Published Online on 24 May 2017

Proc Indian Natn Sci Acad **83** No. 3 September 2017 pp. 681-687

DOI: 10.16943/ptinsa/2017/49025

Research Paper

Purification and Characterization of a Halotolerant and Thermotolerant Lipase Produced from a Novel Bacteria "*Brevibacterium halotolerans* PS4 |KX671556|" and Its Application in Detergent Formulations

PUSHPINDER SHARMA, NIVEDITA SHARMA*, POONAM SHARMA, SHRUTI PATHANIA and SHWETA HANDA

Microbiology Research Laboratory, Deptt. of Basic Sciences, Dr. Y. S. Parmar University of Horticulture and Forestry, Nauni (Solan) Himachal Pradesh 173 230, India

(Received on 15 January 2017; Revised on 27 April 2017; Accepted on 01 May 2017)

An extracellular lipase was isolated and purified from the culture broth of *Brevibacterium halotolerans* PS4 to provide homogeneity, using ammonium sulfate precipitation, followed by chromatographic techniques on Sephadex G-75 column, resulting in a purification factor of 2.98 fold with specific activity of 1016.10 IU/mg. The molecular weight of the purified lipase was estimated by SDS-PAGE to be 80 kDa. The purified lipase had maximal activity within the pH range of 6 to 7, with an optimum pH of 7, and within the temperature range of 35 to 55°C. The purified lipase exhibited not only stable but enhanced maximal activity by Triton X100. The enzyme activity of *Brevibacterium halotolerans* PS4 lipase was enhanced by Ca^{2+} and Mg^{2+}. SDS and metal ions such as Hg^{2+}, Zn^{2+}, Cu^{2+}, Ag^{2+} and Fe^{2+} decreased the lipase activity remarkably. The extracellular lipase from orchard soil isolate was further applied for its application as laundry additives.

Keywords: Extracellular Lipase; Purification; Characterization; *Brevibacterium halotolerans* PS4

Introduction

Lipases are glycerol ester hydrolases (EC: 3.1.1.3), which hydrolyze ester linkages of glycerides at water-oil interface (Garlapati *et al*., 2010). During hydrolysis, lipases pick acyl group from glycerides forming lipase-acyl complex, which then transfers its acyl group to OH group of water (Ramani *et al*., 2010). Lipases are the most important group of industrial bio-catalysts that can be applied both as hydrolases and as synthetases and proved their enormous potential in various biotechnological applications. Unique characters of lipases such as high stability in organic solvents, their broad substrate specificity, and high enantio-selectivity greatly increased their demand in industrial market. The current market scenario of hydrolytic enzymes positioned lipases at the top third rank after proteases and amylases and their annual market is targeted to reach about 590.5million dollars by 2020. Microbial lipases have already established their vast potential regarding their usage in different industries (Bora and Kalita, 2008). In the last decades, the interest in microbial lipase production has increased (Rajesh *et al*., 2010), because of its large potential in industrial applications as additives for foods (flavor modification), fine chemicals (synthesis of esters), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), pharmaceuticals (digestion of oil and fats in foods), leather (removal of lipids from animal skins) and medicine (blood triglyceride assay) (Nadia *et al*., 2010; Padmapriya *et al*., 2011; Sebdani *et al*., 2011). Most of the well studied microbial lipases are inducible extracellular enzymes (Tan *et al*., 2003). They are synthesized within the cell and exported to its external surface or environment. Extracellular lipases have been produced from microorganisms, such as fungi, yeast and bacteria, beside from plants, and animals; whereas commercial lipases have been produced from *Pseudomonas* genus, *Pseudomonas cepacia*, *Pseudomonas alcaligenes* and *Pseudomonas mendocina* (Chigusa *et al*., 1996). In

*Author for Correspondence: E-mail: niveditashaarma@yahoo.co.in

1994, the recombinant lipase "Lipolase", isolated from the fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae* was first introduced by NovoNordisk. Then, in1995, two bacterial lipases 'Lumafast' from *Pseudomonas mendocina* and 'Lipomax' from *Pseudomonas alcaligenes* correspondingly were introduced by Genencor International (Jaeger and Reetz, 1998). Detergent lipases also were originated from *Candida* (Novak *et al*., 1990) and *Chromobacterium* (Nawani *et al*., 1998).

