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Full Length Research Paper

Isolation, purification and properties of lipase from *Pseudomonas aeruginosa*

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Six isolates (Ps1, Ps2, Ps3, Ps4, Ps5 and Ps6) producing lipase were screened from wastewater on a selective medium agar containing Tween 80 or olive oil as the only source of carbon. Isolate Ps_5 showed the highest lipase activity which was later identified as *Pseudomonas aeruginosa*. The effect of media composition was analysed to maximize the production of lipase. The maximum extracellular lipase present in the broth was purified 4 folds with an overall yield of 19.4% through the purification procedure of ammonium sulphate precipitation and diethyl aminoethyl (DEAE) cellulose chromatography. The purified lipase had the maximal activity within the pH range of 6 to 8, with an optimum pH of 7, and within the temperature range of 20 to 35°C, with an optimum temperature for the hydrolysis of olive oil at 30°C. The enzyme activity of *P. aeruginosa* lipase was enhanced by Ca^{2+} and Mg^{2+} but strongly inhibited by heavy metals such as Zn^{2+}, $Cu^{2+}$and Mn^{2+}.

Key words: *Pseudomonas*, lipase, purification, biomass, heavy metals.

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). However, in non-aqueous conditions, these naturally hydrolytic enzymes can transfer acyl groups of carboxylic acids to nucleophiles other than water (Martinelle and Hult, 1995). Thus, lipases can acylate alcohols, sugars, thiols and amines, synthesizing a variety of stereo-specific esters, sugar esters, thioesters and amides (Dellamora-Ortiz et al., 1997; Singh et al., 2003). 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).

Generally, the enzymes of industrial interest are produced in the presence of inducers, but in the case of lipases, the presence of triacylglycerol, surfactants, vegetable oils, oil industry wastes or their hydrolysis products in the culture medium have, in most cases, an inducible effect on lipase production (Damaso et al., 2008). 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). These lipases express different physicochemical properties that depend on metal ions, substrate, pH and temperature. The aim of this study was to analyse the influence of media components on the production of lipase and to purify and characterize the lipases produced on a common production medium using

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Pseudomonas aeruginosa.

MATERIALS AND METHODS

Isolation of producer microorganism

The bacterial strain *Pseudomonas*.sp used in this study was isolated from a wastewater at Sidi bel abbes, Algeria. The isolates were identified on the basis of various morphological, physico-chemical, and biochemical characteristics. Lipolytic bacteria were typically detected and screened through the appearance of clearing zones by using a selective medium (Loo et al., 2006) containing Tween 80 or olive oil as the only carbon source. The diameter ratio of clear zone and colony was measured.

Optimization of lipase production

Four different cultivation media were evaluated for the production of lipase. The composition of the different fermentation media is summarized as follows:

Medium (A): 0.3% yeast extract, 0.1% bacto peptone, 1% olive oil, 0.07% K_2HPO_4, 0.03% KH_2PO_4, 0.05% $MgSO_4$, 0.01% $MnCl_2$, 0.025 % $(NH_4)_2SO_4$ and 0.01 $CaCl_2$ (Li et al., 2004).
Medium (B): 1% Tween 80 was used to replace olive oil in medium (A).
Medium (C): 2% dextrose was added to medium (A).
Medium (D): 2% dextrose was added to medium (B).

The pH of all the production media were adjusted to 7.2 using 0.5 N NaOH before autoclaving at 121°C for 15 min. Enzyme assay was carried out in 500 ml Erlenmeyer flasks containing 94 ml medium inoculated with 6 ml medium in overnight culture. The flasks were incubated at 35°C with a constant shaking at 125 rpm for 3 days.

The cell was separated by centrifugation at 10000 rpm and 4°C for 20 min. The supernatants were collected and used to determine the lipase activity. The isolation expressed the highest activity selected for further study.

Biomass estimation

Biomass concentration was estimated by measuring the optical density at 600 nm using the Spectronic instrument and standard curve previously determined (biomass g/L corresponding to 0,270 × OD_{600}) (Camacho et al., 2009).

