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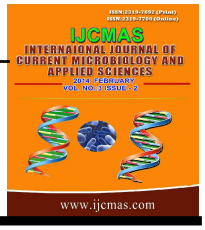

Original Research Article

Effect of parameters on enhanced production of fatty acid methyl esters through immobilized whole cell biocatalyst

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K e y w o r d s

Biodiesel; Whole Cell Biocatalyst; *Rhizopus oryzae*; Novozym 435; Fatty acid methyl ester; trans-esterification

A B S T R A C T

Direct use of intracellular lipase as a whole cell biocatalyst for biodiesel production has been attractive since immobilization can be carried out spontaneously during the process of cell cultivation. In this research, effect of different parameters that affecting the efficiency of enzymatic transesterification using *Rhizopus oryzae* (ATCC 9374) whole cell biocatalyst that was immobilized within biomass support particles and treated with glutaraldehyde solution was investigated. The maximum methyl esters content in the reaction mixture reacheed 84 wt.% using *R. oryzae* whole cell biocatalyst under optimum condition consisted of cuboid form of biomass support particles, olive oil as carbon sources in basal medium, emulsification using ultrasonicated reaction mixtures, 15 wt.% water content and 7 wt.% immobilized biomass support particles, addition of methanol at 0, 4 and 18 h and at the reaction time of 48 h which is remarkably comparable with yield of biodiesel at 90 wt.% obtained with Novozym 435 as the most effective extracellular immobilized lipase. These results suggest that, taking into consideration the simplicity of the lipase production process and usability for successive batch cycles, *R. oryzae* whole cell biocatalysts under the optimal conditions is a promising means of biodiesel production for industrial use.

Introduction

The consideration of reduction of fossil resources, increase in fossil fuel prices and increasing social environmental consciousness has led to a search for fuels which may be produced from renewable sources like plant biomass (Antczak, *et al*., 2009). Some studies have considered the methods that are making it possible to use triglycerides (vegetable oils or animal fats) as a substitute fuel for diesel engines (Fukuda et al., 2001). Directly using vegetable oils or oil blends is commonly

considered impractical due to its high viscosity, acid composition and free fatty acid content (Fukuda et al., 2001). Thus, vegetable oils must be processed so as to decrease viscosity and improve the physical properties of fuels that are necessary to be directly used in today's diesel engines. The most common method to transform oil into biodiesel as short chain alkyl (methyl/ethyl) esters is transesterification that is similar to hydrolysis reaction in which water is replaced by alcohol (Fukuda et al., 2001; Robles-Medina et al., 2009). Biodiesel which is biodegradable and nontoxic has low discharge profile and environmentally advantageous (Bisen et al., 2010). Transesterification reaction can be conducted by lipases as enzymatic biocatalyst. Lipase-catalyzed transesterification has many advantages such as decreasing process stages in biodiesel fuel production, low operation temperature and easy separation of the glycerol by-product without complicated operation stages (Du et al., 2004). The *Candida antarctica* lipase immobilized on acrylic resin available in the market (Novozym 435) was the most effective lipase among any of the extracellular lipases tested for transesterification reaction of vegetable oils (Shimada, et al., 1999; Xu et al., 2003 Du et al., 2004). Although immobilization of extracellular enzyme appeared to be a common method for enzymatic alcoholysis, it requires complex methods for separation, purification and stabilization of lipases which increase the process cost in industrial scale (Ranganathan et al., 2008). Over the recent years, there has been significant interest in the direct use of intracellular lipase as a whole cell biocatalyst for biodiesel production in which the purification and stabilization of the enzyme are not required because immobilization happens naturally during the cell cultivation process (Sun et al., 2009). To present, much researches carried out on use of *R. oryzae* whole cell biocatalyst for transesterification reaction and expressed that *R. oryzae* whole cell biocatalyst has ability to catalyze methanolysis reaction of plant oils such as soybean oil. This reaction could be performed in solvent free system and in the presence of organic solvent. The effect of some parameters on biodiesel production using whole cell biocatalyst has been reported individually. In most studies biocatalyst was added to the reaction mixture based on the number of biomass support particle (BSPs). Ban et al. reported that the optimal amount of water in reaction mixture is 15 wt.% (Ban et al., 2001). Effect of carbon source in basal medium was investigated and olive and soybean oil have shown the best results (Zeng et al., 2006). Effect of cross-linking treatment with glutharaldehyde (GA) on the stability of biocatalyst in consecutive cycle was investigated with Ban et al. and they reported that GA treatment significantly increases the lipase stability (Ban et al., 2002). Hama et al reported that the emulsification of reaction mixture using ultrasonication increased fatty acid methyl esters (FAMEs) content in the reaction mixture (Hama et al., 2007). To our knowledge, all effective parameters have not yet studied in order to improve the FAMEs content comprehensively.