Considering the ever increasing demand for the better lipases in the industry for the search for eco-friendly and economical sources of lipase producing bacteria the present study has been carried out for the purification and the characterization of an extracellular lipase from *Brevibacterium halotolerans* PS4 and its application as a detergent-stable lipase.

Materials and Methods

Microorganism

Brevibacterium halotolerans PS4, accession no. KX671556 identified as a good lipase producer was isolated from the rhizospheric soil of oil seed plants orchard of Distt. Solan of Himachal Pradesh (India). The culture was grown on tributyrinagar medium incubated at 40ºC for 24, and was maintained on nutrient agar slants at 4°C. The media composition comprised (g/l): Beef extract 3; Peptone 5; NaCl 5; Agar 2% and 1% tributyrin.

Production and Purification of Lipase

B. halotolerans PS4 culture was inoculated in tributyrin medium @ 10.0% respectively. Cells were discarded after 72 h of culture by centrifugation (20 min, 12,000 rpm) and the resulting crude enzyme solution (250 mL) was precipitated with solid ammonium sulfate (30-90 % saturation) at 4ºC. The preparations were kept overnight at 4°C and then centrifuged that resulted in separation of precipitates and supernatants. The precipitate obtained after centrifugation was then re-suspended in minimum volume of phosphate buffer (1.0 M, pH 7.0) separately and were refrigerated until further use. The treated supernatant was dialyzed against 0.1M Tris HCl (pH-7.0) buffer with five changes overnight at 4ºC using 14 kDa cut-off dialysis membrane.

The obtained sample was then loaded on a Sephadex G-75 column pre-equilibrated with 0.1M Tris HCl buffer (pH-7.0) and 60 fractions were then eluted with a flow rate of 3 mL/min. The elution profile of proteins was monitored at 280 nm. After 12%-SDS–PAGE analysis, pure and active fractions were stored at –20ºC until used for more biochemical characterization.

Assay for Lipolytic activity (Lawrence et al., 1967)

Lipase activity was measured by titrimetric using olive oil as a substrate. One ml of the culture supernatant was added to the reaction mixture containing 1ml of 0.1M Tris-HCl buffer (pH 8.0), 2.5 ml of deionised water and 3 ml of olive oil and incubated at 37ºC for 30 min. After 30 min, 3 ml of 95% ethanol was added to stop the reaction. Liberated fatty acid was titrated against 0.1M NaOH using phenolphthalein as an indicator. End point was an appearance of pink color.

A unit lipase is defined as the amount of enzyme, which releases one micromole fatty acid per min under specified assay conditions. Enzyme activity was expressed as units per gram (U/g) of dry substrate.

Protein Assay

Protein content was determined following the Lowry protocol (Lowry *et al*., 1951). The purified lipase was analyzed electrophoretically by SDS-PAGE (12%) according to the Laemmli method (Laemmli, 1970).

Characterization of Purified Lipase

Effect of pH

The pH optimum of extra cellular lipase was determined at different pH values from 4 to 11.0. The optimal pH was determined by incubating the enzyme substrate at various pH from 4.0 to 11.0 using different buffers citrate phosphate buffer (pH 4.0-7.0), Tris HCl buffer (pH 8.0) and glycine-NaOH buffer (pH 9.0-11.0) and the enzyme assay was performed to determine the optimal pH titrimetrically using tributyrin as substrate.

Effect of Temperature and Thermal Stability

The temperature optimum of extra cellular lipase was determined at different temperatures ranging from 35-100ºC. To determine the effect of temperature on lipase activity, purified enzyme and substrate were

incubated at various reaction temperatures before starting the experiment and the enzyme assay was performed to determine the optimal temperature titrimetrically using tributyrin as substrate. Thermo stability of the enzyme was determined by incubating purified enzyme in 0.1 M phosphate (pH7.0) buffer at different temperatures (35-100ºC) for 0-180 min. The residual lipolytic activities were then determined using tributyrin as substrate.