Protein determination

Protein was measured photometrically by the method of Bradford with Coomassic protein Assay reagent (Bradford, 1976).

Lipase activity

Lipase activity was determined titrimetrically using olive oil hydrolysis (Borkar et al., 2009), after which 1 ml of enzyme solution was added to the assay substrate containing 10 ml of 10% homogenized olive oil in 10% gum acacia, 2 ml of 0.6% $CaCl_2$ solution and 5 ml of 0.2 mol/L phosphate buffer, with pH 7.2. The enzyme-substrate was incubated at 35°C on an orbital shaker at 125 rpm for 1 h (Syed et al., 2010), after which 20 ml ethanol – acetone (1:1 v/v) was added to stop the reaction. Liberated fatty acids were titrated with 0.1 mol/L NaOH using phenolphthalein as an indicator. The reaction mixture without the enzyme was titrated in the same way and used as blank. One lipase unit was defined as the enzyme that released one micromole (1 µmol) of fatty acid per min under standard assay conditions (Gombert et al., 1999).

Purification of lipase

Lipase purification was carried out at 4°C. The culture medium was centrifuged at 10000 rpm for 20 min to obtain crude enzyme, then the supernatant fluid was subjected to precipitation with ammonium sulphate to 40% saturation and stirred for 2 h. The precipitate was removed by centrifugation. Lipase activity was then determined both in the precipitate and supernatant.

Additional ammonium sulphate was added to the supernatant to bring the saturation to 60%. The precipitate were collected, dissolved in buffer pH 7.2 and dialyzed against same buffer. The enzyme mixture was loaded onto the DEAE cellulose column (15 ×1.5 cm) equilibrated with the phosphate buffer. The enzyme was eluted with a linear gradient of 0 to 1 M NaCl in the same buffer at a flow rate of 1 ml/min. The active fractions that contained lipase enzyme were pooled, dialysed and assessed for protein content. The resulting enzyme was utilised for the characterization of the extracellular lipase.

The protein content at each stage of enzyme purification was determined according to the Bradford (1976) method.

Determination of pH and temperature optimum

The optimum temperature and pH of purified lipase was determined at 20 to 50°C and the pH values ranged from 3 to 10. 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 olive oil as substrate.

The optimal pH was determined by incubating the enzyme-substrate at various pH values (3 to 10) and assayed for lipase activity.

Thermo stability and pH stability of lipase

The thermo stability of the lipase fraction was studied by incubating the purified enzyme at various temperatures (20 to 50°C) for 1 h. The residual lipolytic activities were then determined by using olive oil as substrate.

For pH stability, purified enzyme was incubated using different pH buffers for 1 h. The reaction mixtures were incubated as per standard assay and the residual lipolytic activities were then determined by using olive oil as substrate.

Effect of metal ions on lipase activity

For determining the effect of metal ions on lipase activity, the purified enzyme was preincubated with 1 mM of Ca^{2+}, Mg^{2+}, Mn^{2+}, Cu^{2+} and Zn^{2+} for 1 h at 30°C and the residual activity was determined by using olive oil as substrate.

