PHILIPP AGRIC SCIENTIST
Vol. 101 No. 4, 363-368
December 2018

ISSN 0031-7454

Production of Extracellular Lipase from the Antarctic Bacterial Isolate *Pseudomonas* sp. INK1 by Solid State Fermentation of Soybean Meal

Inkyung Park[1], Jaekoo Lee[1] and Jaiesoon Cho[*]

Department of Animal Science and Technology, Konkuk University, 120 Neungdong-ro, Gwangjin-gu, Seoul 05029, South Korea
[1]These authors equally contributed to this work.
[*]Author for correspondence;e-mail:chojs70@konkuk.ac.kr, Tel: +82-2-450-3375; Fax: +82-2-455-1044

The production of extracellular lipase from the Antarctic bacterial isolate, *Pseudomonas* sp. INK1, by solid state fermentation using agricultural residues as substrate was evaluated. Among the substrates tested, soybean meal was most favorable for the lipase production, increasing the enzyme activity 1.7- and 2.5-fold in comparison with wheat bran and corn flour, respectively, and it was chosen for further work. Maximum lipase production (7,553 ± 235 U/g) was observed at 84 h of fermentation. Lipase production was optimal at moisture content of 71.4%, pH of 6–7, and inoculum size of 6%. The addition of maltose as the carbon source and peptone as the nitrogen source was most effective for enhancing lipase production. This solid state fermentation is meaningful in converting inexpensive agricultural residues into highly valued biotechnological products such as lipase. It may benefit the conventional sector of crop agriculture.

Key Words: lipase, *Pseudomonas* sp., solid state fermentation, soybean meal

INTRODUCTION

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3), which mainly initiate the hydrolysis of insoluble triacylglycerols to free fatty acids, mono and diacylglycerols and glycerol (Rigo et al. 2010), have a broad spectrum of applications in the food, detergent, cosmetic, pharmaceutical and energy industries (Sharma et al. 2001; Ranganathan et al. 2008; Treichel et al. 2010) and they occupy the third major group after proteases and amylases in the global enzyme market (Rigo et al. 2010). Although lipases are ubiquitous in animals (Shimokawa et al. 2005), plants (Villeneuve 2003) and microbes (Ionita et al. 1997; Mahadik et al. 2002; Burkert et al. 2004), microbial lipases are most favorable for industrial applications, due to their ease of mass production and inexpensive fermentation techniques as well as their competent stability and substrate specificity (Contesini et al. 2010; Rajan and Nair 2011).

Recently, solid state fermentation (SSF) characterized by the growth and metabolism of microbes on moist solids without any free flowing water, has been regarded as an efficacious tool to produce diverse bio-products such as feeds, organic acids, bio-pulp, aroma compounds, enzymes, antibiotics, compost, bio-fertilizer and bio-pesticides. It has several merits, including the use of inexpensive agricultural residues, application using simple equipment, and reduced environmental concerns (Balaji and Ebenezer 2008; Chaturvedi et al. 2010; Rajan and Nair 2011). In particular, soybean meal, which is the most important protein supplement of global livestock feeding as the representative agricultural residue of edible oil extraction (Stein et al. 2008), has great potential as substrate for SSF in producing biotechnological products such as enzymes, antibodies, bio-pesticides and vitamins (Rigo et al. 2010). So far, most studies concerning SSF for microbial lipase production have been conducted using fungal strains (Mahadik et al. 2002; Falony et al. 2006; Balaji and Ebenezer 2008) due to their better resistance to low moisture content (Oliveira et al. 2016). However, little data are available on lipase production in SSF using bacterial *Pseudomonas* species, despite the fact that they are prominent sources for industrial applications of the enzyme (Wang et al. 2009). The objective of this work is to evaluate the production conditions of lipase under SSF using soybean meal by the Antarctic bacterial isolate *Pseudomonas* sp. INK1.

MATERIALS AND METHODS

Reagents

The substrate, *p*-nitrophenyl palmitate (pNPP) for lipase assay was obtained from Sigma-Aldrich (St. Louis, MO, USA). Peptone, tryptone, yeast extract, tryptic soy broth and Bacto agar were purchased from BD Bioscience (Sparks, MD, USA). All other chemicals used in this study were of analytical grade and were procured from Sigma-Aldrich.

