content on biodiesel production by whole cells. As shown in Fig. 7, FBC was increased with the increase of water content, and the highest conversion of 92.7%, corresponding to 12.5% of conversion enhancement, was obtained at 3% (w/w) water relative to oil. When the water content was increased further to 5 and 7%, the biodiesel production was decreased consistently but still higher than that without water addition.

3.6. Interaction effect of glycerol and water on biodiesel production

In above sections, initial glycerol content (5%) or initial water content (3%) relative to soybean oil noticeably increased FBC. I further investigated their combinational effect on the FBC as shown in Fig. 8. When 5% glycerol and 3%

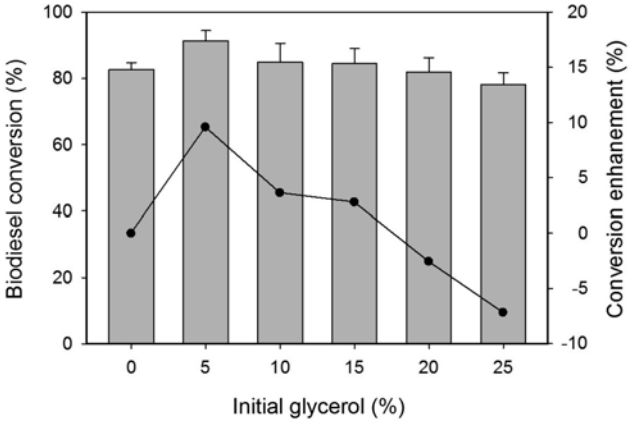

Fig. 6. Effect of initial glycerol addition on the biodiesel production by immobilized cells.

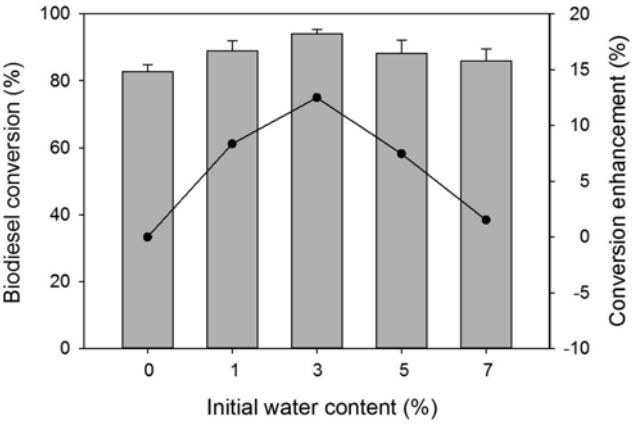

Fig. 7. Effect of initial water addition on the biodiesel production by immobilized cells.

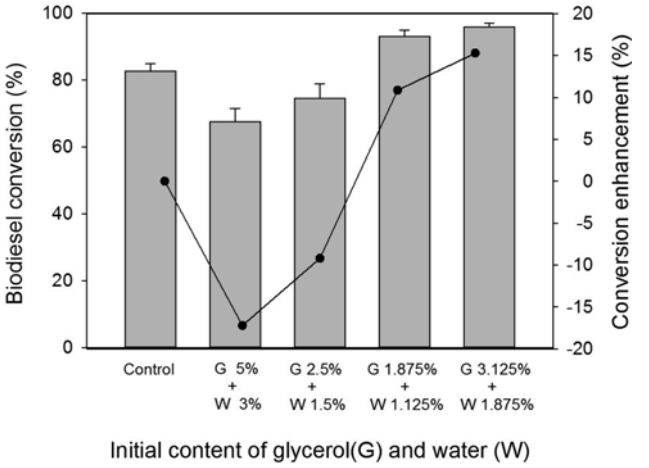

Fig. 8. Combinatorial effect of glycerol and water addition on the biodiesel production by immobilized cells.

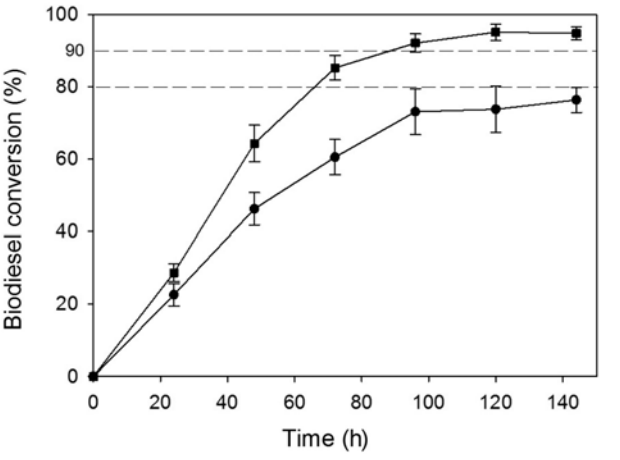

Fig. 9. Enhancement of biodiesel production by whole cells. ●: under initial reaction conditions, ■: under optimum reaction conditions.