RESULTS AND DISCUSSION

Isolation of lipase –producing micro organisms

Six isolates grown in the selective medium were found to

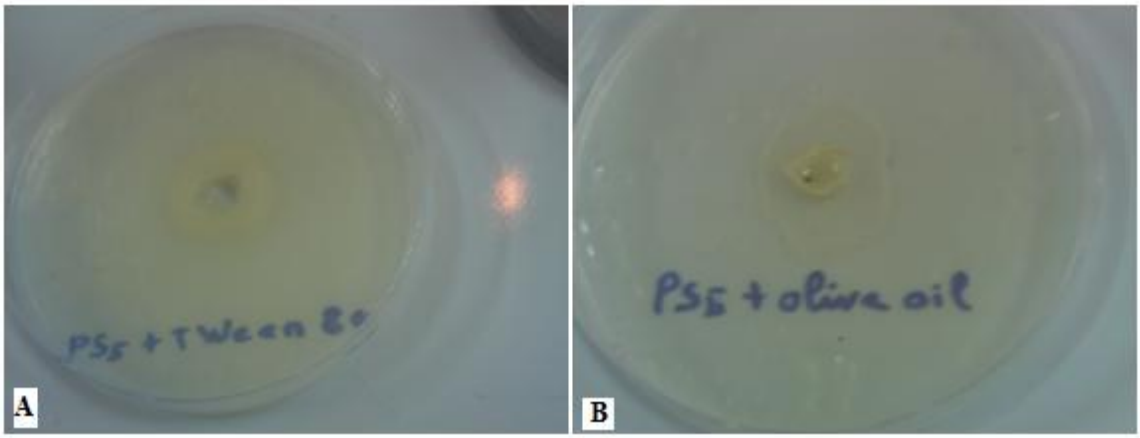

Figure 1. The clear zone displayed by lipase around the *Pseudomonas* sp colony on the emulsion Tween 80-agar (A) and olive oil-agar (B) at 30°C after 96 h of incubation.

Table 1. The diameter ratio of clear zone and colony of the isolated bacterial with addition of Olive oil and Tween 80 to the culture medium.

Bacterial	Diameter ratio of clear zone and colony (mm)	
	Olive oil	Tween 80
Ps1	7	5
Ps2	8	6
Ps3	12	10
Ps4	16	13
Ps5	22	18
Ps6	14	12

produce lipase identified as bacteria (Ps_1, Ps_2, Ps_3, Ps_4, Ps_5 and Ps_6). Their growth showed that they could use olive oil as carbon source and it showed their lipase producing feasibility.

Evaluation of the lipase producing efficiency based on the clear zone around the colony showed that all of them could produce lipase. The result of Ps_5 – whose lipase production was applied on two emulsions (olive oil- agar and Tween 80-agar) and incubated at 35°C for 96 h – was outstanding (Figure 1). Their abilities to produce lipase were evaluated with the diameter ratio of clear zone and colony (Table 1). Ps_5 was the highest with 22 mm of olive oil and 18 mm of Tween 80, whereas Ps_4 was 16 mm of olive oil and 13 mm of Tween 80, and Ps_6 was 14 mm of olive oil and 12 mm of Tween 80 (Table 1). Ps_5 was identified as a member of *Pseudomonas* genus, as such, it was classified as *Pseudomonas aeruginosa*.

Optimization of lipase production by *P. aeruginosa*

Four different media were used for lipase production by *P. aeruginosa*. The results in Figure 2 show that medium (C) with dextrose as carbon and olive oil as inducer of lipase gave the maximum biomass and activity of 18.36 g/L after 48 h. In this present study, lipase production with different compositions was studied to maximize the production of lipase. It was observed that medium (C) presented the highest activity of 37 U/ml with specific enzyme activity of 37.75 U/mg at 35°C after 24 h (Figure 3). Lipase production in medium (A) without dextrose was 16.7% less as compared to lipase production in medium (C); although, medium (D) with Tween 80 and dextrose produced 50% less lipase.

Production studies on medium (B) show a 75% reduction in the production of lipase. Some authors stated that lipase production is greatly affected by the composition of the medium (Lin and Ko, 2005). The results obtained in this study showed that olive oil and dextrose were the most suitable substrate for maximum lipase production by *P. aeruginosa.* Olive oil was the better inducer than Tween 80 under the test conditions, whereas dextrose was the best carbon source for lipase production. In fact, dextrose has been considered as a growth medium for microorganisms providing a satisfactory supply of nutrients for growth. In conclusion, *Pseudomonas aeruginosa* strain was found to be the best lipase producer. Wheat dextrose and olive oil were the