Microorganism and Inoculum

The bacterial strain *Pseudomonas* sp. INK1, described in a previous study (Park and Cho 2012), was used for lipase production. It was routinely grown on tryptic soy agar made up of tryptic soy broth and 1.5% bacto agar at 28 °C for 24 h for inoculum preparation. A single fresh colony of the growth was amplified in a 50 mL falcon tube containing 5 mL of tryptic soy broth at 28 °C for 12 h on a rotary shaker (220 rpm). One percent of the culture was transferred to a 250 mL Erlenmeyer flask containing 50 mL of the medium, followed by incubation for 24 h. This preculture was used as inoculum for further solid state fermentation.

SSF

Commercial quality wheat bran, soybean meal, and corn flour were initially evaluated for selection of an appropriate solid substrate in the fermentation. Briefly, 10 g of the substrate was placed in 250 mL Erlenmeyer flasks. The initial pH and moisture content was 5.5–6.0% and 50%, respectively. After autoclaving at 121 °C for 15 min, the flasks were inoculated with 4% inoculum and incubated at 28 °C with vigorous shaking (220 rpm).

Proximate components of the soybean meal were: dry matter, 89.98%; crude protein, 47.73%; crude fiber, 3.89%; ether extract, 1.52%; acid ether extract, 2.86%; and ash, 6.27%. Unless otherwise stated, SSF was performed solely using 10 g soybean meal (71.4% moisture content) in 250 mL Erlenmeyer flasks with 6% inoculum, followed by incubation at 28 °C. The effects of various physicochemical parameters including substrate moisture content (33–80%), incubation time (0–120 h), pH (5–8) and inoculum size (1–20%) were investigated for the optimum production of lipase by *Pseudomonas* sp. INK1. Additionally, studies were conducted to evaluate the influence of different carbon (glucose, sucrose, galactose, lactose and maltose; final concentrations 1%) and nitrogen (peptone, tryptone, yeast extract, urea, sodium nitrate; final concentrations 1%) sources on enzyme production.

Crude Enzyme Preparation

Enzyme extracts were obtained by mixing a weighed quantity of the fermented matter with 40 mL of cold distilled water, and then shaking the mixture on a rotary shaker (220 rpm) at 28 °C for 1 h. The suspension was centrifuged at 4 °C for 10 min at 5,000 rpm, and the supernatant was used for the lipase assay.

Lipase Assay

Lipase activity was assayed at 50 °C for 15 min in a reaction mixture that consisted of 1.78 mL solution containing 0.1 M NaCl and 0.5% Triton-X100 in 50 mM Tris-HCl (pH 7.4), 20 µL of 13 mM *p*-nitrophenyl palmitate and 0.3 mL of the crude enzyme. The amount of liberated *p*-nitrophenol was monitored spectrophotometrically at 410 nm. One unit (U) of the enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitrophenol per second under the given conditions and was expressed as U/g of dry substrate in SSF.

Statistical Analysis

The results were subjected to a one-way analysis of variance using PROC GLM (SAS 9.4, SAS Institute Inc, Cary, NC, USA) to test for significant differences between treatments with the Duncan's multiple range test. The probability levels used for statistical significance were $P<0.05$ for all tests.

RESULTS AND DISCUSSION

Substrate Selection

Among the substrates tested, soybean meal was most favorable for lipase production by SSF, with significant 1.7- and 2.5-fold increases (both $P<0.05$) of the enzyme activity in comparison with wheat bran and corn flour, respectively (Table 1). Accordingly, it was chosen for further work.

Time Course of Lipase Production by SSF

The highest lipase production (7,553 ± 235 U/g) was found ($P < 0.05$) at 84 h (Fig. 1). After that, it steeply decreased, which may be due to the depletion of substrate as previously observed in the yeast, *Yarrowia lipolytica* (Babu and Rao 2007; Imandi et al. 2013) and with the bacteria, *Bacillus subtilis* (Chaturvedi et al. 2010) and *Bacillus pumilus* (Sangeetha et al. 2011). Lipase production by SSF using pure soybean meal as substrate

Table 1. Lipase synthesis by *Pseudomonas* sp. INK1 on different solid substrates.

Solid Substrate	Enzyme Activity* (U/g)
Wheat bran	337 ± 62[b]
Soybean meal	579 ± 32[a]
Corn flour	231 ± 97[b]

*One unit (U) of the enzyme activity was defined as the amount of enzyme required to liberate 1 nmol of *p*-nitrophenol per second under the given conditions and was expressed as U/g of dry substrate in SSF.