The main objective of this study was to improve of transesterification efficiency of *R. oryzae* (ATCC 9374) whole cell biocatalyst immobilized with BSPs for biodiesel production from soybean oil by optimization of process parameters. Moreover, the FAMEs content of enzymatic transesterification catalyzed by commercially immobilized enzyme

(Novozym 435) in solvent free system were measured to compare the results with those obtained by whole cell biocatalyst.

Materials and Methods

Materials

Refined soybean oil was purchased from Behshahr Industrial Co. (Tehran, Iran). Commercial immobilized lipase from *Candida antarctica* (Novozym 435) was provided as a gift from Novo Nordisk (A.S., Denmark-Tehran Office). Polyurethane foam was obtained from local market. *R. oryzae* ATCC 9374 purchased from PTCC (Persian Type Culture Collection, IROST, Tehran, Iran). Methyl esters of palmitic acid, stearic acid, oleic acid, linoleic acid and linolenic acid methyl ester were purchased from Sigma in analytical grade for chromatography. All other chemicals were of analytical grade.

R. oryzae whole cell biocatalyst preparation

Whole cell biocatalyst experiments were carried out using *R. oryzae* ATCC 9374. The basal culture medium consisted of 70 g polypeptone (50 wt.% pepton, 50 wt.% trypton), 1.0 g $NaNO_3$, 1.0 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$, and 30 g oil (refined olive, soybean and canola oil) in 1l tap water (pH was initially adjusted to 5.6). *R. oryzae* was grown on potato dextrose agar (PDA) slant. Erlenmeyer flask (500 ml) containing 100 ml of the basal medium were inoculated by aseptically transferring spores (about 10^6 spores) from slant, and incubated for 60–72 h at 35°C on an orbital shaker (150 rotation/min) with 0.33 g BSPs subjected to prior sterilization. Reticulated Polyurethane Foam (PUF) with a particle voidage of more than 97% used as BSPs. To examine the effect of BSPs size on transesterification efficiency, BSPs were cut in different size of 6×6×3 mm^3 cuboids and 6 mm^3 cubes; then added to the basal medium. The *R. oryzae* cells became well immobilized within the cavities and pores of BSPs as a natural result of their growth during shake flask cultivation. After that, the immobilized cells were separated from the culture broth by filtration, washed with tap water for 1 minute, dried at 25°C for 24 h and then cross-linked with glutaraldehyde (GA) according to Ban et al. method in order to increase the lipase stability (Ban et al., 2002). In this way the dried cells were treated with a 0.1% (v/v) GA solution at 25°C for 1 h then were shaken in phosphate buffer at 4°C for 5 min, washed with tap water for few minutes, and then dried for 24 h at room temperature. Finally, the GA-treated cells were used as a biocatalyst for transesterification reaction.

Lipase-catalyzed transesterification

R. oryzae whole-cell biocatalyst

The methanolysis reaction took place at 35°C in a 50-ml flask with incubation on an orbital shaker (150 rotations/min). The reaction mixture contained soybean oil, 9.65 g; 0.1M phosphate buffer (pH 6.8), 1.5 ml; 50 BSPs and methanol, 0.35 g which the latter was added stepwise to the reaction mixture three times at 0, 24 and 48 h after reaction started. For full conversion of oil to FAMEs, three molar equivalents of methanol are necessary. To improve the transesterification yield, the effect of two factors contained the amount of water content by substrate weight in the range of 0-25% using 0-0.25 ml phosphate buffer and BSPs weight percent of 1-10 (0.1-1g) on FAMEs content was

investigated. Moreover, methanol was added at different times to study the time course of the reaction.