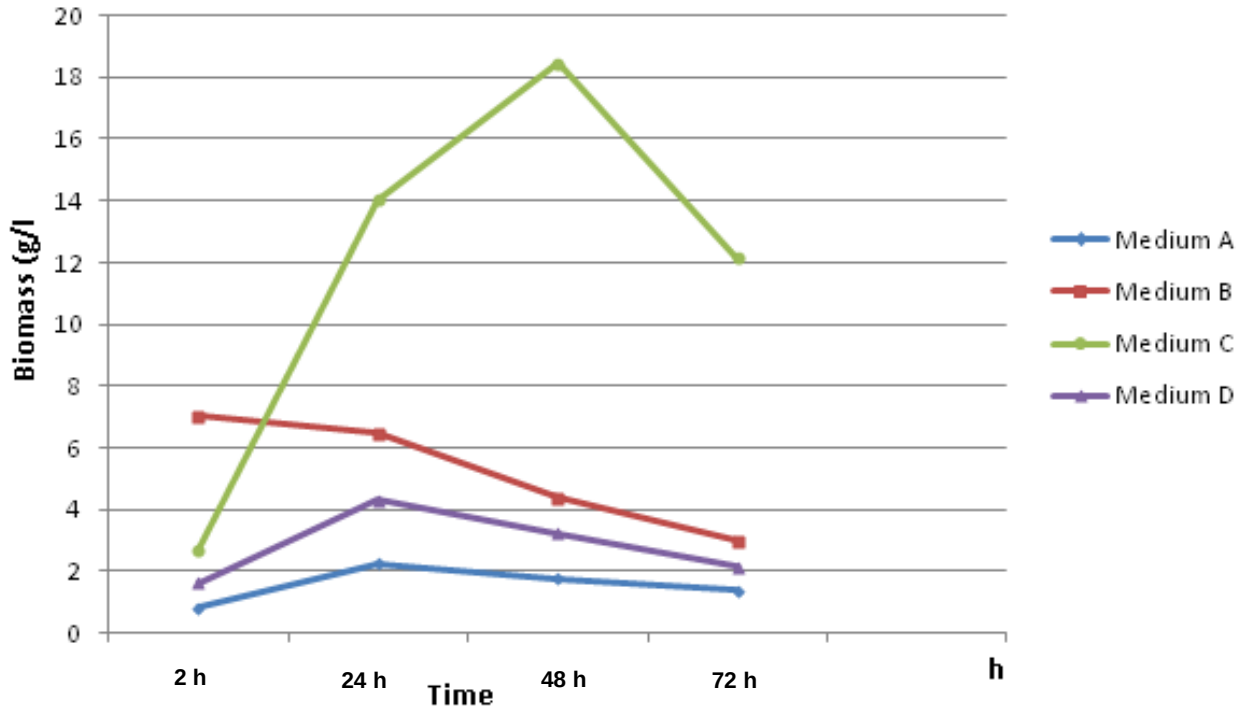

Figure 2. Biomass production by *Pseudomonas* sp in different media (see Materials and Methods).

