Biocatalysis and Biotransformation, 2014; 32(4): 231–235

informa
healthcare

SHORT COMMUNICATION

Effect of ethanol concentrations on rice bran protease activity and ester synthesis during enzymatic synthesis of oleic acid ethyl ester in a fed-batch system using crude rice bran (*Oryza sativa*) lipase

CHUSNUL HIDAYAT[1,2], INDRO PRASTOWO[2,3], PUDJI HASTUTI[1] & RATIH RESTIANI[2]

[1]*Department of Food and Agricultural Product Technology, Faculty of Agricultural Technology, Gadjah Mada University, Yogyakarta, Indonesia,* [2]*Department of Postgraduate Program in Biotechnology, Postgraduate School, Gadjah Mada University, Yogyakarta, Indonesia, and* [3]*Department of Biology Education, Faculty of Teacher Training and Education, Ahmad Dahlan University, Kampus III, Yogyakarta, Indonesia*

Abstract

Crude rice bran lipase was used for the enzymatic synthesis of Oleic Acid Ethyl Ester (OAEE) in a fed-batch system. It was found that rice bran contains protease (4.19 U/g) and lipase (324.03 U/g). The results show that protease inhibits lipase at a low concentration of ethanol (5 μmol/ml), in which lipase esterification activity was only 45.02 U/g. However, protease activity decreased about 11 times at a higher concentration of ethanol (5–50 μmol/ml). It resulted in an increase in the esterification activity of lipase (324.03 U/g). This finding could be used for explaining the effects of ethanol on the enzymatic synthesis of OAEE using crude rice bran lipase in a fed-batch system. When the ethanol concentration in the reaction system was low (≤ 4 μmol/ml), the final OAEE conversion was only 30%. It was caused both by the inhibition of lipase by rice bran protease and by the effect of low ethanol concentration on the equilibrium position of the lipase-catalysed esterification reaction. However, the final OAEE conversion reached 76–92% when higher ethanol concentrations (12.5–50 μmol/ml) were added at initial reaction. It is suggested that the addition of higher ethanol concentrations (12.5–50 μmol/ml) into the reaction system may reduce protease activity and also increase ethanol concentration in the reaction system to be converted into OAEE.

Keywords: *Ethanol concentration, protease activity reduction, ester synthesis, oleic acid ethyl ester; fed-batch; rice bran lipase (*Oryza sativa*)*

Introduction

Lipase (E.C. 3.1.1.3) has shown its potential in catalyzing an esterification reaction (Rajendran et al. 2009; Sangeetha et al. 2011; Adlercreutz 2013). The explorations of low-priced lipase sources have been carried out for industrial applications (Tuter et al. 2003; Haas et al. 2001; Abigor et al. 2002; Natarajan et al. 2010). One of the sources of lipase is rice bran, which can be used as a low-priced biocatalyst for an esterification reaction (Bhardwaj et al. 2001; Chuang et al., 2011 and Shankar 2011). However, crude rice bran may contain protease which may repress lipase esterification activity by reducing the stability of lipase. In fact, the information on the presence of protease in rice bran is still limited and has not been reported yet. On the other hand, it was reported that ethanol could reduce protease activity (Tai and Fu 2003; Shankar 2011).

Enzymatic esterification is mostly carried out in a batch system (Shimada et al. 1999). However, an excess of alcohol concentration frequently occurs in this system which may lead to the permanent inactivation of lipase. Consequently, it can decrease ester synthesis (Al-Zuhair et al. 2007; Fjerbaek et al. 2009). An alternative to overcome this problem is a fed-batch system, in which alcohol is continuously fed into the reaction system at a certain flow rate (Khamseh and Miccio 2011). Meanwhile, an

Correspondence: Indro Prastowo, Department of Biology Education, Faculty of Teacher Training and Education, Ahmad Dahlan University, Kampus III, Jl. Prof. Dr. Soepomo, Janturan, Yogyakarta 55164, Indonesia. E-mail: indro.prastowo25@gmail.com

(Received 22 November 2013; revised 27 April 2014; accepted 11 June 2014)

ISSN 1024-2422 print/ISSN 1029-2446 online
DOI: 10.3109/10242422.2014.934683

introduction of substrates at a certain ratio at initial reaction can improve product synthesis during enzymatic synthesis in a fed- batch system (Sengupta and Modak 2001).