Effect of Metal Ions on Lipase Activity

For determining the effect of different metal ions on lipase activity, the purified enzyme were pre-incubated with 20mM Tris HCl buffer (pH 8.0) was incubated for 30 min with various metal ions (1mM) Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, Co^{2+}, $Zn^{2+}$and the residual activity was determined using tributyrin as substrate under standard assay conditions.

Effect of Media Additives on Lipase Activity

To determine the influence of different additives viz. SDS, EDTA, CTAB, Tween 20, Tween 80, Triton X 100 and Glycerol etc. on lipase activity, purified lipase in 1M phosphate buffer (pH 7.0) was pre-incubated for 30 min at 50ºC and the enzyme assay was performed to determine the effect of media additives titrimetrically, using tributyrin as substrate.

Effect of Organic Solvents on Lipase Activity

To determine the influence of different organic solvents viz. Methanol, Ethanol, Benzene, Chloroform, Xylene and Acetone etc. on lipase activity, purified lipase in 1M phosphate buffer (pH 7.0) was pre-incubated for 30 min at 50ºC and the enzyme assay was performed to determine the effect of media additives titrimetrically using tributyrin as substrate

Shelf Stability of Lipase

Shelf stability of lipase was determined by pre-incubating the enzyme at 4ºC in 20mM TrisHCl buffer (pH 8.0). Enzyme activity was determined every 3 days till 9 days.

Application of Purified Lipase as a Laundry Additive

The potential of purified lipase as a detergent additive was analyzed by its washing performance on white cotton cloth pieces stained with different oils. The different sets were braced as distilled water plus stained cloth with each of viz., olive oil, black grease, butter, vegetable oil, and white grease) were taken as control, distilled water: detergent (100:1) with detergent @7 mg/ml on stained cloth, distilled water: detergent (100:1) with detergent @7 mg/ml plus 2 ml enzyme solution on stained cloth. The above samples were incubated at 50ºC for 15 min. and after incubation the cloth pieces were taken out, rinsed with water and dried. After that visual observation and contrast of different pieces to envision the effect of enzyme in removal of stains was wired.

Results and Discussion

Production and Purification of Lipase

In the present work lipase produced by *Brevibacterium halotolerans* PS4 in the culture broth was subjected to a purification protocol. The crude enzyme was subjected to purification. *B. halotolerans* PS4 lipase exhibited an increase in enzyme activity from 380 IU to 960 IU with a purification fold of 2.50 and 84.2% recovery (Table 1). By increasing the ammonium sulphate concentrations to below 30% and above 90%, a decrease in total activity was obtained. A 3.45 fold increase in lipase after ammonium sulphate precipitation was achieved from *Bacillus subtilis* (Mazhar*et al.*, 2016).

The ammonium sulphate (30-90%) fraction was applied to sephadex G-75 gel filtration column. Many protein peaks were observed and only one activity peak was detected (fractions 6-15) (Fig. 1). Active fractions were pooled and lyophilized. The enzyme activity of pooled fractions was checked by quantitative titrimetric assays. The purification process resulted in 2.94 fold purification factor and a final recovery (yield) of 13.80 % of the enzyme with specific activity of 1016.10IU/mg. The molecular mass of purified lipase was estimated to be 80kDa with only one sub-unit from the relative mobility of proteins on SDS-PAGE (Fig. 2).

Bae *et al.*, 2014 purified lipase from *Pichia lynferdi* iY-7723 with 33 purification fold using chromatographic techniques and the purified lipase represented maximum lipolytic activity. Tripathi *et al.*, 2013 purified lipase from *Microbacterium* sp. by sequential methods of ammonium sulphate precipitation and Sephadex G-75 gel column

Table 1: Purification profile of lipase from *Brevibacterium halotolerans* PS4

Purification step	Total volume	Enzyme activity	Total activity	Total protein	Specific activity	Purification fold	% recovery/ yield
Crude enzyme	300	380	1,14,000	330	345.50	1	100
Ammonium sulphate fractionation (30-90%)	25	960	96000	110	872.72	2.50	84.20
Dialysis	25	980	24500	27.5	890.84	2.58	21.5
Gel exclusion chromatography (Sephadex G-75)	15	1050	15750	15	1016.10	2.94	13.80