[a~b]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments.

and the fungus, *Penicillium simplicissimum* yielded enzyme activity of 21.0 U/g at 48 h of fermentation (Di Luccio et al. 2004), which was much lower than our result (5625 ± 43 U/g).

Effect of Initial Moisture Content in SSF

The maximum yield of lipase was found (P<0.05) when moisture content was 71.4% (Fig. 2), similar to the previous results using *Bacillus subtilis* (Chaturvedi et al. 2010), *Streptomyces* sp. TEM33 (Cadirci et al. 2016), *Curvularia* sp. DHE 5 (El-Ghonemy et al. 2017) or *Rhizopus* sp. (Riyadi et al. 2017). In addition, a drastic reduction in enzyme yield occurred (P<0.05) at very high moisture level (over 75%), which may be associated with the steric hindrance of microorganism growth due to lack of available inter-particle space in substrate and decreased porosity (Rajan and Nair 2011). In contrast, the highest yield was obtained at 80% moisture content for the lipase production by *Yarrowia lipolytica* (Babu and Rao 2007).

Effect of Initial pH on Enzyme Production

The initial pH of the fermentation is one of the most important factors for the optimal growth of microorganism and the efficient production of extracellular enzyme (Prakasham et al. 2006). The highest yield was observed at pH 6 and the effect lasted by pH 7 (Fig. 3). In addition, the optimum pH for the lipase production by SSF using *Pongamia pinnata* seed cake as substrate in *Bacillus pumilus* (Sangeetha et al. 2011) and using wheat bran in *Curvularia* sp. DHE 5 (El-Ghonemy et al. 2017) has been reported to be pH 7.0.

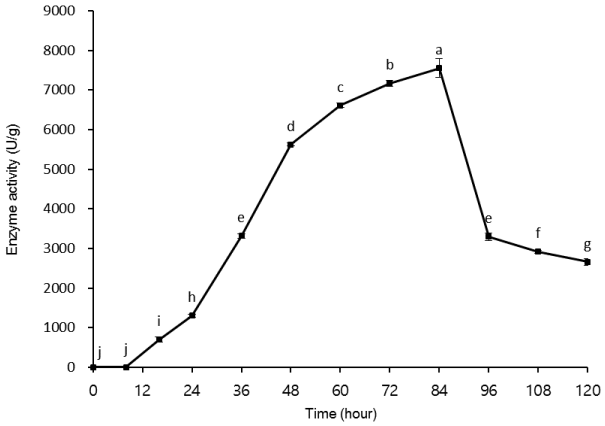

Fig. 1. Time course of the INK1 lipase production in SSF using soybean meal. [a~j]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments.

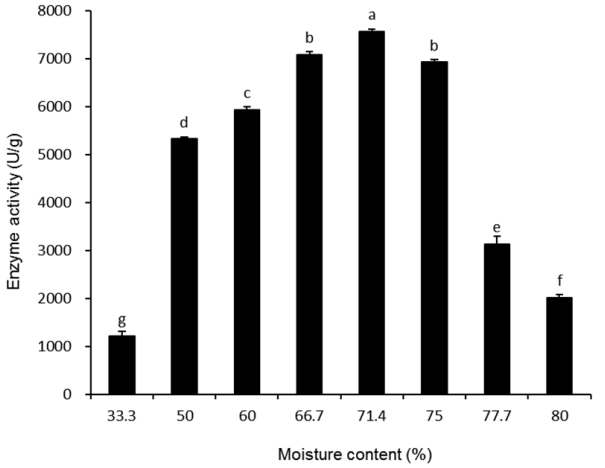

Fig. 2. Effect of initial moisture content on the INK1 lipase production in SSF. [a~g]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments.

Effect of Inoculum Size on Enzyme Production

As shown in Figure 4, the optimal inoculum size for fermentation was 6% (P<0.05). The lipase production was significantly reduced at lower inoculum sizes (1–2%). This may be due to the lack of cell number (Balakrishna et al. 2017). Nevertheless, the highest lipase production of *Rhizopus* sp. using SSF of palm kernel cake was observed with 2% inoculum size (Riyadi et al. 2017). In addition, the enzyme production was reduced at higher inoculum sizes (10–20%) (Fig. 4). This may be due to severe competition among cells toward nutrients (Balakrishna et al. 2017).