In this research, the effect of ethanol concentrations on both protease activity and lipase esterification activity was evaluated. Then, it was further used to explain the effects of ethanol on the enzymatic synthesis of Oleic Acid Ethyl Ester (OAEE) using crude rice bran lipase in a fed-batch system, in which various substrate molar ratios (ethanol/oleic acid) were introduced at initial reaction.

Materials and methods

Fresh rice bran of variety IR-4 was obtained from a local supplier in the Special District of Yogyakarta, Indonesia, and stored at −20°C. Pyridine, oleic acid, acetone, isooctane, ethanol, cupri-acetate, $NaH_2PO_4.H_2O$, $NaH_2PO_4.2H_2O$, Na_2CO_3, and trichloroacetic acid (TCA) were obtained from Merck KGaA (Darmstadt, Germany). Meanwhile, Casein, Folin-Ciocalteu reagent, and SIGMAFAST™ (Protease Inhibitor) were obtained from Sigma Co., (St. Louis, MO, USA).

Crude enzyme was prepared according to Prastowo et al. (2012). Rice bran (20 g) was homogenized in 35-ml cold acetone (−20°C) using a homogenizer (IKA T-50, Germany). Then, the rice bran was defatted in a Soxhlet using acetone for 30 min prior to drying at room temperature and stored at −20°C until used.

The effect of various ethanol concentrations on protease activity and lipase esterification activity was evaluated. Here, the defatted rice bran (29.58 g) was added into a 200 ml casein solution (1 ml casein in 1 ml phosphate buffer, pH 7) and a 200 ml oleic acid solution (3.1 M), each of which contained 0, 2.5, 5, 10, 20, 50 µmol/ml of ethanol concentration. Then, they were incubated at 30°C, and agitated at 1500 rpm for 10 min. Protease activity and lipase esterification activity were further determined according to Cupp-Enyard (2008) and Prastowo et al. (2012), respectively. The unit activity of protease was expressed as the amount of amino acids released from the protein hydrolysis per minute. Meanwhile, the unit activity of lipase was expressed as the amount of free fatty acid that reacted with the ethanol to produce OAEE per minute.

The enzymatic esterification reaction in the fed-batch system was carried out as follows: the ddefatted rice bran (29.58 g) was added into a 200 ml oleic acid solution (3.1 M) and then various concentrations of ethanol were added into the mixtures at the initial reaction (0 h) to obtain certain ratios (0: 1; 0.5: 1; 1: 1; 1.5: 1; and 2.05: 1). The control was an esterification reaction which was carried out at an initial substrate molar ratio (ethanol/oleic acid) of 0:1 with the addition of a protease inhibitor (10 mg/ml). Furthermore, another amount of ethanol was fed at a flow rate of 0.025 ml/min into the reaction system. The mixtures were incubated at room temperature (30°C), and agitated at 1500 rpm for 12 h. The amount of oleic acid, OAEE, ethanol and ester conversion were further determined every 2 h during the reaction. The amount of free fatty acid (FFA) was determined according to Lee et al. (2013), using a UV spectrophotometer (Genesys-20, USA) at 715 nm. The amount of OAEE and ethanol were calculated according to Prastowo et al. (2012), and Sengupta and Modak (2001), respectively. Meanwhile, ester conversion was calculated according to Mat Radzi et al. (2005), as the percentage of formation of ester from the fatty acid.

Results and discussions

Protease activity (4.19 U/g) was found in the rice bran. This finding is very important since it has not been reported yet by any other researchers in any references. Instead, they reported the discovery of genes encoding proteases and peptidases in the stem, leaves, and shoot of rice (not in the bran) (Lee et al. 2004; Chen et al. 2009; Feng and Xue 2006). A low concentration of ethanol (5 µmol/ml) did not have a significant effect on protease activity (Figure 1). Meanwhile, at the same ethanol concentration, lipase only showed a low esterification activity (45.02 U/g) (Figure 1). It is suggested that the low lipase esterification activity (45.02 U/g) may be due to the inhibition of lipase by the rice bran protease, which may not be inhibited by a low ethanol concentration.

