Korean J. Chem. Eng., **30**(2), 405-412 (2013)
DOI: 10.1007/s11814-012-0132-y

Lipase from *Penicillium camembertii* KCCM 11268: Optimization of solid state fermentation and application to biodiesel production

Waraporn Malilas*,, Seong Woo Kang*, Sung Bong Kim*, Hah Young Yoo*, Warawut Chulalaksananukul**, and Seung Wook Kim*,†**

*Department of Chemical and Biological Engineering, Korea University, 1, Anam-dong, Sungbuk-gu, Seoul 136-701, Korea
**Department of Botany, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
(*Received 10 November 2011 • accepted 14 August 2012*)

Abstract−Lipase was produced by *Penicillium camembertii* KCCM 11268 under solid state fermentation (SSF), and the production process was optimized by using statistical experimental designs. The initial moisture content, cultivation time, inoculum size and concentration of basal medium were considered as the factors of optimum conditions for SSF. *P. camembertii* KCCM 11268 was cultivated in SSF using wheat bran as the substrate for lipase production. Under the optimized condition, lipase activity was reached around 7.8 U/ml after eight days fermentation. To partially purify the lipase, ammonium sulfate (80% saturation) was added to the crude lipase solution and concentrated using a diafiltration (VIVAFLOW 50). The concentrated lipase solution from *P. camembertii* KCCM 11268 (PCL) was immobilized on silica gel by cross-linking method. Also, PCL was mixed with a commercial lipase solution from *Candida rugosa* (CRL), and this mixture was co-immobilized on silica gel. The immobilized and co-immobilized lipase activities were 1150.1 and 7924.8 U/g matrix, respectively. Palm oil and methanol were used as substrates and 1 mmol of methanol was added every 1.5 h and 2 times during biodiesel production. The reaction was carried out at temperatures of 30, 40, 50, 60 and 70 °C. The maximum biodiesel conversion by co-immobilized lipase was 99% after 5 h at 50 °C.

Key words: *Penicillium camembertii* KCCM 11268, Solid State Fermentation, Lipase Production, Statistical Experimental Design, Biodiesel Production, Immobilization

INTRODUCTION

Enzymes of industrial interest have traditionally been obtained from submerged fermentation (SmF) because of the advantages of this approach in regards to handling and control. In recent years, SSF has received renewed interest since SSF can produce higher yields or better product characteristics than SmF. Although many microbes can grow on solid substrates, only filamentous fungi can grow to a significant extent in the absence of water [1]. Filamentous fungi are the most ideal and best adapted microorganism for SSF. Fungi have several advantages over unicellular microorganisms in the colonization of solid substrates and utilization of available nutrients, including the hyphal mode of fungal growth and their good tolerance to low water activity and high osmotic pressure conditions [2]. SSFs have been recently been reassessed, especially because of the possibility of using low-cost agro-industrial residues as raw materials [3].

The use of biofuels, such as biodiesel, bioethanol, biobutanol and biohydrogen, from various biomass sources is an efficient strategy to reduce greenhouse gas emissions. In addition, the carbon recycle period of biofuels is shorter than the carbon recycle period from fossil energy. Biodiesel is well developed and has been commercialized as a biofuel. In Korea, biodiesel contains 2% of diesel fuel and the biodiesel content is going to be increased to 10% by 2030. Also, the biodiesel content in several South America countries must be 100% in transport diesel fuel. Biodiesel is a mixture of alkyl esters of fatty acids derived from the transesterification of either edible or inedible oils with alcohol [4,5], and the transesterification reaction is generally performed using a chemical catalyst [6-11]. Enzymatic biodiesel production has been widely studied because of its ease of product separation, no organic solvent system and simple process of biodiesel production. Despite the many advantages of enzymatic biodiesel productions, there are several critical disadvantages. One of these is the higher price of lipase enzymes relative to chemical catalysts, such as KOH and sulfuric acid. Thus, researchers have attempted to develope enzyme immobilization processes to obtain reusable enzyme derivatives [12]. Immobilization enables the recycling of the biocatalyst and hence lowers the cost. In the case of biocatalysts in non-aqueous media, immobilization has also been shown to increase activity. Lipase-catalyzed transesterification is carried out in non-aqueous environments. Thus, many transesterification processes employing lipases have used an immobilized form of the enzyme, and a significant amount of research has been directed at lipase-catalyzed transesterification of triglyceride [13].

Lipase enzymes can produce biodiesel from vegetable oils and alcohols [14]. Lipase enzymes can be classified as 1, 3-specific lipases and non-specific lipases. Oil commonly consists of three fatty acid chains that are linked at three carbons of the glycerol backbone. 1, 3-specific lipase hydrolyzes or esterifies the specific site of glycerol, and non-specific lipase can hydrolyze one of three sites [5]. Previously *Rizopus oryzae* lipase and *Candida rugosa* lipase were employed to produce biodiesel and studies on biodiesel production using these lipases and immobilization of these lipases have been performed

†To whom correspondence should be addressed.
E-mail: kimsw@korea.ac.kr

under various conditions [5,14-16].