Novozym 435 as a biocatalyst

The methanolysis reaction carried out with the immobilized lipase from *Candida antarctica* (Novozym 435) in the solvent-free system in a 50-ml flask at 35°C and 150 rpm. The reaction mixture contained soybean oil, 9.65 g; 0.1 – 0.6 g immobilized lipase and methanol, 0.35 (without any distilled water) which the latter was added stepwise to the reaction mixtures three times at 0, 24 and 48 h after reaction started and for study the time course of the reaction, methanol added at different hours after start of the reaction.

Analytical methods

The FAMEs content in the reaction mixture was analyzed using a GC-3800CP gas chromatography (Varian Crop. Netherlands) connected to a cpsil-5CB capillary column (0.32mm×30 m, Varian, Netherlands). Samples (200 µl) were taken from the reaction mixture at specified time and centrifuged at 13,000 × g for 5 min to obtain the upper organic phase. Eighty micro liters of the upper layer and 20 µl tricaprylin as internal standard were accurately measured and mixed thoroughly in a bottle containing 2 ml hexane as solvent. Also small amount of anhydrous sodium sulfate as dehydrating agent were added. A 1.0 µl aliquot of the treated sample was injected into the gas chromatograph. The column temperature was held at 150 °C for 1 min, raised to 200°C at 20°C/min then increased to 207°C at 1°C/min and finally raised to 300°C at 30°C/min and maintained at this temperature for 24 min. The temperature for injector and flame ionization detector (FID) were set at 270°C and 300°C, respectively.

Results and Discussion

Effect of BSPs size on FAMEs content

R. oryzae cells form a dense film near the surface of the polyurethane foam BSPs and are seldom present inside the particles because of oxygen starvation in immobilized cell culture (Ban et al., 2001). This observation suggests that, in order to improve lipase activity per unit volume of BSP and enhance the volumetric methyl ester production in enzymatic transesterification utilizing *R. oryzae* cells immobilized inside BSPs as whole-cell biocatalyst, the specific surface area of the BSPs should be increased. To examine the effect of BSPs size on methanolysis of soybean oil, cuboid and cube PUFs were used as BSPs and then 50 BSPs were added to the reaction mixture in presence of 15 wt.% water by substrate to weight. Table 1 shows the methanolysis activity of the different size of BSP-immobilized cells after adding methanol in start of the reaction.

The results indicated that in case of using cuboids immobilized BSPs FAMEs content was significantly greater than that of cubes because of the larger specific surface area of cuboid forms of BSPs. Difference in FAMEs content after 4 h of the reaction was more than that of obtained after 12 h which is indicated that the larger surface area the faster transesterification reaction took place. Finally, FAMEs content in both cases reached 33 wt.% as one molar equivalent of methanol was added to the reaction mixture in both cases. Therefore cuboid forms of BSPs were used for further experiments.

Effect of carbon sources in basal medium

Several oils were employed as main carbon sources in the basal medium and their effect on biodiesel production using whole cell biocatalyst were investigated (Zeng et al., 2006). The previous works showed that refined oils achieve higher activity than their crude oils and among them refined soybean oil showed the most FAMEs content in the reaction mixture, and therefore refined soybean oil in further researches has been used (Li et al., 2007a,b; 2008a,b.). However, olive oil has also been suggested with some other scientists to be as a suitable carbon source in basal medium (Ban et al., 2011; 2002; Hama et al., 2004; Oda et al., 2005; Hama et al., 2006). ListeRead phoneticallyTo elucidate the effect of carbon source on transesterification efficiency, various refined oils containing olive, soybean and canola oils were used. Fig. 1 shows the time course of methanolysis catalyzed by cells grown on the different carbon sources. It can be seen that during whole cell catalyzed methanolysis of soybean oil for FAMEs production, the cells cultured with refined olive oil had highest rate of biodiesel production than cells cultured on refined soybean and canola oils. The reason could be that the refined olive oil had the best performance to keep lipase in the cell wall of the biocatalyst.

Effect of emulsification of reaction mixture on FAMEs content

In order to study the effect of emulsifying of reaction mixture on the production of methyl esters, ultrasonicated and non-ultrasonicated reaction mixtures were employed for methanolysis of soybean oil using whole cell biocatalyst. Fig. 2 shows the time course of FAMEs content under both conditions. In the case of emulsification of reaction mixture using ultrasonication, FAMEs content reaches 32 wt.% after 4 h that is 6 wt.% greater than the non- ultrasonicated reaction mixture. Because the lipase catalysis occurs in the interfacial layer between the hydrophobic and hydrophilic phases, the much larger water/oil interface appears to lead in increased access of the substrates to the lipase enzyme (Nielsen et al., 1990). Another reason may be due to the mass transfer resistance which is less when the reaction mixture is emulsified. Consequently, there is not a lot of effort into the transfer of substrate to the cell wall of biocatalyst in the case of ultrasonicated samples. This discovery indicated that emulsification of the reaction mixture before enzymatic transesterification has a beneficial effect on biodiesel production utilizing whole cell biocatalyst. Further experiments were therefore made use of emulsified substrates.