In contrast, protease activity decreased up to 11 times while lipase esterification activity increased up to 324.03 U/g when the ethanol concentration in the

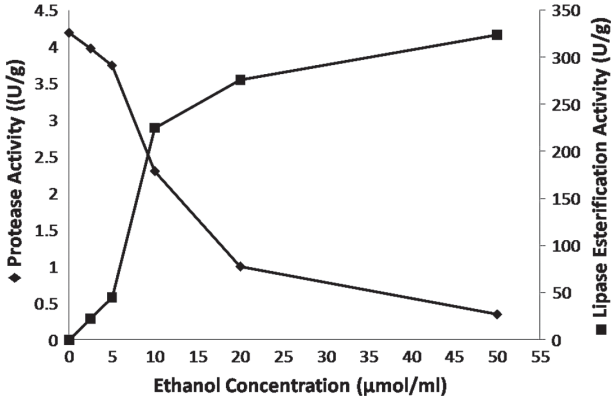

Figure 1. Effect of various ethanol concentrations on protease activity (◆) and lipase esterification activity (■).

system increased from 5 to 50 μmol/ml (Figure 1). It is suggested that rice bran protease is relatively more unstable than rice bran lipase in ethanol at that concentration. It may be due to a higher ethanol concentration (5–50 μmol/ml) that may cause the disruption of hydrogen bonds in the protease structure (Tai and Fu 2003; Shankar 2010). Consequently, protease may lose its 3-dimensional structure and furthermore its activity (Illanes 2008). As protease activity was reduced, the inhibition of lipase by protease could be avoided, and consequently, lipase esterification activity increased up to 324.03 U/g (Figure 1).

OAEE conversion in the system without the initial ethanol addition (initial substrate molar ratio [ethanol/oleic acid] of 0: 1) only increased up to 30% at the end of the reaction, during the enzymatic esterification reaction in the fed- batch system (Figure 2A). Surprisingly, it was still lower than OAEE conversions at the initial molar ratios of 0.5: 1, 1: 1, 1.5: 1 and 2.05: 1 at 2 h (40–58%) (Figure 2B–E). In this system (Figure 2A), the ethanol concentration in the reaction system was very low (≤ 4 μmol/ml) and the molar ratio (ethanol/oleic acid) was no higher than 0.25: 1 during the reaction. In the esterification reaction, ethanol reacts with the fatty acid to produce a fatty acid ethyl ester (Oliveira et al. 2001). Thus, it is quite natural that the rates of the synthesis of ethyl ester and also the final conversion were low as the substrate concentration