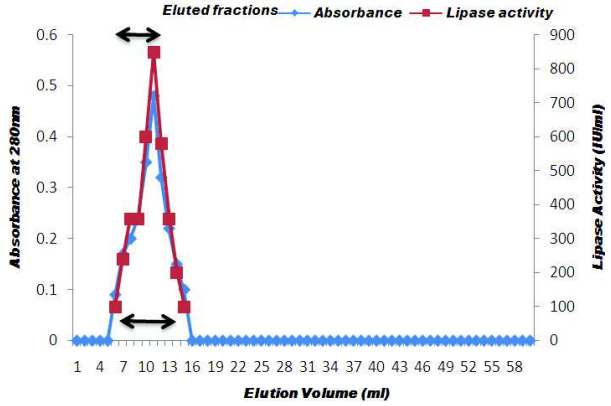

Fig. 1: Protein and enzyme activity profile of fractions of Sephadex G-75 column chromatography of the dialyzed lipase of *Brevibacterium halotolerans* PS4

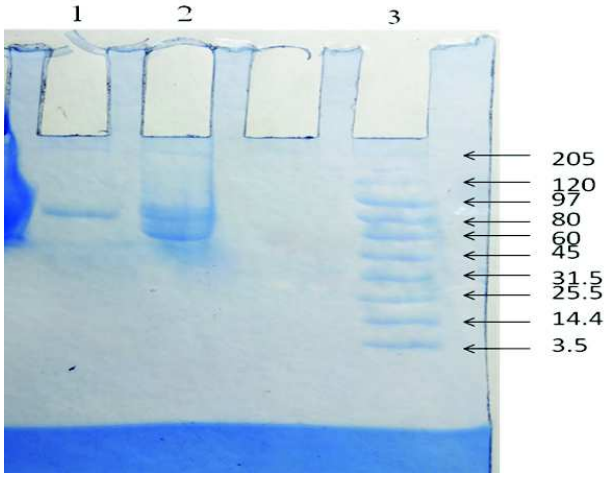

Fig. 2: SDS-PAGE of *Brevibacterium halotolerans* PS4 lipase at various stages of purification. Lane 1: Sephadex column chromatographed enzyme; Lane 2: Crude enzyme; Lane 3: Marker (kDa); Characterization of Purified Lipase

chromatography. This purification procedure resulted in 2.1 fold purification of lipase with a 20.8 % final yield. The purified lipase exhibited maximal hydrolytic activity at a temperature of 50ºC and a pH of 7.0.

Mazhar *et al.* (2016) studied the purification of extracellular lipase from *Bacillus subtilis* and its molecular weight was determined through SDS PAGE which was 41kDa. Ali *et al*. (2016) reported higher molecular weight of around 54 kDa that has been reported from a thermo stable lipase obtained from *Pseudomonas aeruginosa*.

Effect of Temperature

The effect of temperature on purified lipase was investigated at various temperatures ranging from 35ºC, 40ºC…..100ºC for 10 min. The enzyme was found to be completely stable at 55ºC with higher enzyme activity and specific activity (Fig. 3). When the temperature increased above 55°C the activity of the enzyme was affected negatively and gradually reduced. Present results are in close harmony with the recent findings of Ali *et al*., 2016 who studied the effect of temperature on lipase activity. The purified enzyme was active in the range of temperature from 35 to 55ºC with higher enzyme activity and specific activity recorded at 45ºC as compared to control. Similar trends of temperature stability of purified lipase from mesophilic strains have also been noticed

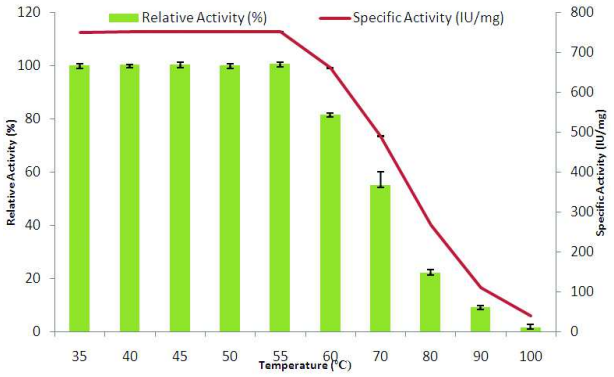

Fig. 3: Effect of temperature on purified lipase of *B. halotolerans* PS4

previously by (Singh *et al*., 2014) and (Daoud *et al*., 2013).

