Available online at BCREC Website: http://bcrec.undip.ac.id

Bulletin of Chemical Reaction Engineering & Catalysis, 11 (3), 2016, 346-353

Research Article

Enzymatic Phorbol Esters Degradation using the Germinated Jatropha Curcas Seed Lipase as Biocatalyst: Optimization Process Conditions by Response Surface Methodology

Avita Kusuma Wardhani, Chusnul Hidayat*, Pudji Hastuti

Graduate Program on Estate Crop Product Technology, Faculty of Agricultural Technology Universitas Gadjah Mada, Jl. Flora, Bulaksumur, Yogyakarta 55281, Indonesia

Received: 22nd December 2015; Revised: 1st April 2016; Accepted: 14th April 2016

Abstract

Utilization of Jatropha curcas seed cake is limited by the presence of phorbol esters (PE), which are the main toxic compound and heat stable. The objective of this research was to optimize the reaction conditions of the enzymatic PE degradation of the defatted Jatropha curcas seed cake (DJSC) using the acetone-dried lipase from the germinated Jatropha curcas seeds as a biocatalyst. Response Surface Methodology (RSM) using three-factors-three-levels Box-Behnken design was used to evaluate the effects of the reaction time, the ratio of buffer volume to DJSC, and the ratio of enzyme to DJSC on PE degradation. The results showed that the optimum conditions of PE degradation were 29.33 h, 51.11 : 6 (mL/g), and 30.10 : 5 (U/g cake) for the reaction time, the ratio of buffer volume to DJSC, and the ratio of enzyme to DJSC, respectively. The predicted degradation of PE was 98.96% and not significantly different with the validated data of PE degradation. PE content was 0.035 mg/g, in which it was lower than PE in non-toxic Jatropha seeds. The results indicated that enzymatic degradation of PE might be a promising method for degradation of PE.

Keywords: jatropha seed cake; phorbol esters; enzymatic degradation; germinated jatropha lipase; response surface methodology

How to Cite: Wardhani, A.K., Hidayat, C., Hastuti, P. (2016). Enzymatic Phorbol Esters Degradation using the Germinated Jatropha Curcas Seed Lipase as Biocatalyst: Optimization Process Conditions by Response Surface Methodology. *Bulletin of Chemical Reaction Engineering & Catalysis*, 11 (3): 346-353 (doi:10.9767/bcrec.11.3.574.346-353)

Permalink/DOI: http://doi.org/10.9767/bcrec.11.3.574.346-353

1. Introduction

Jatropha curcas seed cake contains anti-nutritional factors (trypsin inhibitor, phytic acid, lectin, saponin), and phorbol esters (PE) as the main toxic compound, which known as tumor promoting activity [1-4]. Hence, the presence of PE limits the applications of the seed cake for food and feed consumptions. Currently, the degradation of PE is an important issue to be studied. Various chemicals and physical methods have been studied for the degradation of PE [5-7]. Most of these treatments were non-specific and costly.

PE are defined as "polycyclic" compounds, in which two hydroxyl groups on neighboring carbon atoms are esterified by fatty acids [8]. The structure of PE is analog to fatty acid esters,

* Corresponding Author.
E-mail: chusnul@gadjahmada.edu (C. Hidayat)
Telp: +62-274-549650, Fax: +62-274-549650

especially diacylglycerol [8-10]. Thus, it may be hydrolyzed by lipase. Some researchers reported that the esterase of rat liver, rat plasma, and rat sub-mandibular gland cells could degrade the PE [9,11,12]. Furthermore, *Pseudomonas aeruginosa PseA, B. adusta, P. rufa*, and *Aspergillus niger*, in which they produced lipase, could reduce PE during fermentation [13-15]. Recently, Hidayat [16] reported that PE could be hydrolyzed by rice bran lipase and acetone-dried germinated Jatropha seed lipase [17].