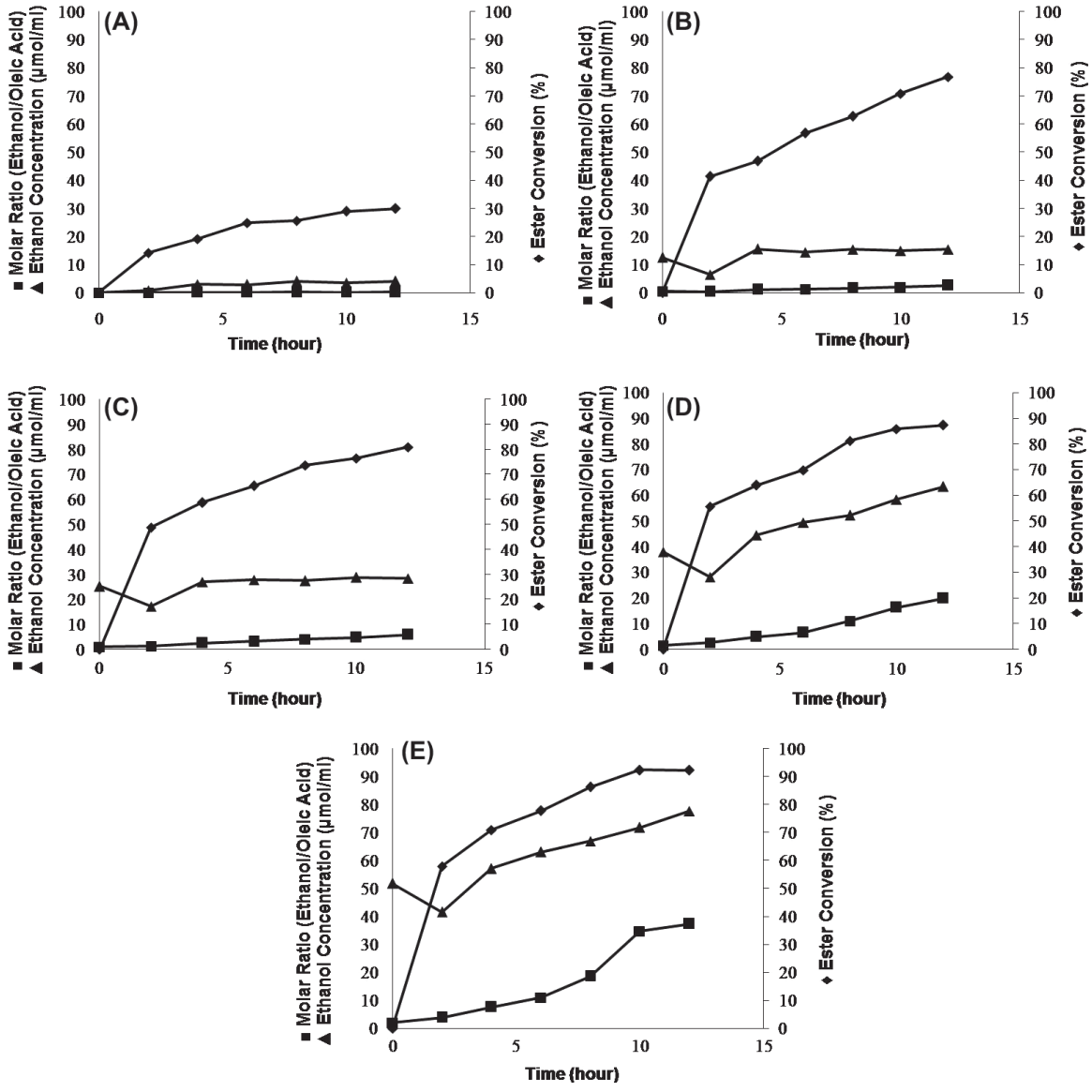

Figure 2. OAEE conversion (◆), ethanol concentration (▲) and substrate molar ratio (ethanol/oleic acid) (■) in the reactions performed at an ethanol flow rate of 0.025 ml/min, at 30°C, at various initial substrate molar ratios (ethanol/oleic acid): 0: 1 (A); 0.5: 1 (B); 1: 1 (C); 1.5: 1 (D); and 2.05: 1 (E) for 12 h.

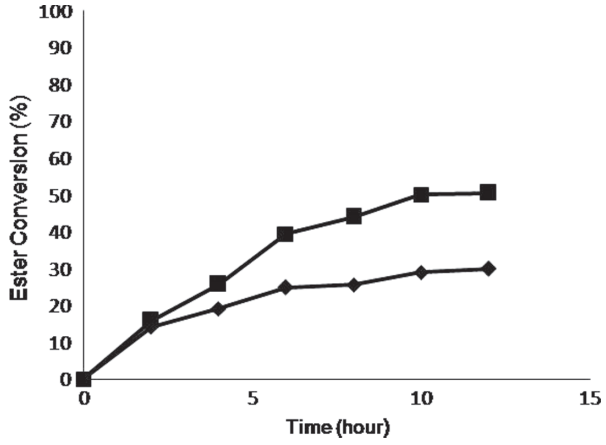

Figure 3. OAEE conversion in the reactions performed at an ethanol flow rate of 0.025 ml/min, at 30°C, at an initial substrate molar ratio (ethanol/oleic acid) of 0: 1, without (◆) and with the addition of a protease inhibitor (■) for 12 h.

(ethanol) in the reaction system was low (≤ 4 μmol/ml). However, Figure 3 shows that the addition of a protease inhibitor into the system without initial ethanol addition (initial molar ratio of 0: 1) increased the OAEE conversion up to 50.49% at the end of the reaction (1.7 times higher than that without the addition of a protease inhibitor). The difference in ester conversion (Figure 3) shows that protease, which was contained in the crude rice bran, was also involved in reducing the ester synthesis rate during the reaction. Protease may work at this level of ethanol concentration (≤ 4 μmol/ml) since a low ethanol concentration (5 μmol/ml) did not show a significant effect on protease activity (Figure 1). This active protease inhibited lipase and resulted in a low lipase esterification activity (45.02 U/g) (Figure 1). Thus, the low esterification activity of lipase due to the inhibition by protease and also the effect of low ethanol concentration on the equilibrium position of the esterification reaction may collaboratively cause a low ester synthesis (30%) in the system without initial ethanol addition (Figure 2A).