In this study, eight strains were screened, and 1, 3-specific lipase production, which is not a commercial enzyme, was evaluated. *Penicillium camembertii* KCCM 11268 strain was selected and lipase production conditions were performed by statistical method. The effects of different variables (initial moisture content, cultivation time, inoculums size and concentration of basal medium) on the process of lipase production in SSF were analyzed using a fractional factorial design (FFD), and the critical factors were further investigated. After FFD experiment, optimization and statistical analysis were performed. Also, partial purification and concentration was performed and immobilization using concentrated lipase and *Candida rugosa* lipase (CRL) was conducted. Finally, biodiesel production using immobilized lipases was performed at various temperatures to determine the optimal process conditions.

EXPERIMENTAL

1. Microorganisms and Commercial Lipase Preparation

Three strains of *Penicillium camembertii* (KCTC 6012, KCCM 11268 and KCCM 60415), one strain of *Rhizomucor pusillus* KCCM 60428 and one strain of *Fusarium solani* were used. *F.solani* was screened from Deciduous Dipterocarp Forest in Nan province, Thailand of soil sample. All strains were cultivated in potato dextrose agar at 30 °C for seven days. The *Candida rugosa* lipase was purchased from Sigma Aldrich.

2. Lipase Production on Submerged Fermentation (SmF)

SmF was carried out by seeding the spore suspension (final concentration of 10^6 spores/ml) in 250 mL Erlenmeyer flasks containing 100 mL of the basal medium solution with the following composition (g/L): $MgSO_4 \cdot 7H_2O$; 0.52, KH_2PO_4; 1.52, $NaNO_3$; 2.00, NaCl; 0.52, $Cu(NO_3)_2 \cdot 3H_2O$; 0.001, $FeSO_4 \cdot 7H_2O$; 0.001, $ZnSO_4$; 0.001, and 1% (v/v) olive oil. The medium pH value was adjusted to 7 and then autoclaved for 15 min at 121 °C. The inocula were incubated at 24 °C for seven days. After incubation, the culture broth was centrifuged at 8,000 g for 20 min and the supernatant was used as extracellular enzyme (lipase).

3. Lipase Production on Solid State Fermentation (SSF)

For SSF, wheat bran (8 g) was used as substrate and humidified with the basal medium. Ammonium sulfate and olive oil at 1% (v/v) were used as nitrogen and carbon sources, respectively. The medium pH value was adjusted to 6. The required concentration (x1, x5 and x9) of basal medium was poured into individual 250 ml Erlenmeyer flasks according to experimental designs, and distilled water was added to adjust the initial moisture content to 50, 60 and 70%. The medium was then autoclaved for 15 min at 121 °C. After cooling, the flasks were inoculated and the contents mixed. The flasks were then incubated at 30 °C under static conditions for 6, 8, or 10 days. After incubation, 100 ml of distilled water was added to each flask and the samples were homogenized at 10,000 rpm for 5 min. The broth was centrifuged at 8,000 g for 20 min and the supernatant was used as extracellular enzyme (lipase).

Table 1. Range of variables at different levels in the fractional factorial design

Independent variables X_i	Levels		
	−1	0	+1
X_1 initial moisture content (%)	50	60	70
X_2 cultivation time (days)	6	8	10
X_3 inoculum size (spores/ml)	10^4	5×10^5	10^6
X_4 concentration of basal medium (magnitude)	×1	×5	×9

4. Experimental Design of SSF

A 2^{4-1} fractional factorial design (FFD) was employed to determine which factors were critical when lipase was produced by *P. camembertii* KCCM 11268. Table 1 shows the coded values for FFD, and the FFD was conducted using twelve sets of experiments containing four center point experiments (Table 3). These experiments were performed in duplicate. The variables were coded according to Eq. (1):

$$\chi_i=(X_i-X_0)/\Delta X_i \quad (1)$$

where x_i is the coded value of an independent variable, X_i is the real value of an independent variable, X_0 is the real value of an independent variable at the center point, and ΔX_i is the step change value. The lipase activity was taken as the dependent variable or response, Y_i. After determining which factors were critical by FFD, statistical analysis and optimization of lipase production was performed using central composition design (CCD) with α-value ($\alpha=(2^n)^{1/4}$) [18]. Table 2 shows the coded values of CCD. The experimental data was empirical fit using polynomial regression, based on analysis of variance (ANOVA). Nineteen experiments were contained in the experimental design (Table 4) [18-21]. The quadratic model for predicting the optimal point was derived according to Eq. (2):

$$y=b_0+\sum b_i x_i+\sum b_{ii} x_i^2+\sum b_{ij} x_i x_j \quad (2)$$

where y is the response variable, b the regression coefficients of the model, and x the coded levels of the independent variable. An SAS 9.1 package was used for the regression analysis of the experimental data obtained. The statistical significance of the second-order model equation was determined based on the F-value and the proportion of variance explained by the model obtained was given by the multiple coefficient of determination, R^2.

Table 2. Results of the regression analysis of the 2^{4-1} fractional factorial design[a]

Independent variables	Levels				
	−1.682	−1	0	+1	+1.682
X_1 initial moisture content (%)	43.18	50	60	70	76.82
X_2 cultivation time (days)	6.318	7	8	9	9.682
X_4 concentration of basal medium (magnitude)	×1.636	×3	×5	×7	×8.364

[a]$R^2=0.999$

Table 3. Experimental design of 2^{4-1} fractional factorial design and the response

Runs	Code levels				Lipase activity (U/ml)
	X_1	X_2	X_3	X_4	
1	−	−	−	−	0.77
2	−	−	+	+	0.22
3	−	+	−	+	0.35
4	+	−	−	+	0.75
5	−	+	+	−	7.25
6	+	−	+	−	1.13
7	+	+	−	−	7.23
8	+	+	+	+	0.98
9	0	0	0	0	7.46
10	0	0	0	0	7.59
11	0	0	0	0	7.48
12	0	0	0	0	7.50

Response surface methodology (RSM) was used to determine the optimal condition of the X_1, X_2 and X_4 variables. The experiment was performed with three independent variables - initial moisture content (X_1), cultivation time (X_2) and concentration of basal medium (X_4) - using a 2^3 full factorial with central composite design (CCD). The coded values corresponding to each factor are present in Table 2.