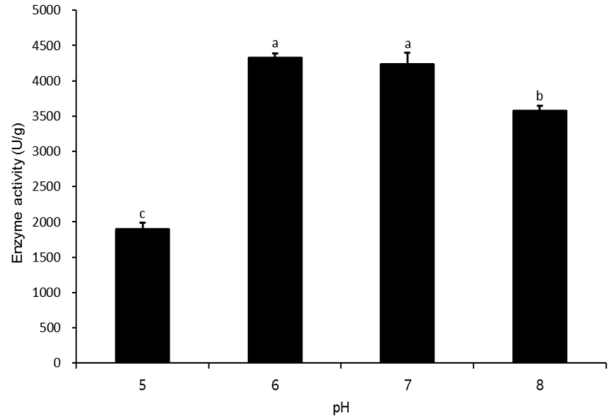

Fig. 3. Effect of initial pH of the fermentation medium on the INK1 lipase production in SSF. [a~c]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments.

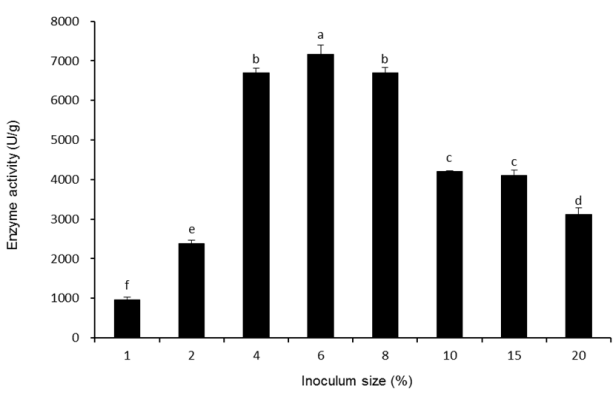

Fig. 4. Effect of inoculum size on the INK1 lipase production in SSF. [a~f]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments.

Effect of Carbon Supplementation on Enzyme Production

Lactose, galactose and maltose were effective in enhancing enzyme activity (all P<0.05) and the maximum yield with maltose was significantly greater than with the other carbon sources (P<0.05) (Fig. 5). In a recent report, the highest lipase production by *Penicillium camemberti* was induced in SSF on rapeseed cake supplemented with lactose (Boratynski et al. 2018). Meanwhile, no remarkable effect was found using sucrose and glucose. Babu and Rao (2007) have reported that glucose had the best impact on the lipase yield in *Yarrowia lipolytica* by SSF.

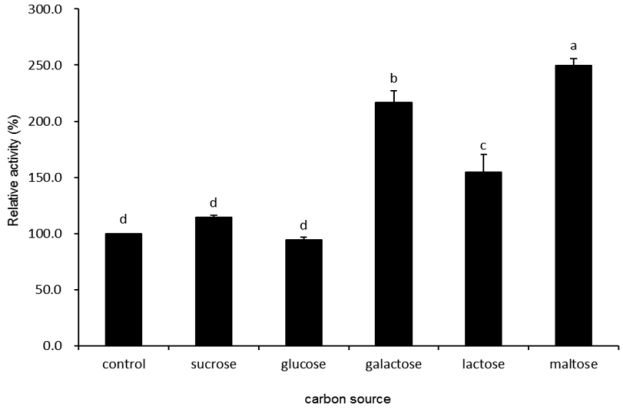

Fig. 5. Effect of different carbon sources on the INK1 lipase production in SSF. Fermentation medium without any carbon supplementation was taken as control. [a~d]Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments. One hundred percent (100%) of the relative activity equates to 1,789 ± 75 (U/g).

Effect of Nitrogen Supplementation on Enzyme Production

Among the nitrogen sources tested, peptone was best (P<0.05) for lipase production (Fig. 6), in agreement with the previous results of SSF in Colletotrichum gloeosporiodes (Balaji and Ebenezer 2008) and *Rhizopus* sp. (Riyadi et al. 2017). Presumably, peptone is relatively rich in amino acids and co-factors essential to enhance the microbial growth (Balaji and Ebenezer 2008). Other organic nitrogen sources such as yeast extract and tryptone are relatively good for enzyme production, which were also founded with *Candida parapsilosis* (Balakrishna et al. 2017). However, urea and sodium nitrate showed no remarkable effect on enhancing lipase activity. Meanwhile, the addition of urea induced the highest lipase yield in *Yarrowia lipolytica* by SSF (Babu and Rao 2007: Imandi et al. 2013).