Effect of pH

Lipase showed the activity in a range of pH (4.0-11.0) and showed maximum activity at pH 7.0. The maximum observed activity of 99.79 were achieved at pH 7.0, proving neutral nature of lipase from *B. halotolerans* PS4 (Fig. 4). The *Bacillus subtilis* PCSIR NL-39 lipase are active in pH range of 3.5-9.0. The previous reports have shown that lipase are mostly produced by bacteria especially *Bacillus* species who's pH is 7.0 (Sirisha *et al*., 2010).

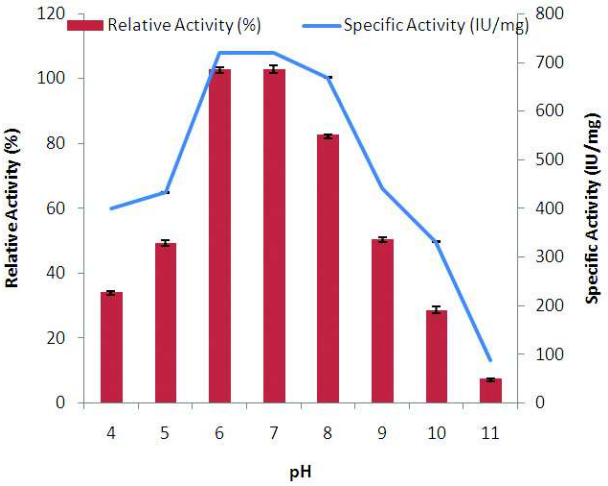

Fig. 4: Effect of pH on purified lipase from *B. halotolerans* PS4

Effect of Metal Ions

Among the metal ions tested, enhancement in the enzyme activity was observed in presence of Ca2+ and Mg^{2+} with 106.64% and 102.81% relative activity when compared to control (Table 2). Similar increase in enzyme activity has been shown by (Bano *et al.*, 2009). A relative activity of 111 and 117% has been reported in the presence of Ca^{2+} and Mg^{2+} respectively. It is suggested that Ca^{2+} increases the thermal stability of the enzyme due to the presence of more binding sites (Ghasemi*et al.*, 2015). These results suggested that Ca^{2+} and Mg^{2+} both stimulants were required for the stability of enzyme.

Effect of Surfactants and Inhibitors

The effect of different detergents on the lipase activity indicated that the enzyme was fairly stable to non-ionic detergents like Triton X. The maximum relative activity observed was 106.64% for *B. halotolerans* PS4 (Table 3). Treatment of ionic detergents like SDS resulted in remarkable loss of enzymatic activity of 9.52% for *B. halotolerans* PS4 lipase. Kiran and Chandra, (2008) also reported retention of 90% activity in the presence of Tween 20, Tween 80 and Triton X-100.

Table 2: Effect of divalent ions on the activity of purified lipase

Divalent ions	Relative activity (%) *B. halotolerans* PS4
Ca^{2+}	106.64
Mg^{2+}	102.81
Cu^{2+}	51.47
Fe^{2+}	21.95
Co^{2+}	9.58
Zn^{2+}	8.57
Control	100.00
SE(m)	0.26
$C.D._{0.05}$	0.80

Table 3: Effect of surfactants on the activity of purified lipase

Surfactant	Relative activity (%) *B. halotolerans* PS4
SDS	9.52
EDTA	22.85
CTAB	33.31
Tween 80	26.67
Triton X 100	106.64
Glycerol	101.92
Control	100.00
SE(m)	0.67
$C.D._{0.05}$	2.07

Effect of Organic Solvents

Stability and activity of enzyme in organic solvents depend not only on the properties and concentration of the organic solvent, but also on the nature of the enzymes. Enzymes being proteins, lose their activity

after addition of organic cosolvents concentrations higher than 10-20%. Therefore, effect of various organic solvents on the enzyme was examined. *B. halotolerans* PS4 exhibited high tolerance to methanol i.e. 70.72 % and 90.47% activity over control (Table 4).