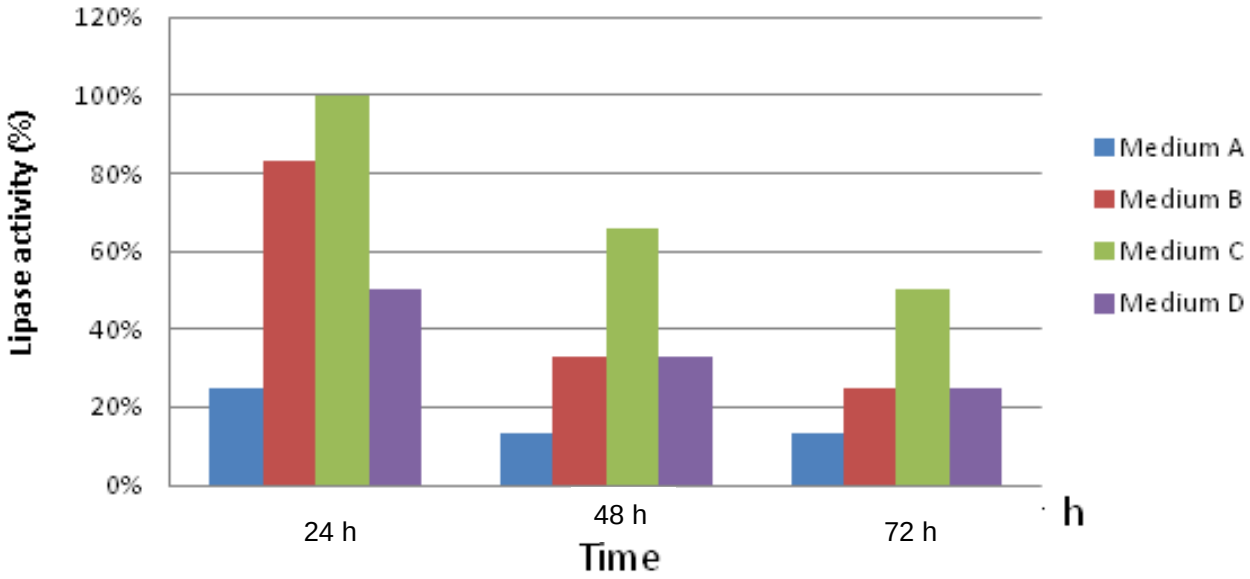

Figure 3. Lipase production by *Pseudomonas* sp in different media (see Materials and Methods).

best substrate and inducer for lipase production by this strain, respectively .

Purification of lipase

The crude enzyme was subjected to purification. The maximal activity, specific activity, and yield (22.5 U/ml, 28.84 U/mg and 60.89%) were detected at 40% ammonium sulphate (Table 2). By increasing the ammonium sulphate concentrations to 60%, a slight decrease in total activity was obtained. The specific activity of 150 U/mg with a yield of 19.45% was obtained after the single step purification on DEAE cellulose chromatography (Table 2).

Table 2. Purification of lipase from *Pseudomonas* sp.

Purification step	Crude protein (mg)	Lipase activity	Specific activity	F. Fold	Yield (%)
Supernatant (crude extract)	76.44	37 U/ml	37.75 U/mg		
$(NH_4)_2SO_4$ 40%	50.7	22.5 U/ml	28.84 U/mg	0.76	60.81
$(NH_4)_2SO_4$ 60%	42.6	16.8 U/ml	24.7 U/mg	0.65	45.4
DEAE cellulose	0.864	7.2 U/ml	150 U/mg	3.97	19.45