5. Lipase Assay

One milliliter of enzyme solution was mixed with 10 ml of the solvent (isooctane containing 10%) and 1 ml of the substrate (soybean oil). The reaction was performed in 50 ml Erlenmeyer flask at 37 ºC and 150 rpm by shaking water bath for 30 min. After reaction, 2 ml of the supernatant was taken to a test tube, and 0.5 ml of cupric acetate-pyridine reagent was added into the test tube and mixed for 1 min. The free fatty acids liberated and dissolved by the isooctane were quantified using a UV spectrophotometer at 715 nm. One unit of lipase activity was defined as the amount of the enzyme required to liberate 1 μmol of the free fatty acid per minute [6].

6. Lipase Partial Purification and Concentration

After determination of lipase activity, the culture broth of the strain was centrifuged at 10,000 rpm for 20 min and the supernatant was prepared as a crude lipase solution. Ammonium sulfate was added to 80% saturation to the crude lipase solution and the resulting suspension was centrifuged at 8,000 rpm for 30 min to obtain the supernatant. The precipitate was suspended in 0.5 M phosphate buffer (pH 6.5) and the solution was concentrated by diafiltration (VIVAFLOW 50) and stored at 4 ºC.

7. Lipase Immobilization Method

One gram of dry silica gel was mixed with 10% of 3-APTES in 20 ml of acetone and incubated at 50 ºC for 2 h with constant mixing. The silica gel was then washed with water and dried at 60 ºC for 2 h. The dried silica gel was then suspended in 20 ml of 0.1 M phosphate buffer solution (pH 8). Two ml of 25% (w/v) glutaraldehyde was incubated in an aqueous solution at 64 ºC for 20 min. The solution was then added to the reaction solution and incubated at 20 ºC for 2 h. The activated silica gel (0.5 g) was mixed with 10 ml of pretreated lipase solution and then incubated at 20 ºC for 24 h to immobilize the lipase. The immobilized lipase was recovered by filtration, washed with water, and then dried overnight at room temperature. The product was set aside for storage at 4 ºC [16].

8. Biodiesel Analytical Method

The methyl ester content in biodiesel produced by immobilized lipase was analyzed using an Agilent 7890A GC System connected to an HP-INNOWAX column (0.25 mm×30 m, HP, USA). The sample injection volume was 1 μl, the injector temperature was 260 ºC, and the oven temperature was increased from 150 ºC to 180 ºC at a rate of 15 ºC/min and increased up to 240 ºC at a rate of 5 ºC/min, and then maintained for 1 min. A flame ionization detector (FID) was used and set to 260 ºC.

9. Biodiesel Production

Biodiesel was produced using palm oil with immobilized pretreated partially purified *P. camembertii* KCCM 11268 lipase (PCL) and co-immobilized (PCL+CRL). 3 mmol of methanol was initially added to the reaction medium containing 3 mmol of palm oil and 0.5 g of the immobilized lipases in 100 mL Erlenmeyer flasks at 200 rpm. An equivalent amount of methanol was then added twice during biodiesel production. The reaction was carried out at different temperatures (30, 40, 50, 60 and 70 ºC). The equation of conversion yield was defined as follows:

$$\text{Conversion yield} = \frac{\text{Moles of FAME}}{\text{Moles of triglyceride} \times 3} \times 100$$

RESULTS AND DISCUSSION

1. Selection of Strain for the Production of Lipase

Eight lipase-producing strains were used to produce lipase in SmF. Olive oil, which was used as an inducer, was added to the liquid medium. After seven days of fermentation, the lipase activity was determined by monitoring the oleic acid released from the soybean oil, which was used as a substrate, according to a method described by Lee et al. [6]. Fig. 1 shows the results of the lipase activity assays. The activities of *R. pusillus* KCCM 60428 and *P. camembertii* KCTC 6012 were not competitive, and no notable lipase activity was observed from *P. camembertii* KCCM 60415 and *Fisarium solani*. In the case of *P. camembertii* KCCM 11268, the activity was found to be 0.027 U/ml, which was the highest activity of all strains tested

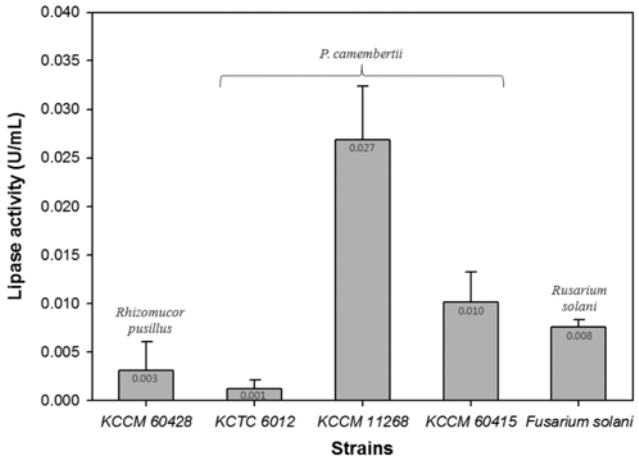

Fig. 1. Comparison of lipase activity produced by fungi in SmF (seven days, 24 ºC and 10^6 spores/ml).