Enzymatic PE degradation is affected by some factors. Therefore, the process conditions have to be optimized. The objective of this research was to optimize the reaction conditions of PE degradation of the defatted jatropa curcas seed cake (DJSC). Factors that affect on PE degradation, such as: the reaction time, the ratio of buffer volume to DJSC, and the ratio of enzyme to DJSC on PE degradation, were evaluated by Response Surface Methodology (RSM) using three-factors-three-levels Box-Behnken design.

2. Materials and Method

2.1. Materials

Jatropha curcas seeds were obtained from local supplier in Yogyakarta. Methanol, n-hexane, acetone, NaH_2PO_4, Na_2HPO_4, acetic acid, CH_3COONa, disodium tetraborate were obtained from Merck KGaA (Darmstadt, Germany). Petroleum ether was obtained from J.T. Baker (USA). Standard phorbol-12-myristate-13-acetate (PMA) was obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Acetone-dried germinated jatropha curcas lipase

The germinated jatropha curcas seeds were prepared according to the method of Hidayat [17], with some modifications. Seeds were soaked in phosphate buffer at pH 7 containing daconyl fungicide (1.5 g/L) at room temperature for 12 h. It was further removed from the fungicide solution, air-dried, and dehydrated at room temperature (RH >90%) for 24 h. The soaked seeds were spread onto tray, which was covered with cotton. They were allowed to germinate. The seeds were harvested when the length of germinated seeds was approximately 2 - 2.5 cm.

The germinated seeds were carefully cracked to remove the shells. The seeds kernels (20 g) were crushed in 35 mL of cold acetone (4 °C) using a homogenizer at 5200 rpm for 10 min. The suspension was filtered through a filter paper and washed with cold acetone (4 °C) until the filtrate was colorless. The residue was air-dried at room temperature to get the acetone powder as the crude germinated Jatropha seed (CGJS) lipase, which was further kept at -18 °C.

2.3. Defatted jatropha seed cake

Defatted jatropha seed cake (DJSC) was prepared according to the Adebowale [18] method, with some modifications. The seeds were shelled. The kernel was separated and grinded using a homogenizer. It was dried at 50 °C for 24 h. Kernel powder was pressed using a hydraulic press at a pressure of 450 kg/cm^2. Furthermore, the pressed cake was crushed with a blender and sieved using 40 mesh sieves. Oil in the pressed cake powder was extracted using hexane to produce DJSC.

2.4. Effects of buffer pH on phorbol esters degradation

CGJS lipase powder (1 g) was added into Erlenmeyer containing 5 g DJSC and 40 mL buffer at various pH (5, 6, 7, 8 and 9). The suspensions were incubated in a shaker water bath at 30 °C and 120 strokes/min for 24 h. The reactions were terminated by placing the Erlenmeyer's into ice bath. Seed cake residues were obtained by filtering the suspensions. PE in the residue was further extracted and analyzed by TLC method. The percentage of PE degradation was calculated according to Equation (1).

$$\%\ Degradation = \frac{(C_0 - C)}{C_0} \times 100\% \quad (1)$$

where C_0 is the initial content of PE (mg/g) and C is the content of PE after hydrolysis (mg/g).

2.5. Effects of reaction time on phorbol esters degradation

CGJS lipase powder (1 g) was added into Erlenmeyer containing 5 g DJSC and 40 mL phosphate buffer pH 7. The suspensions were incubated in a shaker water bath at 30 °C and 120 strokes/min for 8, 12, 16, 20 and 24 h. The reactions were terminated by placing the Erlenmeyer's into ice bath. Seed cake residues were obtained by filtering the suspensions. PE in the residue was further extracted and analyzed by TLC method.

2.6. Effects of buffer volume to DJSC ratio on phorbol esters degradation

CGJS lipase powder (1 g) was added into Erlenmeyer containing 5 g DJSC and various amounts of phosphate buffer pH 7 (40, 50, 60, 70, 80 mL). The suspensions were incubated in a shaker water bath at 30 °C and 120 strokes/min for 24 h. The reactions were stopped by placing the Erlenmeyer into ice bath. Seed cake residues were obtained by filtering the suspensions. PE in the residue was further extracted and analyzed by TLC method.