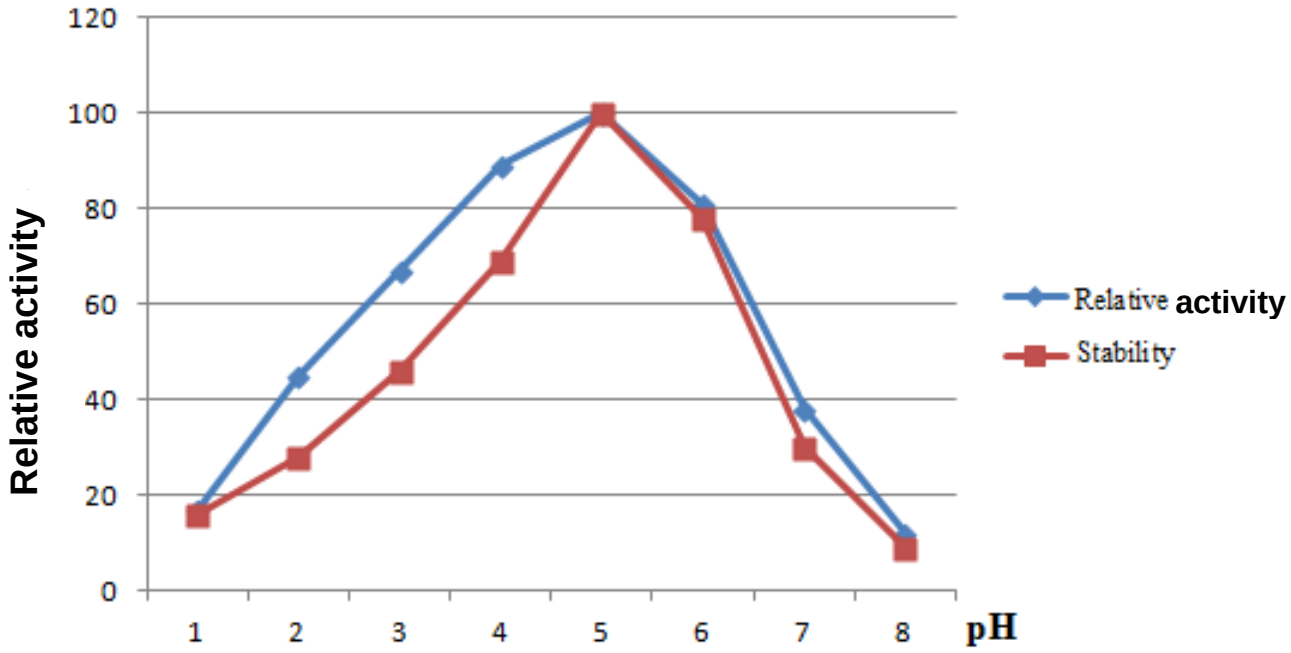

Figure 4. Effect of pH on the relative activity and stability of *Pseudomonas* sp lipase.

pH and temperature optimum

Initial pH of the culture broth is one of the most critical environmental parameters affecting both growth and lipase production. The results show that *P. aeruginosa* was able to grow in the pH range of 6 to 8 (Figure 4) and reached the maximum lipase activity of 42 U/ml at pH 7. These data are in agreement with those of Kojima and Shimizu (2003), who reported that the maximum lipase activity of *Pseudomonas fluorescens* HU 380 was detected at pH 7.

The results of this study show that maximum lipase activity was detected at 30°C (Figure 5). A decrease in the lipolytic activity was observed at above 35°C and completely ended after 45°C. Such results are similar to those reported for many bacterial species (Gilbert et al., 1991).

pH and temperature stability

The pH stability of the lipase was determined by the activity retained at different pH from 3 to 10 after 1 h of incubation. The pH stability curve showed that the lipase was stable at pH 6 to 8 (Figure 4). The stability data showed a decline in lipase activity below 6 and above 8, however, 70 and 80% relative activity was retained at this pH. Kojima and Shimizu (2003) stated that majority of bacterial lipase presented optimal pH stability in the range of 6 to 8 and were unstable at pH values above 8.

Maximal thermo stability of the lipase was observed in the temperature range of 25 to 35°C (Table 3). The enzyme was found to be completely stable at 30°C after 1 h. At 35°C, the enzyme maintained 81% stability 1 h after the initial activity.

Effect of metal ions on lipase activity

Among the metal ions tested, enhancement of the enzyme activity was observed in the presence of Ca^{2+} with 116% and Mg^{2+} with 107% relative activity when compared to the control (Table 3). These ions have been reported to play the role of lipase cofactor (Dong et al.,