Effect of water content and weight of biocatalyst on reaction mixture

Water content is a key factor that influences the activity of an enzyme in a non-aqueous medium because lipases have the unique activating feature at the interface between an aqueous phase and an organic phase (Zheng et al., 2010).

Fig. 3 shows the FAMEs content in the reaction mixture using whole cell biocatalyst at different water content and weight of biocatalyst. Biodiesel in the reaction mixture (after 96 h) increases while water content ratio increases up to 15 wt.% and then decreases at higher ratio. Therefore, the optimum water content for immobilized *R. oryzae* whole cell was obtained as 15 wt. % that confirm the

Table.1 FAMEs content (wt.%) at different size of BSPs

Time (h)	Cuboid BSPs	Cubic BSPs
4	29	21
8	30	27
12	33	31

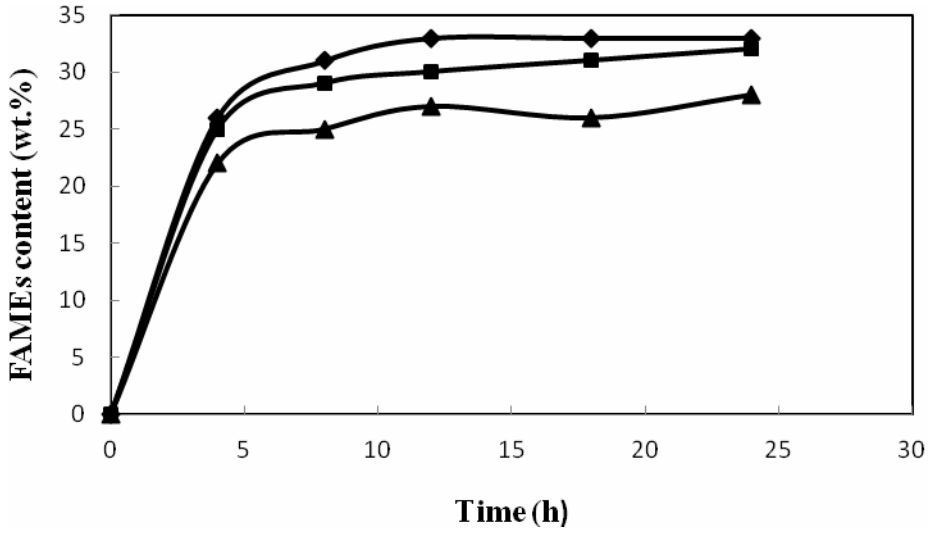

Fig.1 FAMEs content in methanolysis of soybean oil at different carbon source in cell cultures (50 BSPs as catalyst and reaction temperature at 35 °C), olive oil (♦), soybean oil (■), canola oil (▲).