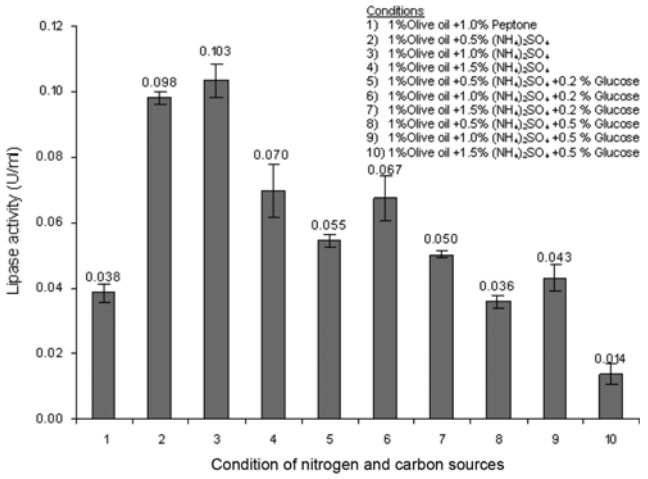

Fig. 2. Effect of nitrogen and carbon sources on the production of lipase by *P. camembertii* KCCM 11268 (PCL) in SmF for seven days, 30 °C and 10^6 spores/ml).

(Fig. 1); thus, this strain was used in the further studies.

2. Effect of Carbon and Nitrogen Source on Lipase Production

Carbon is a critical nutrient and energy source for cells. Nitrogen sources, including organic nitrogen and inorganic nitrogen, play an important role in the synthesis of enzymes. This is because inorganic nitrogen sources can be used quickly, while organic nitrogen sources are used for the production of cell growth factors and amino acids, which are needed for cell metabolism and enzyme synthesis. Various concentrations of carbon and nitrogen sources of both organic and inorganic compounds were used during lipase production from *P. camembertii* KCCM 11268. Oilve oil, peptone, $(NH_4)_2SO_4$ and glucose were employed as carbon and nitrogen sources. Fig. 2 shows the effect of nitrogen and carbon sources on the production of lipase by *P. camembertii* KCCM 11268 in SmF. When olive oil and $(NH_4)_2SO_4$ were used, lipase activity was higher than when olive oil with peptone or olive oil, $(NH_4)_2SO_4$ and glucose was used. The maximum lipase activity was obtained when 1% of olive oil and 1% of $(NH_4)_2SO_4$ was employed during the lipase production. Olive oil has been reported to be a good inducer and glucose is reported to be a bad inducer of lipase production form *P. camembertii* in SmF [23]. Since glucose was a bad inducer, lipase concentrations in the culture broth produced by *P. camembertii* KCCM 11268 did not increase.

3. Statistical Analysis and Optimization of Lipase Production

For efficient production of lipase using *P. camembertii* KCCM 11268, which produces high lipase titers, the medium composition and culture condition were optimized using wheat bran in SSF. The four variables most important to SSF were identified (Table 1) and the effect of each variable on the production of lipase by *P. camembertii* KCCM 11268 was investigated. ANOVA was employed to determine significant variables. The experimental design and the results of the 2^{4-1} fractional factorial design are shown in Table 3. In these experiments, the initial moisture content, cultivation time, inoculums size and basal medium concentration ranged from 50-70%, 6-10 days, 10^4-10^6 spores/ml and 1-9 times, respectively. The lipase activity varied from 0.22-7.59 U/ml under the conditions tested.

Table 4. Range of variables at different levels for CCD and the response

Runs	Code levels			Lipase activity (U/ml)
	X_1	X_2	X_3	
1	−1	−1	−1	2.71
2	+1	−1	−1	7.30
3	−1	+1	−1	2.67
4	+1	+1	−1	7.34
5	−1	−1	+1	0.76
6	+1	−1	+1	1.29
7	−1	+1	+1	1.22
8	+1	+1	+1	7.23
9	−1.682	0	0	0.58
10	+1.682	0	0	1.94
11	0	−1.682	0	1.73
12	0	+1.682	0	2.84
13	0	0	−1.682	1.70
14	0	0	+1.682	0.71
15	0	0	0	7.34
16	0	0	0	7.29
17	0	0	0	7.16
18	0	0	0	7.24
19	0	0	0	7.21

Table 5. Experimental design of the 2^3 fractional factorial with CCD

Factors	Mean square	F-value	P-vale
X_1	35.81	11,679.80	<0.0001
X_2	20.94	6,830.85	<0.0001
X_3	0.03	9.24	0.0559
X_4	24.75	8,073.27	<0.0001
$X_1 \times X_2$	0.01	3.43	0.1612
$X_1 \times X_3$	18.62	6,074.14	<0.0001
$X_1 \times X_4$	0.09	27.82	0.0133
$X_2 \times X_3$	0	0	0
$X_2 \times X_4$	0	0	0
$X_3 \times X_4$	0	0	0
Model	17.01	5,547.29	<0.0001