2.7. Effects of enzyme to DJSC ratio on phorbol esters degradation

Various amounts of CGJS lipase powder (10, 20, 30, 40, 50 U) were added into Erlenmeyer containing 5 g DJSC and 40 mL phosphate buffer pH 7. The suspensions were incubated in a shaker water bath at 30 °C and 120 strokes/min for 24 h. The reactions were stopped by placing the Erlenmeyer in ice bath. Seed cake residues were obtained by filtering the suspensions. PE in the residues was further extracted and analyzed by TLC method.

2.8. Extraction of phorbol esters

PE in the seed cake was extracted by the method of Saetae and Suntornsuk [19], with some modifications. DJSC samples (5 g) were added into flasks containing 20 mL methanol. The mixture was stirred at 150 rpm for 5 min. It was then filtered using filter paper. The residue and the extract were collected separately. The extraction process was repeated four times. The extract fractions of all five extractions were pooled together and rotary-evaporated at 50 °C to obtain the concentrated PE fractions.

2.9. Analysis of phorbol esters

PE were analyzed and quantified by Thin Layer Chromatography (TLC) according to the method of Demmissie and Lele [20]. The TLC analysis was performed on 20 cm × 20 cm TLC silica gel G60F254 plate (Merck KGaA, Germany). The plates were pre-washed with methanol and activated at 50 °C for 5 min. Samples were applied at 15 mm from the base of the plate using a micropipette. The chamber was saturated with mobile phase (petroleum ether : acetone of 3 : 2 (v/v)) for 30 min prior to plate development. The quantification of PE was performed using a CAMAG TLC scanner 3 'dummy' S/N (1.14.16) controlled under CAMAG winCATS planar chromatography software at 263 nm. A calibration curve was performed using phorbol-12-myristate-13-acetate as a standard. The results were expressed as equivalent to phorbol-12-myristate-13-acetate.

2.10. Optimization of phorbol esters degradation by response surface methodology

RSM using three-factors-three-levels Box Behnken design was used to optimize the reaction conditions of PE degradation. Reaction time (X_1), ratio of buffer volume to DJSC (X_2) and enzyme to DJSC (X_3) were selected as the independent variables. PE degradation (Y) was considered as the response. The experimental ranges and levels of the independent variables are given in Table 1.

3. Results and Discussion

3.1. Effects of buffer pH on phorbol esters degradation

The effects of pH on PE degradation are shown in Figure 1. PE degradation increased 19.93% with an increase in buffer pH from 5 to 7. It decreased when buffer pH further increased to 9. Thus, the highest PE degradation was obtained at pH 7. These results correlated with the optimum pH of CGJS lipase (pH 7) for the hydrolysis of acyl glycerol (data not shown). Based on these results, pH 7 was selected as the pH condition for further experiments and selected as the center point of buffer pH for RSM analysis.

Table 1. Experimental ranges and levels of the independent variables in the Box-Behnken experimental design

Variables	Symbol	Unit	Coded Level		
			-1	0	1
Reaction time	X_1	h	24	28	32
Ratio of buffer volume to DJSC	X_2	mL/g	40:6	50:6	60:6
Ratio of enzyme to DJSC	X_3	(U/g)	20:5	30:5	40:5

3.2. Effects of reaction time on phorbol esters degradation

The effects of the reaction time on PE degradation are shown in Figure 2. PE degradation increased from 86.68 to 98.10% with an increase in the reaction time from 8 to 28 h. Degradation of PE reached equilibrium at the reaction time 28 h. PE content in DJSC was 0.08 mg/g for the reaction time 12 h. Since PE content in the non-toxic Jatropha seed is 0.11 mg/g [2], the degradation of PE in DJSC for 12 h was enough to full fill the requirement for non-toxic Jatropha seed cake. Further increase in the reaction time to 32 h did not significantly affect PE degradation. Hence, 28 h was considered to be the optimum reaction time and it was selected as the centre point of reaction time for RSM analysis.