water were initially placed together, FBC was significantly decreased to 68.2%, corresponding to -17.2% of biodiesel conversion. When half of them (2.5% of glycerol and 1.5% of water) were fed together, FBC was also decreased significantly to 74.8%, corresponding to -9.2% of conversion enhancement. On the contrary, when the total amount of glycerol and water was 3 or 5%, maintaining their ratio with 5:3, FBC was remarkably increased to 91.4 or 95.0%, respectively. These increases were higher than that from sole glycerol (90.3%) or sole water (92.7%) additions. There should be interaction effects among support number, glycerol content and water content on biodiesel production. In particular, glycerol and water contents are closely related because both of them are hydrophilic compounds, dissolving methanol to prevent methanol inhibition on immobilized whole cells. However, while water is believed to be essential to maintain the activity of the cells, glycerol in excess may cause adverse effect.

Based on these overall results, it was concluded that 100 pieces of supports with immobilized cells, initial glycerol (3.125% relative to soybean oil) and water (1.875% relative to soybean oil) contents, 4.5 equivalent molar ratio of methanol to soybean oil, 4 aliquot and 24 h of feeding interval was the optimal reaction condition for biodiesel production. It should be noted that since chemical treatment required intensive labor and cost despite of non-negligible increase in FBC, it seemed to be impractical from an economic viewpoint, thereby it was excluded. I finally compared the biodiesel production after optimization with that before optimization of reaction conditions.

As can be seen in Fig. 9, the biodiesel conversions at 24 h of reaction were 22.6% for initial reaction conditions and 28.7% for optimal reaction conditions. Considering the theoretical maximum conversions for the first aliquot are 50% for initial reaction conditions and 37.5% for optimal reaction conditions, both IBCR and biodiesel conversion under optimal reaction conditions were significantly enhanced compared with those under initial reaction conditions. The enhancement was noticeable for the first 48 h, that is, IBCR for the first 48 h was 1.34%/h under optimal reaction conditions, which was 52.3% higher than that under initial reaction conditions. As the dashed lines indicate, the biodiesel conversion under initial reaction conditions could not reach 80% but it took only about 90 h to reach 90% of biodiesel conversion under optimal reaction conditions. The FBCs were 95.1 and 86.5% for optimal and initial reaction conditions, respectively. Finally, it should be noted that the biodiesel conversion over 95% was achieved when the optimal reaction conditions were applied to the production of biodiesel.

4. Conclusion

We previously suggested optimal methods to prepare immobilized whole cell biocatalyst including microorganism strain, immobilization media and drying method. However, the biodiesel conversion rate and final biodiesel conversion needed improving. In this study, I devised several methods to enhance biodiesel production. The increase in the number of supports onto which whole cells were immobilized significantly increased biodiesel conversion rate and biodiesel conversion, but biodiesel production was rather inhibited when the volume ratio of supports to reaction mixture was over 18.0 or 2.4% of reaction mixture based on apparent or actual volume of supports, respectively due to mass transfer limitation . The molar ratio of methanol to soybean oil was 4.5, and the optimal aliquot number of methanol and

feeding interval were found to be 4 and 24 h, respectively. The chemical treatment of immobilized cells with 0.1% of chloroform increased biodiesel conversion from 82.4 to 90.5%. Intermittent glycerol removal during biodiesel production did not provide any positive results while initial glycerol at 3% (w/w) increased biodiesel production to 90.3%. Initial water addition also increased biodiesel production, and the highest biodiesel conversion of 92.7% was achieved at 3% (w/w) water content. I further investigated the effect of the mixture of glycerol and water, and the biodiesel conversion was remarkably increased to 95.0% at the total mass of 5% with their ratio of 5:3. In brief, by optimizing reaction conditions and adding glycerol and water, biodiesel conversion rate and biodiesel conversion were significantly increased by 26.8 and 24.1%, respectively.

As stated by many researchers, biodiesel production using whole cells has serious drawbacks of low biodiesel production rate and conversion although it has been considered as the promising clean and economical technology for biodiesel production. This study shows that biodiesel production using whole cells could be enhanced by optimizing reaction conditions and by addition glycerol and water, which preventing methanol inhibition on enzymes or whole cells, and maintain enzyme activity inside the whole cells. This study is believed to facilitate biodiesel production by whole cells, and more advanced methods should be devised further to make biodiesel production by whole cells more feasible.