Meanwhile, at initial molar ratios of 0.5: 1, 1: 1, 1.5: 1 and 2.05: 1, higher concentrations of ethanol (12.5–50 μmol/ml) were added into the reaction system at initial reaction (Figure 2B–E). Figure 1 shows that an increase in ethanol concentration from 5 to 50 μmol/ml resulted in both a drastic decrease in protease activity (11 times) and a drastic increase in lipase esterification activity up to 324.03 U/g. It is suggested that the addition of higher ethanol concentrations (12.5–50 μmol/ml) may cause the inactivation of rice bran protease and avoid the inhibition of lipase. Thus, it may enable lipase to show high esterification activity (up to 324.03 U/g). On the other hand, the addition of higher ethanol concentrations (12.5–50 μmol/ml) into the reaction system may also increase the synthesis rate of OAEE since shifting the esterification reaction towards the formation of OAEE (Oliviera et al. 2001). This addition consequently resulted in a drastic increase in OAEE conversions (40–58%) in the first 2 h of the reaction (Figure 2B–E). However, from the first 2 h to the end of the reaction, OAEE conversions increased slowly (0.03–13%) to 76–92% at the end of the reaction (Figure 2B–E). During this period, substrate molar ratios (ethanol/oleic acid) were relatively higher than 2.05: 1 (Figure 2C–E) - except for 0.5: 1, in which it was from the 8th h to the end of the reaction (Figure 2B). It was reported that lipase esterification activity showed a decrease at the substrate molar ratio (ethanol/oleic acid) higher than 2.05: 1 (unpublished data) (Prastowo et al. 2012). It is suggested that the amount of ethanol at that level may have reduced lipase esterification activity (Prastowo et al. 2012; Goddard et al. 2000).

Conclusions

The low synthesis of OAEE (30%) in the system without the initial addition of ethanol was caused by both the inhibition of lipase by rice bran protease and the effect of low ethanol concentration on the equilibrium position of the lipase-catalysed esterification reaction. However, the addition of higher ethanol concentrations (12.5–50 μmol/ml) to the initial reaction resulted in an increase in the final ester conversion up to 76–92%. It is suggested that the addition of higher ethanol concentrations (12.5–50 μmol/ml) into the reaction system may reduce protease activity and also increase ethanol concentration in the reaction system to be converted into OAEE. Thus, ethanol concentration can be used not only to reduce protease activity but also to improve ester synthesis during enzymatic synthesis of OAEE in a fed-batch system using crude rice bran lipase.

Declaration of interest: The authors report no declarations of interest. The authors alone are responsible for the content and writing of the paper.

References

Abigor RD, Uadia PO, Foglia TA, Haas MJ, Scott K, & Savary BJ. 2002.Partial purification and properties of lipase from germinating seeds of Jatrophacurcas L. J Am Oil Chem Soc 79:1123–1126.

Adlercreutz P. 2013. Immobilisation and application of lipases in organic media. Chem Soc Revs 42:6406–6436.

Al-Zuhair S, Ling FW, Jun LS. 2007.Proposed kinetic mechanism of the production of biodiesel from palm oil using lipase. Process Biochem 42:951–960.

Bhardwaj K, Raju A, Rajasekharan R. 2001. Identification, purification, and characterization of a thermally stable lipase from rice bran.A new member of the (phospho) lipase family. Plant Physiol 127:1728–1738.

Chen J, Ouyang Y, Wang L, Xie W, Zhang Q. 2009. Aspartic proteases gene family in rice: gene structure and expression, predicted protein features and phylogenetic relation. Gene 442:108–118.

Chuang HH, Chen PT, Wang WN, Chen YT, Shaw JF. 2011. Functional proteomic analysis of rice bran esterases/lipases and characterization of a novel recombinant esterase. J Agric Food Chem 59:2019–2025.