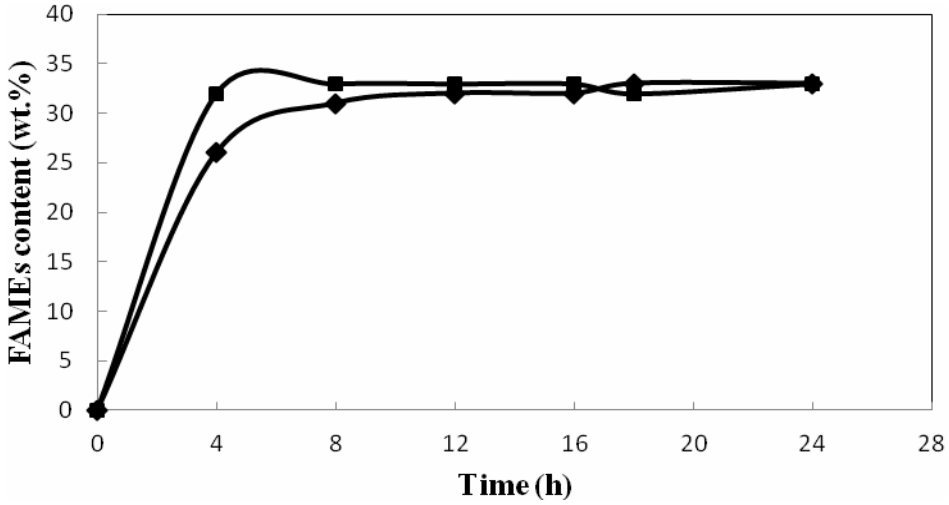

Fig.2 FAMEs content in methanolysis of soybean oil using ultrasonicated (■) and non-ultrasonicated (◆) reaction mixtures (50 BSPs as catalyst and reaction temperature at 35 °C)

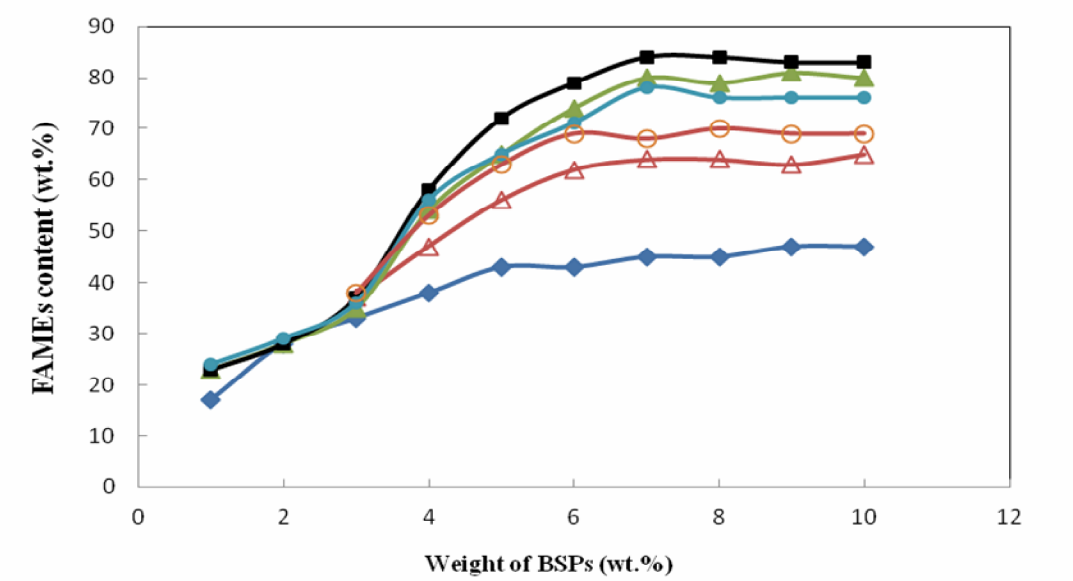

Fig.3 FAMEs content in methanolysis of soybean oil at different water content and weight of BSPs after 96 h (addition of methanol 0, 24, 48 h and reaction temperature at 35 °C), 0% water (◆), 5%water (△), 10%water (▲), 15% water (■), 20%water (●), 25% water (○).