3.3. Effects of buffer volume to DJSC ratio on phorbol esters degradation

Effects of the ratio of buffer volume to DJSC on PE degradation are shown in Figure 3. PE degradation increased from 92.10 to 98.54% with an increase in the ratio of buffer volume to DJSC from 40:6 to 50:6. Further increase in the ratio of buffer volume to DJSC to 80:6 did not affect significantly on PE degradation. This was in accordance with the work of Mustafa [unpublished data] about the effects of water content on enzymatic hydrolysis of jatropha curcas oil by CGJS lipase. It was reported that the forming of free fatty acids increased with an increase in water content from 40 to 65%. Further increase in water content to 75% did not affect significantly on the forming of free fatty acids. Hence, 50:6 mL/g was considered to be the best ratio of buffer volume to DJSC and it was selected as the centre point of ratio of buffer volume to DJSC.

3.4. Effects of enzyme to DJSC ratio on phorbol esters degradation

Effects of the ratio of enzyme to DJSC on PE degradation are shown in Figure 4. PE degradation increased with an increase in the ratio of enzyme to DJSC from 10:5 to 30:5. Further increase in the ratio of enzyme to DJSC to 50:5 did not have significantly effect on PE degradation. PE content in DJSC was 0.06 mg/g for the ratio of enzyme to DJSC of 10:5. Thus, it was enough to full fill the requirement for non-toxic Jatropha seed cake. Hence, the enzyme to DJSC of 30:5 U/g cake was considered as the best ratio of enzyme to DJSC and selected as the center point of the ratio of enzyme to DJSC for RSM analysis.

3.5. Optimization of phorbol esters degradation by response surface methodology

Data of PE degradation from the combination of independent variables are given in Table 2. Degradation of PE was in the range between 90.02 and 98.74 %. Combination of three center points for each independent variable (0,0,0), which was the target of the optimum conditions, showed the highest percentage of PE degradation (±98 %).

Multiple regression of the experimental data showed that data fitted well with a second-order polynomial equation. A mathematical regression model for PE degradation using the coded

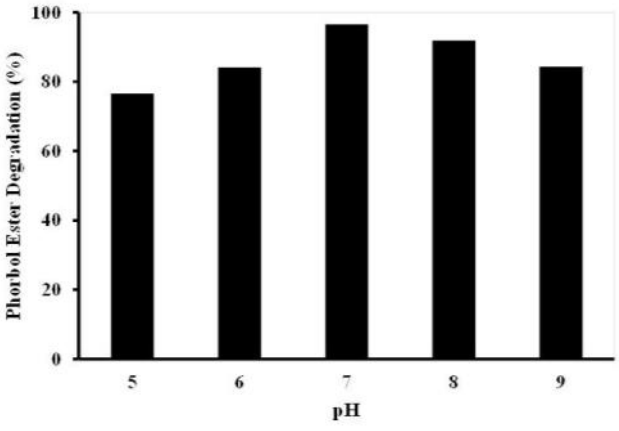

Figure 1. Effects of buffer pH on PE degradation. About one g CGJS lipase powder was added into suspension containing 5 g DJSC and 40 mL phosphate buffer at various pH. It was incubated at 30 °C and 120 strokes/min for 24 h

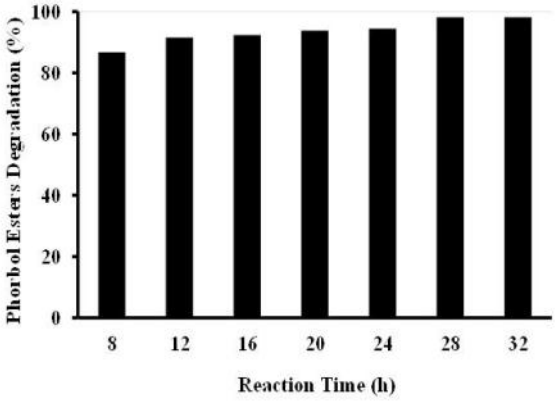

Figure 2. Effects of the reaction time on PE degradation. About one g CGJS lipase powder was added into suspension containing 5 g DJSC and various amount of phosphate buffer pH 7. It was incubated at 30 °C and 120 strokes/min for various reaction times