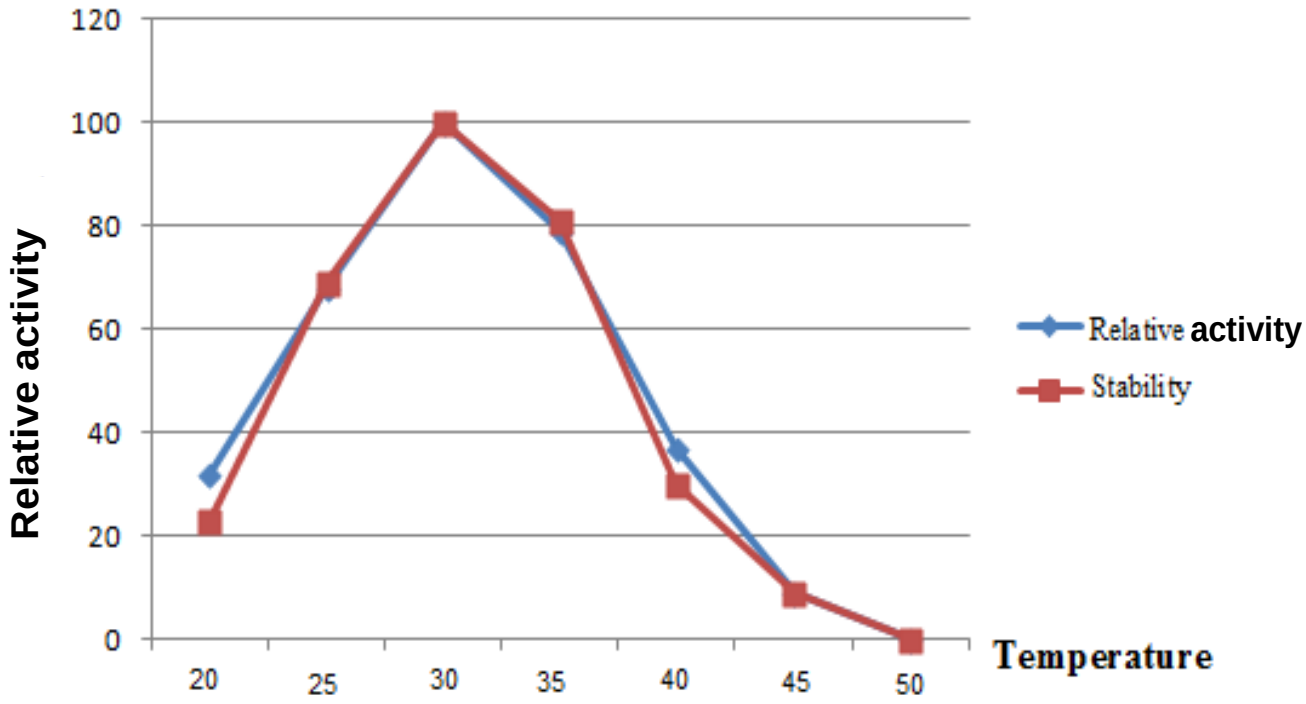

Figure 5. Effect of temperature on the relative activity and stability of *Pseudomonas* sp lipase.

Table 3. Effect of different metal ions on enzyme activity.

Metal ion used	Concentration used (mM)	Remaining activity (%)
Control	none	100
Ca^{2+}	1	116
Mg^{2+}	1	107
Mn^{2+}	1	36
Cu^{2+}	1	31
Zn^{2+}	1	26

1999). Calcium ion, in particular, was used in Ca^{2+} binding processing to create an impact on the position specificity of the active site (Surinenaite et al., 2002). Same lipases produced by *Pseudomonas* sp. have been found to be Ca^{2+} dependent; however, Ca^{2+} exerted an inhibitory effect on *Pseudomonas* sp. strain (Borkar et al., 2009). The hydrolytic activity of the enzyme was inhibited by heavy metals such as Zn^{2+}, Mn^{2+} and Cu^{2+} with 36% relative activity.

Conclusion

Out of the 36 bacterial isolates obtained from the wastewater at Sidi Bel abbes (Algeria), 6 exhibited lipase activity. *Pseudomonas aeruginosa* proved to be the best lipase producer. Various physicochemical parameters were studied to determine the optimum conditions for its lipase production. The obtained results showed that the medium composition (1 bacto peptone, 3 yeast extract, 20 dextrose, 10 olive oil, 0.7 k_2HPO_4, 0.3 KH_2PO_4, 0.5 $MgSO_4$, 0.1 $MnCl_2$ g/lL) for lipase production was at pH 7, 30°C and 125 rpm in 100 and 500 ml flasks. The purified lipase showed optimal activity in a wide range of temperature and pH values, due to its pronounced thermal stability, as well as the preservation of its activity and stability.

It was concluded that *Pseudomonas aeruginosa* could be used as a new potent microbial source of lipase. Further studies are recommended on the use of other bacteria and strains of *Pseudomonas* sp for much better lipolytic activity.

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REFERENCES

Bradford MM (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254.

Bora L, Kalita MC (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.

Borkar PS, Bodade RG, Rao SR, Khobragade CN (2009). Purification and characterization of extracellular lipase from a new strain-*Pseudomonas aeruginosa* SRT9. Braz. J. Micobiol. 40: 358-366.

Camacho RM, Mateos JC, Reynoso OG, Prado LA, Codova J (2009). Production and characterization of esterase and lipase from *Haloarcula marismortui*. J. Ind. Microbiol. Biotechnol. 36: 901-909.