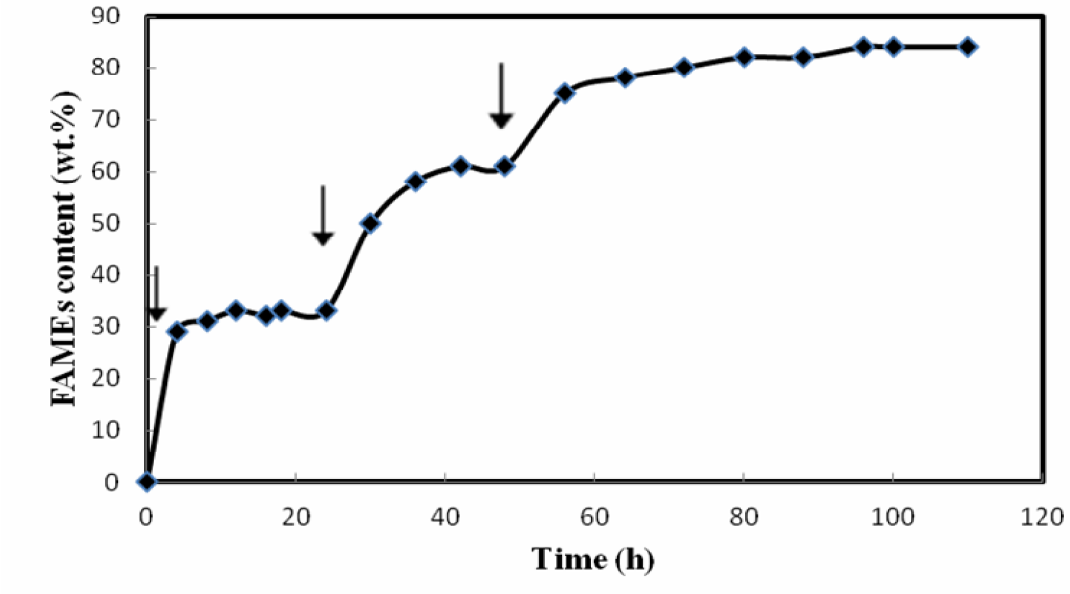

Fig.4 FAMEs content in methanolysis of soybean oil (addition of methanol at 0, 24 and 48 h). arrows shows the time of methanol feeding.

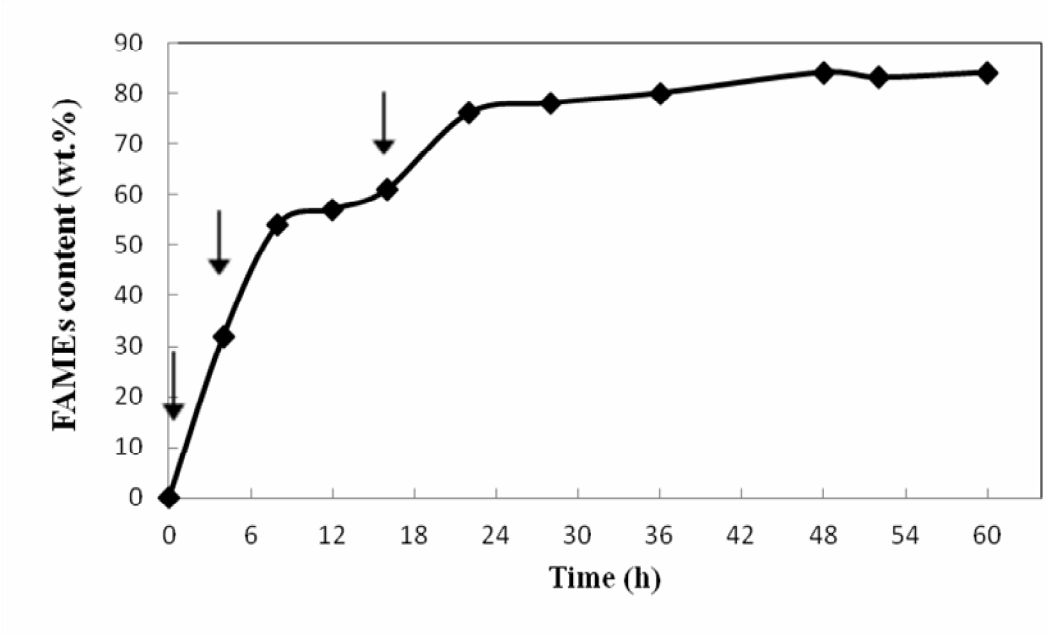

Fig.5 FAMEs content in methanolysis of soybean oil with new strategy (addition of methanl at 0, 4 and 18 h). arrows shows the time of methanol feeding.