factors is given as follows:

$$Y = 98.5843 + 1.5843X_1 + 0.7708X_2 + 0.3851X_3 - 2.7812X_{11} - 1.6653X_{22} - 1.9582X_{33} - 1.2385X_{12} - 0.8363X_{13} + 0.4381X_{23} \quad (1)$$

where Y is the predicted response of PE degradation. The reaction time (X_1), the ratio of buffer volume to DJSC (X_2), and the ratio of enzyme to DJSC (X_3) are the coded variables.

Figure 5a shows that the interaction effect between the reaction time and the ratio of buffer volume to DJSC (X_{12}) was significant (p-value (0.014) < 0.05), when the ratio of enzyme to DJSC was kept constant at 30:5 (U/g cake). On the other hand, Figure 5b shows that the interaction effect between the reaction time and the ratio of enzyme to DJSC (X_{13}) was not significant (p-value 0.054 > 0.05), when the ratio of buffer volume to DJSC was constant at 50:6 (mL/g). Figure 5c also shows that the interaction effect between the ratio of buffer volume to DJSC and the ratio of enzyme to DJSC (X_{13}) was not significant (p-value 0.246 > 0.05), when the reaction time was constant at 28 h.

The ANOVA of the regression model demonstrated that the model was highly significant based on the Fisher's F-test (F = 23.23) and a very low p-value (p-value (0.001) < 0.05). The lack of fit of the model was not significant only at the 1% level (p-value > 0.001). The residual points on the plot fell fairly close to the straight line (data not shown), which represented a normal distribution of the residual [21]. In this case, R^2 was 0.97 indicated that 97.66% of the variability in the response could be explained by the model. R^2-adj was 0.93, which was very close to the R^2 value. All of these indicated that the model was accurately in describing the experimental data.

A response optimization plot is useful in determining the operating conditions that will result in a desirable response. The optimum conditions of PE degradation based on a response optimization plot were X_1 = 0.33; X_2 = 0.11; and X_3 = 0.01 for a predicted PE degradation of 98.96% (Y) and the desirability score was 0.95 under these conditions. When the coded values were converted into the actual values, the optimum conditions were 29.33 h, 51.11:6 (mL/g cake), and 30.10:5 (U/g cake) for the reaction time, the ratio of buffer volume to DJSC, and the ratio of enzyme to DJSC, respectively.

Validation of the predicted reaction conditions was conducted to confirm the validity of model. The result showed that the percentage of PE degradation was 98.91% and not significantly different from the predicted value. Based on the optimum reaction conditions, PE degradation by CGJS lipase could decrease PE content from 3.18 mg/g to 0.035 mg/g. Thus, PE content was lower than that of non-toxic variety of Jatropha curcas seed reported by Makkar [2], in which the value was 0.11 mg/g. Therefore, the target of the detoxification to reduce PE content to a safety level was successfully achieved.

4. Conclusions

The highest PE degradation was obtained at pH 7. The optimum reaction conditions of PE degradation by RSM were a reaction time of

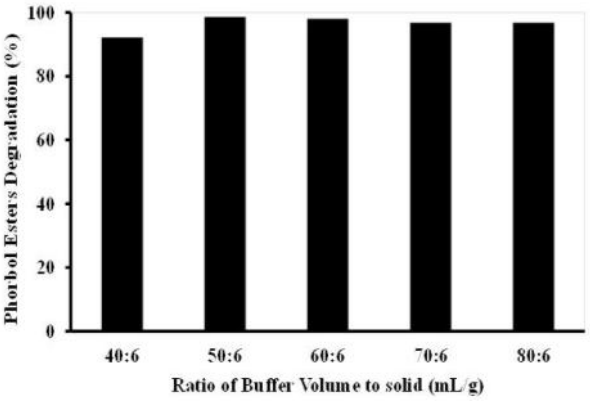

Figure 3. Effects of buffer to DJSC ratio on phorbol esters degradation. About one g CGJS lipase was added into suspension containing 5 g DJSC and various amount of phosphate buffer pH 7. It was incubated at 30 °C and 120 strokes/min for 24 h.