Cupp-Enyard C. 2008. Sigma's non-specific protease activity assay-casein as a substrate. J Vis Exp, (19).

Feng Y, Xue Q. 2006. The serine carboxypeptidase like gene family of rice (Oryza sativa L. ssp. japonica). Funct Integr Genomics 6:14–24.

Fjerbaek L, Christensen KV, Norddahl B. 2009. A review of the current state of biodiesel production using enzymatic transesterification. Biotechnol Bioeng 102:1298–1315.

Goddard R, Bosley J, Al-Duri B. 2000. Esterification of oleic acid and ethanol in plug flow (packed bed) reactor under supercritical conditions: Investigation of kinetics. J Supercrit Fluids 18:121–130.

Haas MJ, Cichowicz DJ, Dierov JK. 2001. Lipolytic activity of California-laurel (Umbellulariacalifornica) seeds. J Am Oil Chem Soc 78:1067–1071.

Illanes A. 2008. Enzymes as catalyst: structure and functionality relationship. In: Illanes A, ed. Enzyme biocatalyst, principles and applications. Berlin: Springer. pp. 1–8.

Khamseh AA, Miccio M. 2011. Comparison of batch, fed-batch and continuous well-mixed reactors for enzymatic hydrolysis of orange peel wastes. Process Biochem.

Lee S, Jung KH, An G, Chung YY. 2004. Isolation and characterization of a rice cysteine protease gene, OsCP1, using T-DNA gene-trap system. Plant Mol Biol 54:755–765.

Lee JH, Kim SB, Yoo HY, Lee JH, Han SO, Park C, Kim SW. 2013. Co-immobilization of Candida rugosa and Rhyzopusoryzae lipases and biodiesel production. Korean J Chem Eng 1–4.

Mat Radzi S, Basri M, BakarSalleh A, Ariff A, Mohammad R, Abdul Rahman MB, Abdul Rahman RNZR. 2005. High performance enzymatic synthesis of oleyloleate using immobilised lipase from Candida Antarctica. Electron J Biotechnol 8.

Natarajan P, Kanagasabapathy D, Gunadayalan G, Panchalingam J, Sugantham PA, Singh KK, & Madasamy P. 2010. Gene discovery from Jatrophacurcas by sequencing of ESTs from normalized and full-length enriched cDNA library from developing seeds. BMC Genomics 11:606.

Oliveira AC, Rosa MF, Aires-Barros MR, Cabral JMS. 2001. Enzymatic esterification of ethanol and oleic acid—a kinetic study. J Mol Catal B Enzym 11:999–1005.

Prastowo I, Hidayat C, & Hastuti P. 2012. Production and optimization of oleic acid ethyl ester synthesis using lipase from rice bran (Oryza sativa L.) and germinated jatropha seeds (Jatrophacurcas L.) by response surface methodology. Indon J Biotechnol 17:17–26.

Rajendran A, Palanisamy A, Thangavelu V. 2009. Lipase catalyzed ester synthesis for food processing industries. Braz Arch Biol Technol 52:207–219.

Sangeetha R, Arulpandi I, Geetha A. 2011. Bacterial lipases as potential industrial biocatalysts: an overview. Res J Microbiol 6:1–24.

Sengupta S, Modak JM. 2001. Optimization of fed-batch bioreactor for immobilized enzyme processes. Chem Eng Sci 56: 3315–3325.

Shankar S. 2011. Stability of Protease in Organic Solvent, Detergent and Denaturant, PhD Thesis, University of Pune, India. 107–121.

Shimada Y, Watanabe Y, Samukawa T, Sugihara A, Noda H, Fukuda H, Tominaga Y. 1999. Conversion of vegetable oil to biodiesel using immobilized Candida Antarctica lipase. J Am Oil Chem Soc 76:789–793.

Tai DF, Fu SL. 2003. Bromelain catalyzed synthesis of peptides in organic solvent. J Chin Chem Soc Taip 50:179–183.

Tuter M, Secundo F, Riva S, Aksoy HA, & Ustun G. 2003. Partial purification of Nigella sativa L. seed lipase and its application in transesterification reactions. J Am Oil Chem Soc 80:43–48.