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

Damaso MCT, Passianoto MA, Freitas SC, Freire DMG, Lago RCA, Couri, S (2008). Utilization of agro industrial residues for lipase production by solid state fermentation. Braz. J. Microbial. 39: 676-681.

Dellamora-ortiz GM, Martins RC, Rocha WL, Dias AP (1997). Activity and stability of a *Rhizomucor miehei* lipase in hydrophobic media. Biotechnol. Appl. Biochem. 26:111-116.

Dong H, Gao S, Han S, Cao S (1999). Purification and characterization of a *Pseudomonas* sp. Lipase and its properties in non-aqueous media. Biotechnol. Appl. 30: 251-256.

Garlapati VK, Vundavilli PR, Banerjee R (2010). Evaluation of lipase production by genetic algorithm and particle swarm optimization and their comparative study. Appl. Biochem. Biotechnol. 162(5): 1350-1361.

Gilbert JE, Cornish A, Jones CW (1991).Purification and properties of extracellular lipase from *Pseudomonas aeruginosa* EF2. J. Gen. Microbiol. 137: 2223-2229.

Gombert AK, Pinto AL, Castillo LR, Freire DMG (1999). Lipase production by *Penicillium restrictum* in solid-state fermentation using babassu oil cake as substrate. Process Biochem. 35: 85-90.

Kojima Y, Shimizu S (2003). Purification and characterization of the lipase from *Pseudomonas fluorescens* HU 380. J. Biosci. Bioeng.96: 211-226.

Li CY, Cheng CY, Chen TL (2004). Fed- batch production of lipase by Acinetobacter radioresistens using tween 80 as carbon source. Biochem Eng.J. 19: 25- 31.

Lin ES, Ko HC (2005). Glucose stimulates production of the alkaline thermostable lipase of the edible Basidiomycete *Antrodia cinnamomea*. Enzyme Microb. Tech. 37: 261-265.

Loo JL, Lai OM, Long K, Ghazali HM (2006). Identification and characterization of a locally isolated lipolytic microfungus *Geotrichum candidum*. Malaysian J. Microbiol. 2: 22-29.

Martinelle M, Hult K (1995).Kinetics of acyl transfer reactions in organic media catalysed by *Candida Antarctica* lipase B. Acta, Biochim. Biophys. 1251: 191-197.

Nadia N, Nehad ZA, Elsayed AE, Essam MA, Hanan MA (2010). Optimization of lipase synthesis by *Mucor racemosus* - Production in a triple impeller bioreactor. Malaysian J. Microbiol. 6: 7-15

Padmapriya B, Rajeswari T, Noushida E, Sethupalan DG, Venil CK (2011). Production of Lipase Enzyme from *Lactobacillus* spp. and Its Application in the Degradation of Meat. World Appl. Sci. J. 12: 1798-1802.

Rajesh EM, Arthe R, Rajendran R, Balakumar C, Pradeepa N, Anitha S (2010). Investigation of lipase production by *Trichoderma reesei* and optimization of production parameters. J. Environ. Agric. Food Chem., 9(7):1177-1189.

Ramani K, Kennedy LJ, Ramakrishnan M, 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 RM, Ardakani MR, Ghezelbash GR, Sadrinasab M (2011). Phylogenic Characterization of Lipase Producing *Bacillus* Strains Isolated from Persian Gulf Sediments. Aust. J. Basic Appl. Sci. 5(4): 121-126.

Singh SK, Felse AP, Nunez A, Foglia TA, Gross RA (2003). Regioselective enzyme-catalyzed synthesis of phospholipid esters,amides, and multifunctional monomers. J. Organic Chem. 68: 5466-5477.

Surinenaite B, Bendikiene V, Juodka B, Bachmatova I, Marcinkevichiene L (2002). Characterization and physicochemical properties of a lipase from *Pseudomonas mendocina* 3121-1. Biotechnol Appl. Biochem. 36:47-55.

Syed MN, Iqbal S, Bano S, Khan AB, Ali-ul-Qader S, Azhar A (2010). Purification and characterization of 60 kD lipase linked with chaperonin from *Pseudomonas aeruginosa* BN-1. Afr. J. Biotechnol. 9: 7724-7732.

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