On the basis of these experimental values, statistical testing was carried out using Fisher's statistical test for regression to measure the mean square due to error and the influence of each controlled factor on the tested models. Also, the *P*-value corresponding to the *F*-value indicates the probability that differences between calculated and tabulated statistics were due only to random experimental error. Table 5 shows the results of ANOVA when FFD was used. When ANOVA analysis reflects the significance of a model with a confidence level greater than 99% ($P<0.01$) in lipase production, the *F*-value and *P*-value were 5,547 and <0.0001, respectively, as shown in Table 5 [18-22]. The *F*-values of X_1, X_2, X_4 and X_3 were approximately 11,679, 6,830, 8,073 and 9.24, respectively. Relatively larger *F*-value means that the factor is more significant and more effective on the result than smaller F-values. The ANOVA results showed that initial moisture content (X_1), cultivation time (X_2) and concen-

Table 6. Statistical analysis of the models used for lipase production at different initial moisture content, cultivation times and basal medium concentrations[a]

Source	Sum of squares	Degrees of freedom	Mean square	*F*-value	*P*-vale
Model	116.37	9	12.93	3.20	0.0491
Error	36.37	9	4.04		
Total	152.74	18			

[a]R^2=0.762

tration of basal medium (X_4) were the three most important variables for the production of lipase. There was no evidence of any interactions between these factors.

Response surface methodology (RSM) was used to determine the optimal condition and the results are presented in Table 4. The center point (0) was 60% of the initial moisture content, eight days of cultivation time and a basal medium concentration of five times.

Regression analysis was performed to fit the response function to the experimental data. As shown in Table 6, the *F*-value and *P*-value of CCD results were 3.20 and 0.0491, respectively. Quadratic models were obtained for the supplement combinations X_1*X_2 (Eq. (3)), X_1*X_4 (Eq. (4)), and X_2*X_4 (Eq. (5)) through statistical analysis of the data. These models describe lipase activity ([LA], expressed in U/ml) as a function of the normalized statistically significant variables.

$$LA=7.149+1.324\,X_1+0.605\,X_2-1.568\,X_1^2-1.206\,X_2^2+0.695\,X_1X_2 \quad (3)$$

$$LA=7.149+1.324\,X_1-0.819\,X_4-1.568\,X_1^2-1.587\,X_4^2-0.340\,X_1X_4 \quad (4)$$

$$LA=7.149+0.605\,X_2-0.819\,X_4-1.206\,X_2^2-1.587\,X_4^2+0.800\,X_2X_4 \quad (5)$$

where X_1=coded value of initial moisture content,
X_2=coded value of cultivation time,
X_4=coded value of concentration of basal medium.

Fig. 3 shows a three-dimensional diagram and a contour plot of the calculated response surface. The maximum points were the optimum conditions for the lipase production process [18-22]. The optimal X_1-X_2, X_1-X_4 and X_2-X_4 values for lipase production were as follows: x_1=0.309, x_2=0.192, and x_4=−0.138 in coded value. For calculation purposes, the normalized, coded values X_1, X_2 and X_4 were defined as: $x_1=(X_1-60)/10$, $x_2=(X_2-8)/1$ and $x_4=(X_4-5)/2$. According to these results, the optimal initial moisture content, cultivation time and concentration of basal medium for lipase production were calculated to be 63.1%, 8.2 days and 4.7 times, respectively.

Consequently, an inoculum size of 5×10^5 spores/ml, initial moisture content of 63%, cultivation time of eight days and basal medium concentration of five times were optimum for lipase production.

4. Confirmation of Optimization

These optimal conditions (initial moisture content of 63%, cultivation time of eight days and basal medium concentration of five times) were then experimentally confirmed in duplicate. The time course profile of lipase activity is shown in Fig. 4. The lipase activity was increased as the cultivation time passed and the lipase activity was reached approximately 7.83 U/ml at eight days of fermentation, after which the activity decreased. This result is notable compared to other reports of 4.99 U/ml of *Rhuzomucor pusillus* and 2.67 U/