Acknowledgements

This work was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2015R1D1A1A01060540). The author greatly appreciates this support. The author also thanks Young Wook Go, Pansuwan Supaporn and Jin Seon Kyoung for their technical assistance.

References

1. Kwon, M. H. and S. H. Yeom (2015) Optimization of one-step extraction and transesterification process for biodiesel production from the marine microalga *Nannochloropsis* sp. KMMCC 290 cultivated in a raceway pond. *Biotechnol. Bioproc. Eng.* 20: 276-283.
2. Atadashi, I. M., M. K. Aroua, A. Z. Abdul, and N. Sulaiman (2013) The effects of catalysts in biodiesel production: A review. *J. Ind. Eng. Chem.* 19: 14-26.
3. Lee, J. H., S. B. Kim, H. Y. Yoo, Y. J. Suh, G. B. Kang, W. I. Jang, J. W. Kang, C. H. Park, and S. W. Kim (2013) Biodiesel production by enzymatic process using Jatropha oil and waste soybean oil. *Biotechnol. Bioproc. Eng*. 18: 703-708.
4. Go, Y. W. and S. H. Yeom (2014) Application of pseudo-two phase partitioning bioreactor (P-TPPB) to the production of biodiesel. *Bioproc. Biosyst. Eng*. 37: 269-275.
5. Kyeong, J. S. and S. H. Yeom (2014) Preparation of immobilized whole cell biocatalyst and biodiesel production using a packed-bed bioreactor. *Bioproc. Biosyst. Eng.* 37: 2189-2198.
6. Lateef, F. A., O. D. Onukwuli, U. C. Okoro, P. M. Ejikeme, and P. Jere (2014) Some physical properties and oxidative stability of biodiesel produced from oil seed crops. *Kor. J. Chem. Eng.* 31: 725-731.
7. Ranganathan, S. V., L. Marasimhan, and K. Muthukumar (2008) An overview of enzymatic production of biodiesel. *Bioresour. Technol*. 99: 3975-3981.
8. Li, L., W. Du, D. Liu, L. Wang, and Z. Li (2006) Lipase-catalyzed transesterification of rapeseed oils for biodiesel production with a novel organic solvent as the reaction medium. *J. Mol. Catal. B: Enz.* 43: 58-62.
9. Fjerbaek, L., K. V. Christensen, and K. V. Norddahl (2009) A review of the current state of biodiesel production using enzymatic transesterification. *Biotechnol. Bioeng.* 102: 1298-1315.
10. Chen, H. C., H. Y. Ju, T. T. Wu, Y. C. Liu, C. C. Lee, C. Chang, Y. L. Chung, and C. J. Shieh (2011) Continuous production of lipase-catalyzed biodiesel in a packed-bed reactor: Optimization and enzyme reuse study. *J. Biomed. Biotechnol.* doi: 10.1155/2011/950725.
11. Jeon, D. J. and S. H. Yeom (2011) Comparison of methods for preventing methanol inhibition in enzymatic production of biodiesel. *Kor. J. Chem. Eng*. 28: 1420-1426.
12. Shimada, Y., Y. Watanabe, A. Suglihara, and Y. Tominaga (2002) Enzymatic alcoholysis for biodiesel fuel production and application of the reaction to oil processing. *J. Mol. Catal. B: Enz.* 17: 133-142.
13. Samukawa, T., M. Kaieda, T. Matsumoto, K. Ban, A. Kondo, Y. Shimada, H. Noda, and H. Fukuda (2000) Pretreatment of Immobilized *Candida antarctica* lipase for Biodiesel fuel production from plant oil. *J. Bioresour. Bioeng.* 90: 180-183.
14. Hama, S., H. Yamaji, T. Fukumizu, T. Numata, S. Tamalampudi, A. Kondo, H. Noda, and H. Fukuda (2007) Biodiesel-fuel production in a packed-bed reactor using lipase-producing *Rhizopus oryzae* cells immobilized within biomass support particles. *Biochem. Eng. J.* 34: 273-278.
15. Talukder, M. M. R., J. C. Wu, T. B. van Nguyen, N. M. Fen, and Y. L. S. Melissa (2009) Novozym 435 for production of biodiesel from unrefined palm oil: Comparison of methanolysis methods. *J. Mol. Cat. B: Enz.* 60: 106-112.
16. Jeon, D. J. and S. H. Yeom (2010) Two-step bioprocess employing whole cell and enzyme for economical biodiesel production. *Kor. J. Chem. Eng.* 27: 1555-1559.
17. Jin, G., T. J. Bierma, C. G. Hamaker, R. Mucha, V. Schola, J. Stewart, and C. Wade (2009) Use of a whole-cell biocatalyst to produce biodiesel in a water-containing system. *J. Environ. Sci. Health, Part A.* 44: 21-28.
18. Jin, G., T. J. Bierma, C. G. Hamaker, R. Rhykerd, and L. A. Loftus (2008) Producing biodiesel using whole-cell biocatalysts in separate hydrolysis and methanolysis reactions, *J. Environ. Sci. Health, Part A*. 43: 589-595.
19. Arai, S., K. Nakashima, T. Tanino, C. Ogino, A. Kondo, and H. Fukuda (2010) Production of biodiesel fuel from soybean oil catalyzed by fungus whole-cell biocatalysts in ionic liquids. *Enz. Microbial. Technol.* 46: 51-55.
20. Lee, J. H., S. B. Kim, H. Yoo, J. H. Lee, S. O. Han, C. H. Park, and S. W. Kim (2013) Co-immobilization of *Candida rugosa* and *Rhyzopus oryzae* lipases and biodiesel production. *Kor. J. Chem. Eng.* 30: 1335-1338.
21. Tamalampudi, S., M. R. Talukder, S. Hama, T. Numata, A. Kondo, and H. Fukuda (2008) Enzymatic production of biodiesel from Jatropha oil: A comparative study of immobilized-whole