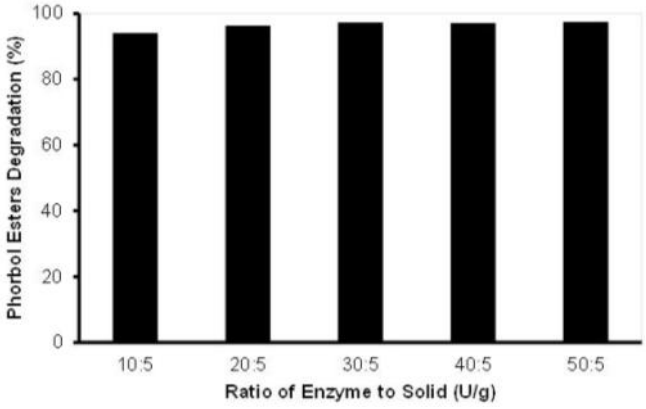

Figure 4. Effects of enzyme to DJSC ratio on PE degradation. Various amount of CGJS lipase was added into suspension containing 5 g DJSC and 40 mL phosphate buffer pH 7. It was incubated at 30 °C and 120 strokes/min for 24 h

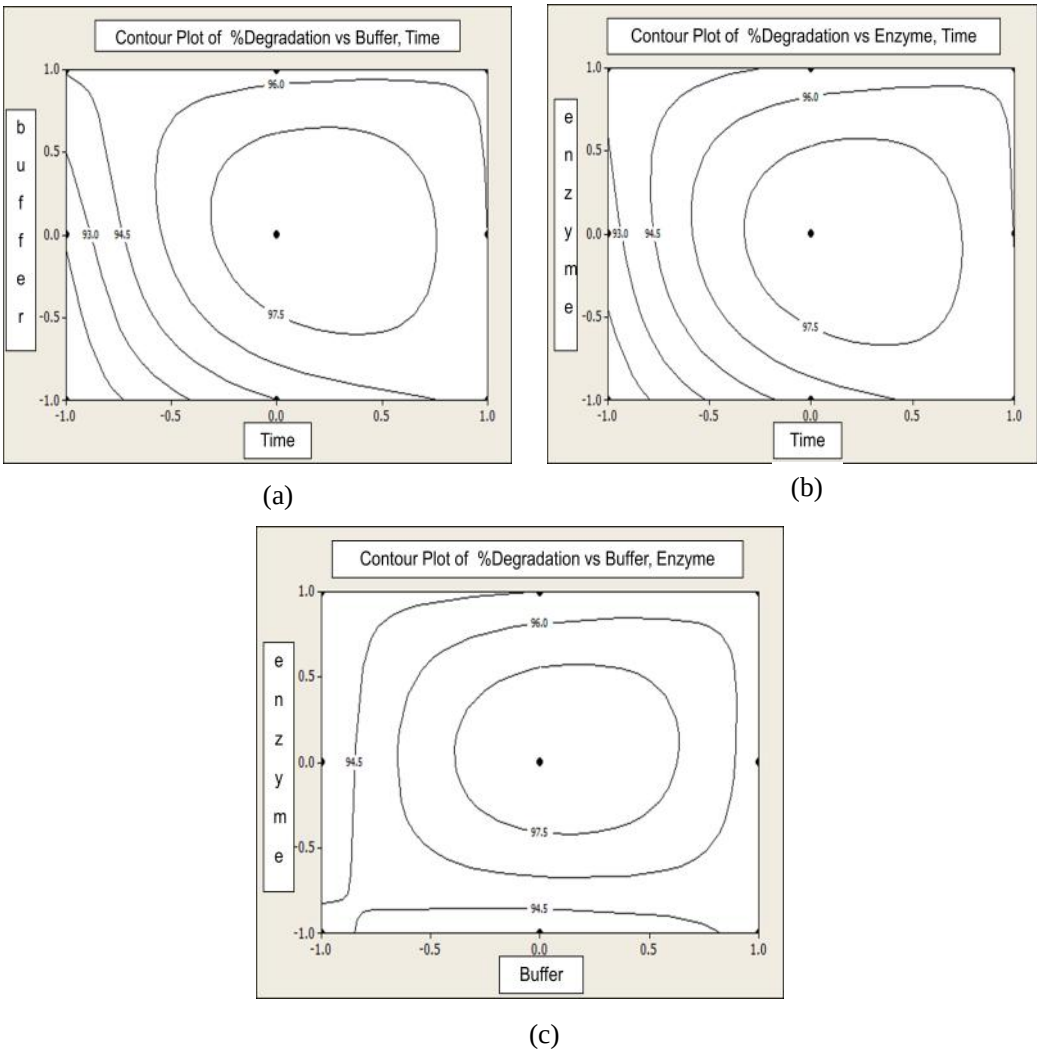

Figure 5. Contour plots of the interactive effect of two independent variables on the degradation of PE: (a) the interactive effect between the reaction time and the ratio of buffer volume to DJSC; (b) the interactive effect between the reaction time and the ratio of enzyme to DJSC; (c) the interactive effect of the ratio of buffer volume to DJSC and the ratio of enzyme to DJSC

Table 2. Experimental values of the Box-Behnken design

No	Reaction time	Ratio of buffer to DJSC	Ratio of enzyme to DJSC	PE Degradation (%)
	X_1	X_2	X_3	Y
1	-1a	-1	0	90.02
2	-1	1	0	94.61
3	1	-1	0	96.14
4	1	1	0	95.78
5	-1	0	-1	90.17
6	-1	0	1	93.22
7	1	0	-1	96.14
8	1	0	1	95.85
9	0	-1	-1	95.03
10	0	-1	1	93.92
11	0	1	-1	95.13
12	0	1	1	95.76
13	0	0	0	98.43
14	0	0	0	98.58
15	0	0	0	98.74

29.33 h, the ratio of buffer volume to DJSC of 51.11:6 (mL/g), and the ratio of enzyme to DJSC of 30.10:5 (U/g cake), for a predicted PE degradation of 98.96%. PE content was 0.035 mg/g, in which it was lower than PE content in non-toxic Jatropha seeds. The results indicated that enzymatic degradation of PE may a promising method to degrade PE in Jatropha seed cake.