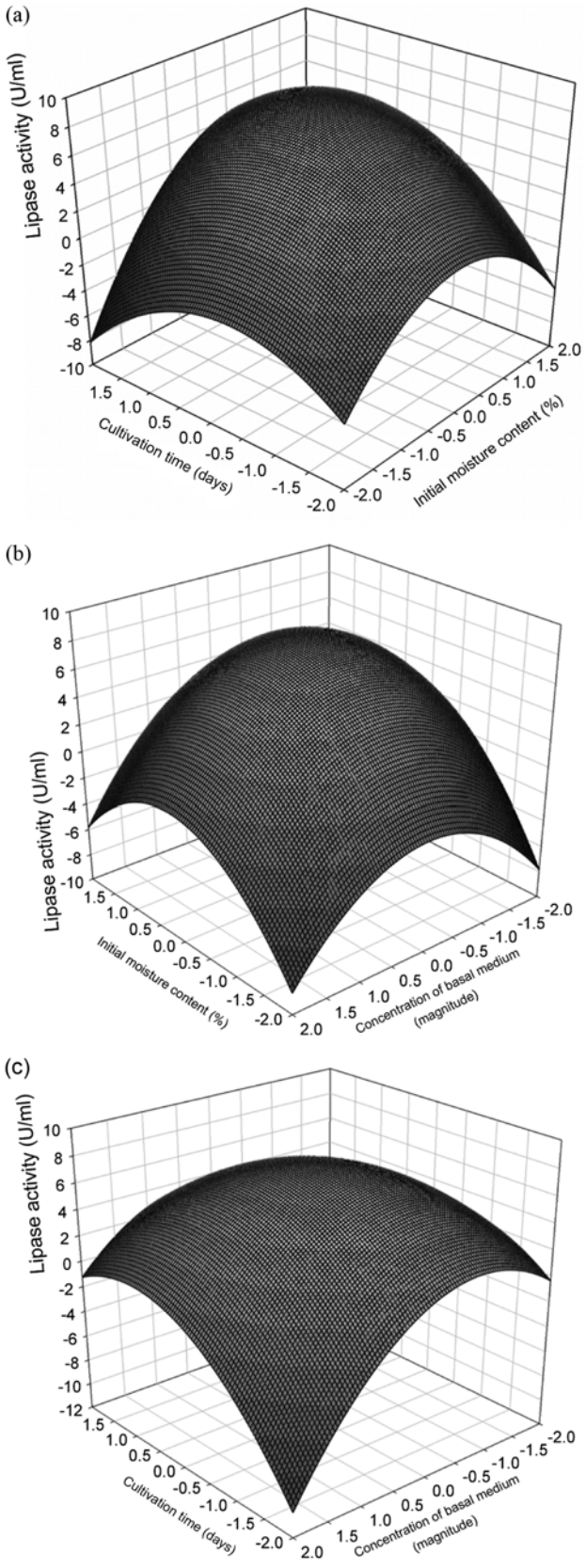

Fig. 3. Three-dimensional response surface plot of the central composite design experiment: (a) initial moisture content and cultivation time, (b) initial moisture content and concentration of basal medium, and (c) cultivation time and concentration of basal medium for lipase activity.

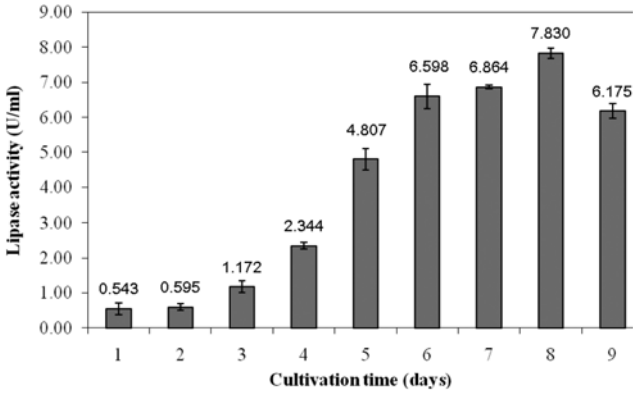

Fig. 4. Time course of lipase production by *P. camembertii* KCCM 11268 in SSF. Experimental conditions: initial moisture content 63%, inoculums size 5×10^5 spores/ml of wheat bran, five-times concentration of basal medium.

ml of *R. rhizopodiformis*, which are research references of Cordova et al. [17]. The strong correlation between experimental and statistical results confirms the validity of the response model and the existence of an optimal point. In addition, the results of the statistical analysis were reliable in regards to practical use and utilization of real processes.

5. Lipase Purification and Immobilization

Partially purified and concentrated PCL was obtained by ammonium sulfate and diafiltration. Table 7 presents the results of purification and concentration. The total protein amount was 316.18 mg after centrifugation at 8,000 rpm and 73.93 mg after ammonium sulfate treatment. The specific activity of the PCL was 1.64 U/mg. The specific activity was 37% higher than the supernatant (1.19 U/mg) before ammonium sulfate treatment.

The concentrated crude PCL was immobilized on activated silica gels for 24 h using a cross-linking method. Previous studies have shown that enzyme activity could be increased by co-immobilization [24]. The concentrated crude PCL was mixed with the commercial CRL. This mixture was also immobilized on activated silica gels for 24 h using a cross-linking method (co-immobilized lipase). Fig. 5 shows the lipase activity of immobilized PCL and co-immobilized lipase were determined to be 1,150.1 and 7,924.8 U/g matrix, respectively. The CRL is non-specific [25] and PCL is 1, 3-specific [26] lipase. The activity of co-immobilized lipase was approximately seven-fold higher than the activity of immobilized PCL (Fig. 5). Enzymatic biodiesel production is based on transesterification, and when 1, 3-specific lipase and non-specific lipase were utilized, the reaction rate dramatically increased and as well as productivity due to the removal of the rate determining step of the acyl-migration reaction. Thus, the co-immobilized lipase activity was higher than single immobilized PCL.

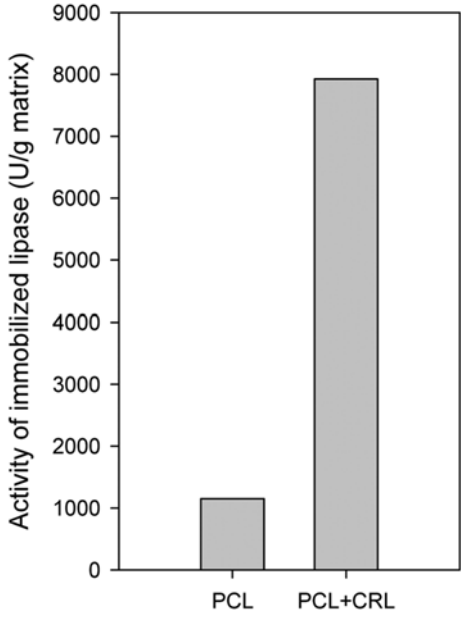

Fig. 5. The lipase activities of immobilized PCL and co-immobilized lipase (PCL+CRL) after 24 h of immobilization (CRL: Lipase from *C. rugosa*, PCL: Lipase from *P. camembertii*).