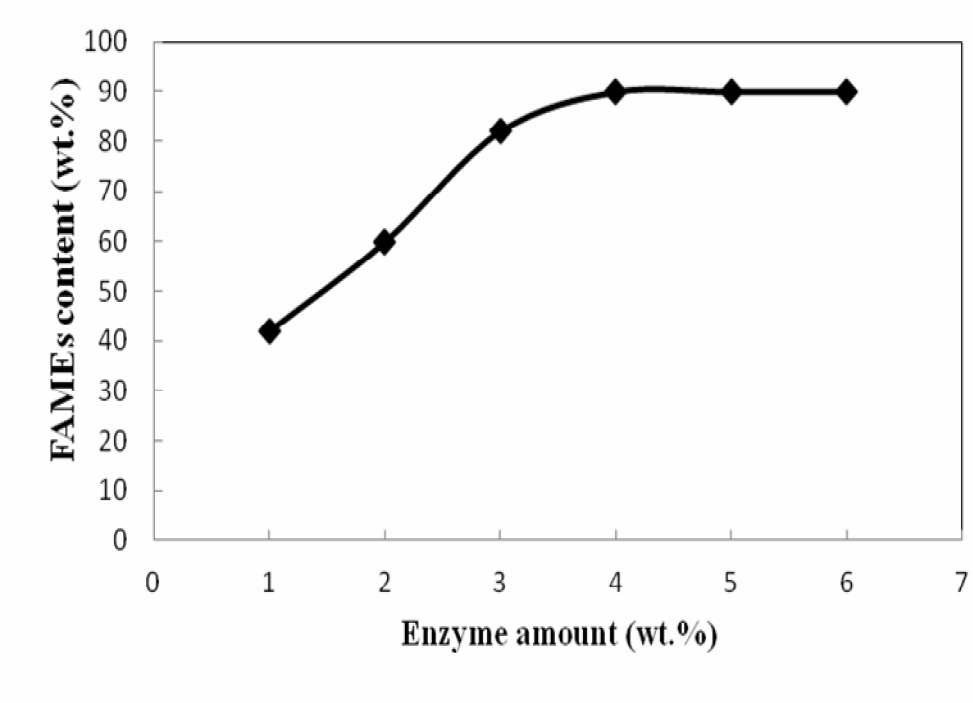

Fig.6 FAMEs content in methanolysis of soybean oil at different amounts of Novozyme 435 as a biocatalyst where methanol was added at 0, 4 and 18 h after the reaction started

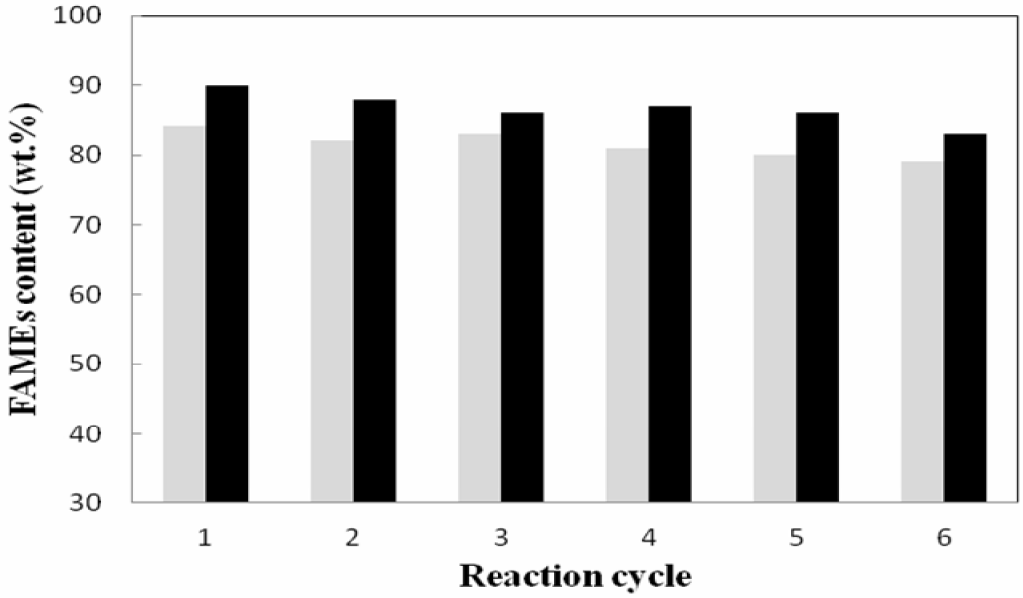

Fig.7 FAMEs content in methanolysis of soybean oil using GA treated *R. oryzae* whole-cell (gray) and Novozym 435 (black) as biocatalyst for repeated reaction cycles (addition of methanol in 0, 4 and 18

previous results (Ban et al., 2001). However, this parameter limited the activity of Novozym 435 for methanolysis reaction since the reaction mixture was closely maintained anhydrous (Tamalampudi et al., 2008). Water content of more than 500 ppm in soybean oil has been reported to lower the rate of biodiesel production utilizing Novozym 435 as biocatalyst (Shimada et al., 2002). Although it is widely reported that lipases require an optimal small amount of water to preserve their activity in organic media which should be determined for any specific reaction system, Novozym 435 seems to have enough content of water in itself to preserve the catalytic adaptation (Ognjanovic et al., 2009).