References

[1] Makkar, H.P.S., Becker, K. (2009). Jatropha curcas, a promising crop for the generation of biodiesel and value-added coproducts. European. *Journal of Lipid Science and Technology*, 1: 773-787.

[2] Makkar, H.P.S., Aderibigbe, A.O., Becker, K. (1998). Comparative evaluation of nontoxic and toxic varieties of Jatropha curcas for chemical composition, digestibility, protein degradability and toxic factors. *Food Chemistry*, 62: 207-215.

[3] Makkar, H.P.S., Becker, K., Sporer, F., Wink, M. (1997). Studies on nutritive potential and toxic constituents of different provenances of Jatropha curcas. *Journal of Agricultural and Food Chemistry*, 45: 3152-3157.

[4] Aitken, A. (1986). The biochemical mechanism of action of phorbol esters. In: Evans F.J. (Ed.), Naturally Occurring Phorbol Esters. CRC Press, Boca Raton, pp. 271-288.

[5] Devappa, R.K., Darukeshwara, J., Raj, K.R., Narasimhamurthy, K., Saibaba, P., Bhagya, S. (2008). Toxicity studies of detoxified Jatropha meal (Jatropha curcas) in rats. *Food and Chemical Toxicology*, 46: 3621-3625.

[6] Martinez-Herrera, J., Siddhuraju, P., Francis, G., Davila-Ortiz, G., Becker, K. (2006). Chemical composition, toxic/antimetabolic constituents, and effects of different treatments on their levels, in four provenances of Jatropha curcas L. from Mexico. *Food Chemistry*, 96: 80-89.