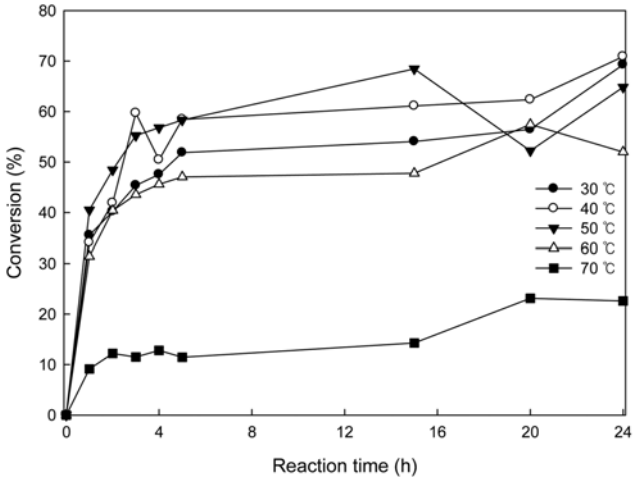

Fig. 6. Biodiesel production at various temperatures by immobilized PCL.

6. Biodiesel Production

Biodiesel production was performed with the immobilized lipase. The reaction time for biodiesel production was reduced by optimizing the biodiesel production conditions. Mixture systems have been previously used to increase mass transfer [6]. Lifka and Ondruschka [27] reported that temperature was important for biodiesel pro-

Table 7. Data of the partially purified and concentrated PCL

The lipase from *P. camembertii*	U/ml	Total volume (ml)	Total activity (IU)	Total protein (mg)	Specific activity (U/mg)
8,000 rpm supernatant	0.626	600	375.428	316.18	1.19
8,000 rpm 80% A. S. Precipitant	1.215	100	121.455	73.93	1.64

A.S.: ammonium sulfate

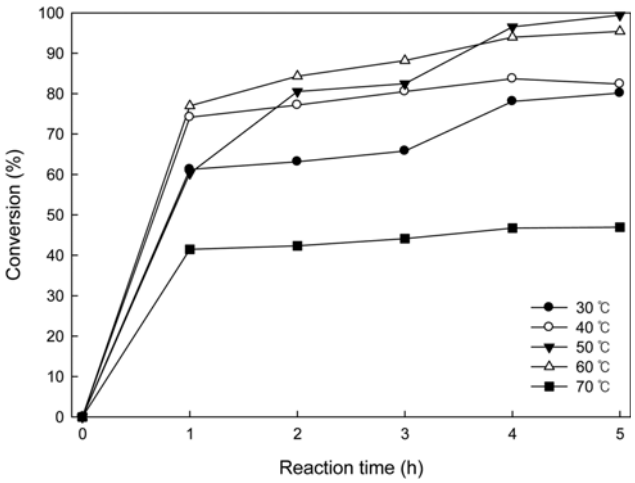

Fig. 7. Biodiesel production at various temperatures by co-immobilized lipase (PCL+CRL).

duction. Therefore, in this study, the optimal temperature for both immobilized PCL and co-immobilized PCL and CRL was investigated to improve biodiesel productivity.

Biodiesel production using immobilized PCL was performed at 200 rpm for 24 h. Fig. 6 shows the effect of temperature on biodiesel production using immobilized lipase. Biodiesel productivity was the lowest at 70 °C. Lipase activity seemed to decrease by thermal inhibition or changes in protein structure. The reaction rate and productivity at 60 °C are higher than at 70 °C but still lower than the other temperatures. The initial reaction rate was highest at 50 °C. Co-immobilized lipase was also investigated (Fig. 7). As was observed for the PCL, the reaction rate and productivity of 70 °C sample was lower than others' temperatures and the parameters were very low at 30 °C. The conversion of biodiesel at 60 °C was approximately 90%. In addition, the reaction rate at 50 °C was relatively low, but the conversion was approximately 95% after 5 h. Thus, the optimal temperature for biodiesel production using immobilized PCL and co-immobilized lipase (PCL+CRL) were 40 °C at 24 h and 50 °C at 5 h, respectively.