In conclusion, SSF strategy allows the conversion of inexpensive agricultural residues into highly valued biotechnological products such as lipase. It may extend the conventional sector of crop agriculture. Further study for scale up of the lipase production will be warranted.

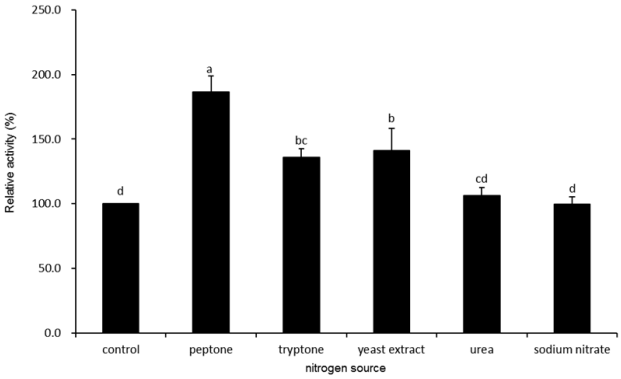

Fig. 6. Effect of different nitrogen sources on the INK1 lipase production in SSF. Fermentation medium without any nitrogen supplementation was taken as control. $^{a\sim d}$Means lacking common superscripts differ significantly (P<0.05). Data were expressed as mean ± standard errors from three experiments. One hundred percent (100%) of the relative activity equates to 3,039 ± 158 (U/g).

REFERENCES CITED

BABU IS, RAO GH. 2007. Lipase production by *Yarrowia lipolytica* NCIM 3589 in solid state fermentation using mixed substrate. Res J Microbiol 2: 469–474.

BALAJI V, EBENEZER P. 2008. Optimization of extracellular lipase production in *Colletotrichum gloeosporioides* by solid state fermentation. Ind J Sci Technol 1:1 –8.

BALAKRISHNA K, SWARUPARANI G, HANUMALAL N, BINDU S, BHIMA B. 2017. Plausible exploitation of Jatropha de-oiled seed cake for lipase and phytase production and simultaneous detoxification by *Candida parapsilosis* isolated from poultry garbage. Bioresour Technol 225: 215–224.

BORATYNSKI F, SZCZEPANSKA E, GRUDNIEWSKA A, GNILKA R, OLEJNICZAK T. 2018. Improving of hydrolases biosynthesis by solid-state fermentation of *Penicillium camemberti* on rapeseed cake. Sci Rep 8:10157. Doi: 10.1038/s41598-018-28412-y.

BURKERT JFM, MAUGERI F, RODGRIGUES MI. 2004. Optimization of extracellular lipase production by *Geotrichum* sp. using factorial design. Bioresour Technol 91: 77–84.

CADIRCI BH, YASA I, KOCYIGIT A. 2016. *Streptomyces* sp. TEM 33 possesses high lipolytic activity in solid-state fermentation in comparison with submerged fermentation. Prep Biochem Biotechnol. 46: 23–29.

CHATURVEDI M, SINGH M, MAN CR, PANDEY S. 2010. Lipase production from *Bacillus subtilis* MTCC 6808 by solid state fermentation using ground nut oil cakes as substrate. Res J Microbiol 5: 725–730.

CONTESINI FJ, LOPES DB, MACEDO GA, NASCIMENTO M, CARVALHO P. 2010. *Aspergillus* sp. lipase: potential biocatalyst for industrial use. J Mol Catal B Enzym 67: 163–171.

DI LUCCIO M, CAPRA F, RIBEIRO NP, VARGAS GDLP, FREIRE DMG, DE OLIVEIRA D. 2004. Effect of temperature, moisture, and carbon supplementation on lipase production by solid state fermentation of soy cake by *Penicillium simplicissimum*. Appl Biochem Biotechnol 113–116: 173–180.

EL-GHONEMY DH, EL-GAMAL MS, TANTAWY AE, ALI TH. 2017. Extracellular alkaline lipase from a novel fungus *Curvularia* sp. DHE 5: optimisation of physicochemical parameters, partial purification and characterisation. Food Technol Biotechnol 55: 206–217.

FALONY G, ARMAS JC, MENDOZA JCD, HERNANDEZ JLM. 2006. Production of extracelluar lipase from *Aspergillus niger* by solid state fermentation. Food Technol Biotechnol 44: 235–240.