Table 4: Effect of organic solvents on the activity of purified lipase

Organic solvent	Relative activity (%) *B. halotolerans* PS4
Ethanol	83.83
Methanol	90.47
Acetone	83.82
Benzene	70.47
Chloroform	51.43
Xylene	55.29
Control	100.05
SE (m)	0.29
$C.D._{0.05}$	0.90

Shelf Stability

Shelf stability of the enzyme was studied at 4°C and at room temperature for 9 days. *B. halotolerans* PS4 was found to be quite stable with a relative activity of 98.63% upto 6 days at 4°C. which further receded to 52.77% on day 9. On the other hand at room temperature, decline in lipase activity was more i.e. 54.39% on 9^{th} day by *B. halotolerans* PS4.

Application of Lipase

Lipases have long been incorporated as a bio-builder into heavy dirty detergents to hydrolyze and remove lipolytic materials in stained clothes. The enzyme based detergents have better cleansing properties as compared to synthetic detergent. The enzymes in the detergents do not lose their activity after removing stain. The enzyme containing detergents also improve the fabric quality and keeping color bright (Kumar *et al*., 2016). The purified lipase from *Brevibacterium halotolerans* PS4 have shown thermo stability, halotolerance, as well as, broader pH range resistance, therefore in the present work purified lipase was proven for its proficiency to act in detergent formulations. The additive effect of purified enzyme in water along with branded detergents notably improved the cleansing of grease, butter, vegetable oil, olive oil and grease (white) as compared to the control. The high competence of lipase to erase the stain was proven and hence the lipase of *Brevibacterium halotolerans* PS4 is strongly recommended as an efficient additive for detergent industry.

Conclusion

In the present work, purified lipase from novel isolate *Brevibacterium halotolerans* PS4 showed excellent activity over a vast range of temperatures and pH values. Moreover, because of its pronounced thermal stability as well as shelf stability and stability in organic solvents, this enzyme could be of symbolic biotechnological benefit, particularly in laundry additives. As purification of lipase from a new strain of *Brevibacterium halotolerans* PS4 is first time reported and further research will focus on new characteristics of this enzyme for its adoption in detergent industry. Overall, the obtained data suggested that purified lipase from *Brevibacterium halotolerans* PS4 may be explored as a future candidate in the detergent formulations.

Acknowledgement

The authors would like to thank Department of Environment, Science and Technology, Govt. of Himachal Pradesh, India for their financial support to this research work.

References

Ali Y, Hanna M A and Leviticus L I (2016) Emissions and power characteristics of diesel engines on methyl soyate and diesel fuel blends *Bioresour Technol* **52** 185-195

Bae J H, Kwon M H, Kim I H, Hou C T and Kim H R (2014) Purification and characterization of a cold-active lipase from *Pichia lynferdii* Y-7723: pH-dependant activity deviation *Biotechnol Bioprocess Eng* **19**851-857

Bano S, Syed M.N, Iqbal S, Khan A B, Ali-ul-Qader S and Azhar A (2010) Purification and characterization of 60 kD lipase linked with chaperon in from *Pseudomonas aeruginosa* BN-1 *Afr J Biotechnol* **9** 724-7732

Bora L and Kalita M C (2008) Production of thermostable alkaline lipase on vegetable oils from a thermophilic *Bacillus* sp. DH4, characterization and its potential applications as detergent additive *J Chem Technol Biot* **83** 688-693

Chigusa K, Hasegawa T, Yamamoto N and Watanabe Y (1996) Treatment of wastewater from oil manufacturing plant by yeasts *Water Sci Technol* **34** 51-58

Daoud F B O, Kaddour S and Sadoun T (2013) Adsorption of cellulase *Aspergillus niger* on a commercial activated carbon: kinetics and equilibrium studies *Colloid Surf B Biointerfaces* **75** 93-99

Garlapati V K, Vundavilli P R and Banerjee R (2010) Evaluation of lipase production by genetic algorithm and particle swarm optimization and their comparative study *Appl Biochem Biotechnol* **162** 1350-1361