CONCLUSIONS

The objectives of this study were to culture fungi in submerged culture for lipase production and optimize the SSF conditions by using statistical experimental design. And purify the lipase from the culture medium and immobilize the concentrated lipase on silica gel by using a cross-linking method for biodiesel production. At first, eight mold cultures were cultivated for the production of lipases in submerged fermentation (SmF) using 1% (v/v) olive oil as substrate. The maximum lipase activity was obtained from *P. camembertii* KCCM 11268. SSF conditions for lipase production were optimized by using statistical experimental designs. The results of optimized conditions are follows: inoculum size of 10^5 spores/ml, the initial moisture content of 63% and five-times concentration of basal medium. After optimization, confirmation was conducted and lipase activity reached about 7.8 U/ml after eight days fermentation. This result is notable compared to other reports of 4.99 U/ml of *Rhuzomucor pusillus* and 2.67 U/ml of *R. rhizopodiformis*. The crude PCL solution was partially purified using ammonium sulfate and diafiltration. The concentrated PCL was immobilized on silica gel by using a cross-linking method and utilized for the biodiesel production. In addition, co-immobilized lipase (PCL+CRL) was evaluated. The activities of immobilized PCL and co-immobilized lipase were 1,150.1 and 7,924.8 U/g matrix, respectively. The palm oil and methanol were used as substrates and 1 mmol of methanol was added to the initial reaction medium every 1.5 h, 3 times during biodiesel production. The reaction was carried out at different temperatures. The maximum biodiesel conversion by co-immobilized lipase was 99% after 5 h at 50 °C. Improved microbial strains for the overproduction of industrial products have been the hallmark of all commercial fermentation processes.

ACKNOWLEDGEMENTS

This work was supported by the Advanced Biomass R&D Center (ABC) of Global Frontier Project funded by the Ministry of Education, Science and Technology (ABC-2011-0031360), and was supported by grants from the Korea Foundation for Advanced Studies (KFAS).

REFERENCES

1. R. G. S. Couto and A. M. Sanroma, *J. Food Eng*., **76**, 291 (2006).
2. M. Raimbault, *Electron. J. Biotechnol*., **1**, 234 (1998).
3. N. Pérez-Guerra, A. Torrado-Agrasar, C. López-Macias and L. Pastrana, *Agric. Food Chem*., **2**, 343 (2003).
4. M. Oda, M. Kaieda, S. Mana, H. Yamaji, A. Kondo, E. Izumoto and H. Fukuda, *Biochem. Eng. J*., **45-51**, 23 (2005).
5. J. H. Lee, S. B. Kim, C. Park, B. Tae, S. O. Han and S. W. Kim, *Biochem. Biotehcnol*., **365-371**, 161 (2010).
6. D. H. Lee, C. Park, J. M. Yeo and S. W. Kim, *J. Ind. Eng. Chem*., **777-782**, 12 (2006).
7. A. V. L. Pizarro and E. Y. Park, *Process Biochem*., **1077-1082**, 38 (2003).
8. C. J. Shieh, H. F. Liao and C. C. Lee, *Bioresour. Technol*., **103-106**, 88 (2003).
9. Y. Shimada, Y. Watanabe, T. Samukawa, A. Sugihara, H. Noda and H. Fukuda, *J. Am. Oil Chem. Soc.*, **789-793**, 76 (1999).
10. Y. Shimada, Y. Watanabe, A. Sugihara and Y. Tominaga, *J. Mol. Catal. B-Enzym*., **133-142**, 17 (2002).
11. P. M. Neilsen, J. Brask and L. Fjerbaek, *Biotechnol. Bioeng*., **692-700**, 110 (2008).
12. D. H. Lee, J. M. Kim, H. Y. Shin and S. W. Kim, *Biotechnol. Bioprocess Eng*., **522-525**, 11 (2006).
13. A. F. Hsu, K. Jones, T. A. Fogolia and W. M. Marmer, *Biotechnol. Appl. Biochem*., **181-186**, 36 (2002).
14. S. Shweta, S. Shweta and M. N. Gupta, *Energy Fuels*., **154-159**, 18 (2004).
15. J. H. Lee, S. B. Kim, C. Park and S. W. Kim, *Bioresour. Technol*., **s66-s70**, 101 (2010).
16. J. H. Lee, S. B. Kim, S. W. Kang, Y. S. Song, C. Park and S. W. Kim, *Bioresour. Technol.*, **2105-2108**, 102 (2011).
17. J. Cordova, M. Nemmaoui, M. Ismaïli-Alaoui, A. Morin, S. Roussos, M. Raimbault and B. Benjilali, *J. Mol. Catal. B Enzymatic*, **5**, 75 (1998).

18. J. H. Lee, D. H. Lee, J. S. Lim, B. H. Um, C. Park and S. W. Kim, *J. Microbiol. Biotechnol*., **18**, 1927 (2008).
19. S. B. Kim, J. H. Lee, K. K. Oh, S. J. Lee, J. Y. Lee, J. S. Kim and S. W. Kim, *Biotechnol. Biopro. Eng*., **16**, 725 (2011).
20. K. Sunitha, J. K. Lee and T. K. Oh, *Bioproc. Eng*., **21**, 477 (1999).
21. B. H. Um and S. H. Bae, *Korean J. Chem. Eng*., **28**, 1172 (2011).
22. J. Li, L. Liu, G. Du, J. Chen and W. Tao, *Korean J. Chem. Eng*., **27**, 1233 (2010).
23. A. Amrane, V. Prstel and Y. Prigent, *J. Gen. Appl. Microbiol*., **251-5**, 49 (2003).
24. S. F. Torabi, K. Khajeh, S. Ghasempur, N. Ghaemi and S. O. R. Siadat, *J. Biotechnol.*, **111-20**, 131 (2007).
25. R. Gupta, P. Rathi and S. Bradoo, *Crit. Rev. Food Sci. Nutr.*, **635-44**, 43 (2003).
26. H. S. Krishna and G. N. Karanth, *Catal. Rev.*, **499-591**, 44 (2002).
27. J. Lifka and B. Ondruschka, *Chem. Eng. Technol*., **1156-9**, 27 (2004).