[7] Aregheore, E.M., Becker, K., Makkar, H.P.S. (2003). Detoxification of a toxic variety of Jatropha curcas using heat and chemical treatments, and preliminary nutritional evaluation with rats. *South Pacific Journal of Natural Science,* 21: 50-56.

[8] Goel, G., Makkar, H.P.S., Francis, G., Becker, K. (2007). Phorbol esters: structure, biological activity, and toxicity in animals. *International Journal of Toxicology*, 26: 279-288.

[9] Mentlein, R. (1986). The tumor promoter 12-O-tetradecanoyl phorbol 13-acetate and regulatory diacyl glycerols are substrates for the same carboxyl esterase. *Journal of Biological Chemistry*, 261: 7816-7818.

[10] Cabot, M.C. (1985). Hydrolysis of novel diacylglycerol analogs and phorbol diesters by serum lipase. *Biochemical and Biophysical Research Communications*, 833(2): 330-335.

[11] Kadner, S.S., Katz, J., Levitz, M., Finlay, T.H. (1985). The 65-kDa phorbol-diester hydrolase in mouse plasma is esterase 1 and is immunologically distinct from the 56-kDa phorbol-diester hydrolase in mouse liver. *Journal of Biological Chemistry*, 260: 15604-15609.

[12] Fleming, N., Bilan, P.T., Sliwinski-Lis, E. (1986). Effects of a phorbol ester and diacylglycerols on secretion of mucin and arginine esterase by rat submandibular gland cells. *Pflugers Arch*, 406: 6-11.

[13] Joshi, C., Mathur, P., Khare, S.K. (2011). Degradation of phorbol esters by Pseudomonas aeruginosa PseA during solid-state fermentation of deoiled jatropha curcas seed cake. *Bioresource Technology*, 102: 4815-4819.

[14] de Barros, C.R.M., Ferreira, L.M.M., Nunes, F.M., Bezerra, R.M.F., Dias, A.A., Guedes, C.V., Cone, J.W., Marques, G.S.M., Rodrigues, M.A.M. (2011). The potential of white-rot fungi to degrade phorbol esters of Jatropha curcas L. seed cake. *Engineering in Life Sciences*, 11(1): 107-110.

[15] Belewu, M.A., Belewu, K.Y., Ogunsola, F.O. (2010). Nutritive value of dietary fungi treated jatropha curcas kernel cake: Voluntary intake, growth and digestibility coefficient of goat. *Agriculture and Biology Journal of North America*, 1(2): 135-138.

[16] Hidayat, C., Hastuti, P., Wardhani, A.K., Nadia, L.S. (2014). Method of phorbol ester degradation in Jatropha curcas L. seed cake using rice bran lipase. *Journal of Bioscience and Bioengineering*, 177(3): 372-374.

[17] Hidayat C., Hastuti P., Utazmi S., Wardhani A.K., Pradipta D.S. (2014). Enhancing indigenous lipase activity of germinated Jatropha curcas L. seeds for the enzymatic degradation of phorbol ester. *Biocatalysis and Agricultural Biotechnology*, 3(3): 71-76.

[18] Adebowale, Y.A., Adebowale, K.O. (2008). Emulsifying property of mucuna flour and protein isolates. *Journal of Food Technology*, 6(2): 66-79.

[19] Saetae, D., Suntornsuk, W. (2010). Antifungal activities of ethanolic extract from jatropha curcas seed cake. *Journal of Microbiology and Biotechnology*, 20: 319-324.

[20] Demissie, A.G., Lele, S.S. (2010). Bioassay-assisted identification of phorbol ester from Jatropha curcas (Linn.) tissue culture. *International Journal of Pharma and Bio Sciences*, 1: 1-7.

[21] Zulkali, M.M.D., Ahmad, A.L., Norulakmal, N.H. (2006). Oryza sativa L. husk as heavy metal adsorbent: Optimization with lead as model solution. *Bioresource Technology*, 97: 21-25.