IMANDI SB, KARANAM SK, GARAPATI HR. 2013. Use of Plackett-Burman design for rapid screening of nitrogen and carbon sources for the production of lipase in solid state fermentation by *Yarrowia lipolytica* from mustard oil cake (*Brassica napus*). Brazil J Microbiol 44: 915–921.

IONITA A, MOSCOVICI M, POPA C, VAMANU A, POPA O, DINU L. 1997. Screening of yeast and fungal strains for lipolytic potential and determination of some biochemical properties of microbial lipases. J Mol Catal B Enzym 3: 147–151.

MAHADIK ND, PUNTAMBEKAR US, BASTAWDE KB, KHIRE JM, GOKHALE D. 2002. Production of acid lipase by *Aspergillus niger* in solid state fermentation. Process Biochem 38: 715–721.

OLIVEIRA F, MOREIRA C, SALGADO JM, ABRUNHOSA L, VENANCIO A, BELO I. 2016. Olive pomace valorization by *Aspergillus* species: lipase production using solid-state fermentation. J Sci Food Agric 96: 3583–3589.

PARK I, CHO J. 2012. Extracellular lipase of the Antarctic bacterial isolate *Pseudomonas* sp. INK1 as a potential tool for improving the flavor quality of dairy products. Afr J Agri Res 7: 2502–2508.

PRAKASHAM RS, RAO S, SARMA PN. 2006. Green gram husk-an inexpensive substrate for alkaline protease production by *Bacilllus* sp. in solid state fermentation. Bioresour Technol 97: 1449–1454.

RAJAN A, NAIR AJ. 2011. A comparative study on alkaline lipase production by a newly isolated

Aspergillus fumigatus MTCC 9657 in submerged and solid state fermentation using economically and industrially feasible substrate. Turk J Biol 35: 569–574.

RANGANATHAN SV, NARASIMHAN SL, MUTHUKUMAR L. 2008. An overview of enzymatic production of biodiesel. Bioresour Technol 99: 3975–3981.

RIGO E, NINOW JL, LUCCIO MD, OLIVEIRA JV, POLLONI AE, REMONATTO D, ARBTER F, VARDANEGA R, DE OLIVEIRA D, TREICHEL H. 2010. Lipase production by solid state fermentation of soybean meal with different supplements. LWT Food Sci Tech 43: 1132–1137.

RIYADI FA, ALAM MZ, SALLEH MN, SALLEH HM. 2017. Optimization of thermostable organic solvent-tolerant lipase production by thermotolerant *Rhizopus* sp. using solid-state fermentation of palm kernel cake. 3. Biotech 7: 300. Doi: 10.1007/s13205-017-0932-1.

SANGEETHA R, GEETHA A, ARULPANDI I. 2011. *Pongamia pinnata* seed cake: A promising and inexpensive substrate for production of protease and lipase from *Bacillus pumilus* SG2 on solid state fermentation. Indian J Biochem Biophys 48: 435–439.

SHARMA R, CHISTI Y, BANERJEE UC. 2001. Production, purification, characterization, and applications of lipases. Biotechnol Adv 19: 627–662.

SHIMOKAWA Y, HIRATA K, ISHIDA T, KOJIMA Y, INOUE N, QUERTERMOUS T, YOKOYAMA M. 2005. Increased expression of endothelial lipase in rat models of hypertension. Cardiovasc Res 66: 594–600.

STEIN HH, BERGER LL, DRACKLEY JK, FAHEY JR GC, HERNOT DC, PARSONS CM. 2008. Nutrition properties and feeding values of soybeans and their coproducts. In: Johnson A, White PJ, Galloway R. editors. Soybeans: Chemistry, Production, Processing, and Utilization. AOCS Press, Urbana, IL. USA. p. 613–660.

TREICHEL H, DE OLIVEIRA D, MAZUTTI MA, DI LUCCIO M, OLIVERA JV. 2010. A review on microbial lipases production. Food Bioprocess Technol 3: 182–196.

VILLENEUVE P. 2003. Plant lipases and their applications in oils and fat modification. Eur J Lipid Sci Technol 105: 308–317.

WANG SL, LIN YT, LIANG TW, CHIO SH, MING LJ, WU PC. 2009. Purification and characterization of extracellular lipases from *Pseudomonas monteilii* TKU009 by the use of soybeans as the substrate. J Ind Microbiol Biotechnol 36: 65–73.