Ghasemi S, Rasoul A, Kazemi G, Zarrini, Morowvat M H and Kargar M (2015) Isolation and characterization of some moderately halophilic bacteria with lipase activity *Microbiol* **80** 483-487

Jaeger K E and Reetz T M (1998) Microbial lipases from versatile tools for biotechnology *Trends Biotechnol* **16** 396-403

Kiran K K and Chandra T S (2008) Production of surfactant and detergent-stable, halophilic, and alkalitolerant alpha-amylase by a moderately halophilic *Bacillus* sp. strain TSCVKK *Appl Microbiol Biotechnol* **77** 1023-1031

Kumar S G, Gurramkonda C, Rather G, Muniramanna G S C, Mangamuri U K, Podha S and Choi Y L (2016) Glucoamylase from a newly isolated *Aspergillusniger* FME: detergent-mediated production, purification and characterization *J Korean Soc Appl Biol Chem* **56** 427-433

Laemmli U K (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage *Nature* **227** 680-685

Lawrence R C, Fryer T F and Reiter B (1967) A rapid method for the quantitative estimation of microbial lipases *Nature* London **21** 1264

Lowry O H, Rosebrough N J, Farr A L and Randall R J (1951) Protein measurement with the Folin-Phenol Reagent *J Biol Chem* **193** 265-275

Mazhar H, Abbas N, Ali S S, Hussain Z and Ali S (2016) Purification and characterization of lipase production from *Bacillus subtilis* PCSIR-NL39 *J Biol Chem Research* **33** 546-558

Nadia N, Nehad Z A, Elsayed A E, Essam M A and Hanan M A (2010) Optimization of lipase synthesis by *Mucorracemosus* - Production in a triple impeller bioreactor *Malaysian J Microbiol* **6** 7-15

Nawani N, Dosanjh N S and Kaur J (1998) A novel thermostablelipase from a thermophilic *Bacillus* sp.: characterization and esterification studies *Biotechnol Lett* **20** 997-1000

Novak J, Kralova B, Demnerova K, Prochazka K, Vodrazka Z, Tolman J, Rysova D, Smidrkal J and Lopata V (1990) Enzyme agent based on lipases and oxido reductases for washing, degreasing and water reconditioning *European Patent* **355** 228

Padmapriya B, Rajeswari T, Noushida E, Sethupalan D G and Venil C K (2011) Production of lipase enzyme from *Lactobacillus* spp. and its application in the degradation of meat *World Appl Sci J* **12** 1798-1802

Rajesh E M, Arthe R, Rajendran R, Balakumar C, Pradeepa N and Anitha S (2010) Investigation of lipase production by*Trichodermareesei*and optimization of production parameters *J Environ Agric Food Chem* **9** 1177-1189

Ramani K, Kennedy L J, Ramakrishnan M and Sekaran G (2010) Purification, characterization and application of acidic lipase from *Pseudomonas gessardii*using beef tallow as a substrate for fats and oil hydrolysis *Process Biochem* **45** 1683-1691

Sebdani R M, Ardakani M R, Ghezelbash G R and Sadrinasab M (2011) Phylogenic characterization of lipase producing *Bacillus* strains isolated from Persian Gulf Sediments *Aust J Basic Appl Sci* **5** 121-126

Singh R, Tripathi R D, Dwivedi S, Kumar A, Trivedi P K and Chakrabarty D (2014) Lead bioaccumulation potential of an aquatic macrophyte *Najasindica* are related to antioxidant system *Bioresour Technol* **101** 3025-3032

Sirisha E, Rajasekar N and Lakshmi M N (2010) Isolation and optimization of lipase producing bacteria from oil contaminated soils *Advan Biol Res* **4** 249-252

Tan T, Zhang M, Wang B, Ying C and Deng I (2003) Screening of high lipase producing *Candida* sp and production of lipase by fermentation *Process Biochem* **39** 459-465

Tripathi P, Niyonzima F N and More S (2013) Detergent-compatible proteases: microbial production, properties, and stain removal analysis *Prep Biochem Biotechnol* **45** 233-258.