To our knowledge, certain number of BSPs was added to the reaction mixture (Ban et al., 2001; Hama et al., 2007; Oda et al., 2005). Sine cell concentration in all BSPs may not be identical, while decided to add BSPs based on weight fraction criterion. Fig. 3 shows that by increasing weight of BSPs up to 7 wt.%, methyl esters production increased and remained approximately constant up to BSPs amount of 10 wt.%. The highest methyl ester content of 84 wt.% was attained when the reaction mixture contained 1.5 ml buffer solution (15 wt.% water by substrate to weight) and 0.7 g BSPs (7 wt.% BSPs by substrate to weight). Because acyl migration occurs with intracellular lipase of immobilized cell in whole cell biocatalyst and water can increase cell permeability, in the presence of additional water the rate of transesterification reaction catalyzed by *R. oryzae* whole cell biocatalyst increases. However, the excess water decreases production of methyl esters because it may act as a competitive inhibitor for lipase-catalyzed transesterification (Tamalampudi et al., 2008).

Time course of transesterification

The time course of methanolysis of soybean oil with stepwise addition of methanol at 0, 24 and 48 h was studied. Fig. 4 shows the times course of FAMEs production at different times after reaction started with this feeding strategy. The reaction continues up to 96 h that is relatively long time, while at the time duration of 4 to 24 h and 36 to 48 h the FAMEs production had not much progress. It seems that methanol has been completely used in transesterification reaction at those time duration. In order to reduce time of reaction progress, new strategy of methanol addition was employed. Methanol was added stepwise at 0, 4 and 18 h after the reaction. The FAMEs content of 84 wt.% was attained during 48 h with this feeding strategy which is a time saving and cost effective method for biodiesel production (Fig. 5).

Enzymatic methanolysis reaction using Novozym 435 as biocatalyst

To compare the FAMEs content by Novozym 435 as biocatalyst with that of *R. oryzae* whole cell biocatalyst, the same feeding strategy was employed. Methanol was added stepwise to the reaction mixture at the start of the reaction, 4 h and 18 h. As depicted in Fig. 6 the effect of various amounts of Novozym 435 on the FAMEs production were investigated. The methyl esters production yield increased when the amount of Novozym 435 was increased up to 4 wt.% and maintained approximately constant up to Novozym 435 amount of 6 wt.%. Therefore 4 wt.% of Novozym 435 based on the weight of substrates was considered as optimum amount and used for further experiments. FAMEs content in the reaction mixture reached 90 wt.% after 48 h which was comparable to the FAMEs content obtained using *R. oryzae* whole cell biocatalyst.

Repeated use of BSP-immobilized cells and Novozym 435 for methanolysis of soybean oil

In order to test the stability of Novozym 435 and *R. oryzae* whole cell treated by GA as biocatalyst, both were separated from the reaction mixture by filtration and were used directly for the next cycle. The time of methanolysis using *R. oryzae* whole cell and Novozym 435 are kept constant at 48 h for each reaction cycle (Fig. 7). The results showed that there was almost no considerable decrease in production of methyl esters even after six batches of methanolysis reaction using both lipases as biocatalysts and then they have good potential to be used for repeated batch cycles.

The effect of some parameters on the efficiency of enzymatic transesterification using *R. oryzae* (ATCC 9374) whole cell biocatalyst immobilized within biomass support particles and treated with GA solution was investigated and finally compared with Novozym 435 under optimum conditions. The FAMEs content was increased significantly compared with the initial reaction condition. The maximum FAMEs content in the reaction mixture reaches 84 wt.% using *R. oryzae* whole cell biocatalyst in optimum condition of reaction after 48 h which is remarkably comparable with 90 wt.% biodiesel yield using Novozym 435. The optimum water content for *R. oryzae* was obtained as 15 wt.% whereas this parameter inhibits the activity of Novozym 435 for methanolysis. The water content could be a limiting factor when waste oils are used as feedstock for biodiesel production because they contain small

amounts of water. In summary, *R. oryzae* whole cell is more applicable and because of its usability for successive batches cycles, has a great potential to be used for biodiesel production in industrial scale.

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