cell and commercial lipases as a biocatalyst. *Biochem. Eng. J.* 39: 185-189.
22. Li, W., W. Du, and D. Liu (2007) *Rhizopus oryzae* IFO 4697 whole cell catalyzed methanolysis of crude and acidified rapeseed oils for biodiesel production in *tert*-butanol system. *Proc. Biochem*. 42: 1481-1485.
23. Sun, T., W. Du, and D. Liu (2011) Comparative study on stability of whole cells during biodiesel production in solvent-free system. *Proc. Biochem.* 46: 661-664.
24. Matsumoto, T., S. Takahashi, M. Kaieda, M. Ueda, A. Tanaka, H. Fukuda, and A. Kondo (2001) Yeast whole-cell biocatalyst constructed by intracellular overproduction of *Rhizopus oryzae* lipase is applicable to biodiesel fuel production. *Appl. Microbiol. Biotechnol.* 57: 515-529.
25. Du, W., W. Li, T. Sun, X. Chen, and D. Liu (2008) Perspectives for biotechnological production of biodiesel and impacts. *Appl. Microbiol. Biotechnol*. 79: 331-337.
26. Yoshida, A., S. Hama, N. Tamadani, H. Fukuda, and A. Kondo (2012) Improved performance of a packed-bed reactor for biodiesel production through whole-cell biocatalysis employing a high-lipase-expression system. *Biochem. Eng. J.* 63: 76-80.
27. Yoshid, A., S. Hama, N. Tamadani, H. Noda, H. Fukuda, and A. Kondo (2012) Continuous production of biodiesel using whole-cell biocatalyst: Sequential conversion of an aqueous oil emulsion into anhydrous product. *Biochem. Eng. J.* 68: 7-11.
28. Sim, J. H., A. H. Kamaruddin, and S. Bhatia (2009) Effect of mass transfer and enzyme loading on the biodiesel yield and reaction rate in the enzymatic transesterification of crude palm oil. *Energy Fuels*. 23: 4651-4658.
29. Lukovic, N., K. J. Zorica, and D. Bezbradica (2011) Biodiesel Fuel Production by Enzymatic Transesterification of Oils: Recent Trends, Challenges and Future Perspectives, Alternative Fuel, Dr. Maximino Manzanera (Ed.), ISBN: 978-953-307-372-9, InTech.
30. Le, W., W. Du, D. Liu, and Y. Yao (2008) Study on factors influencing stability of whole cell during biodiesel production in solvent-free and *tert*-butanol system. *Biochem. Eng. J*. 41: 111-115.
31. Hong, W. P., J. Y. Park, K. Min, M. J. Ko, K. Park, and Y. J. Yoo (2011) Kinetics of glycerol effect on biodiesel production for optimal feeding of methanol. *Kor. J. Chem. Eng*. 28: 1908-1912.
32. Shieh, C. J., H. F. Liao, and C. C. Lee (2003) Optimization of lipase-catalyzed biodiesel by response surface methodology. *Bioresour. Technol.* 88: 103-106.