Plant Archives Vol. 19 No. 2, 2019 pp. 4379-4385 **e-ISSN:2581-6063 (online), ISSN:0972-5210**

PURIFICATION AND CHARACTERIZATION OF LIPASE PRODUCTION FROM *PSEUDOMONAS AERUGINOSA* AND STUDY EFFECT OF SILVER NANOPARTICLE IN ACTIVITY OF ENZYME IN APPLICATION OF BIOLOGY

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Abstract

P. aeruginosa was isolated from burns patient to ensure that the isolate was belonging to *P. aeruginosa* various tests are made such as staining techniques, biochemical assay, morphological and sensitivity test. The gram stain and biochemical test result show rod pink gram negative bacteria and ensure that the isolate was belong to (*P. aeruginosa*). Optimization education for bacterial growth were done by used more than pH and temperature and it was found that the best conditions for the production and increase the number of bacteria at pH 5.5 with bacterial number (4.5×10^8 cells) and Temperature 37°C with bacterial number (5.25×10^8 cells). Extracellular enzyme was extracted by cool centrifuge 10000 × g for 20 min in 4°C, the cell pellet was re-used as a crude enzymatic extract and discard supernatant cell. Purification of Lipase was accomplished by using Salt precipitation, Ion exchange and gel filtrations chromatographic techniques. The result shows that, gel filtration has optimal specific activity and purification fold at (4U/ml), purification fold 5.7 times.

Key word: Isolation, Identification, Bacteria, *Pseudomonas aeruginosa*, Lipase, Triglycerides.

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).

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. These lipases express different physicochemical properties that depend on metal ions, substrate, pH and temperature and to purify and characterize the lipases produced on a common production medium using *Pseudomonas aeruginosa*.

The aim of the study

1. Purification of the molecular enzyme by salting out by ammonium sulphate deposition.
2. Final (Holly) purification by ion exchange chromatography and gel filtration chromatography.
3. Measurement of triglycerides in blood with the use of the enzyme Lipase, prepared and purified from *P. aeruginosa* and Silver Nanoparticle.

Materials and Methods

Patients, specimens, collection

Through the period extending from November 2018 till December 2018, 8 Clinical specimens comprising; UTI, otitis media patients and burns were collected as swabs in sterilized containers and then test the best isolate for growth by spectrophotometer and was found that the optimal isolate for enzyme production was burns isolate.

Samples identification

All the bacterial isolates were examined for gram stain ability (Leboffe and Pierce, 2012), shape and color of the cells were observed by light microscope using oil emersion, the collected specimens were streak plate technique is used for the isolation into pure culture of the organisms (mostly bacteria), from mixed population. The *P. aeruginosa* was streaked over the agar surface. Some individual bacterial cells are separated and well-spaced from each other. As the original sample is diluted by streaking it over successive quadrants and then incubate at 37°C for 24h., the number of organisms decreases and will show the bacterial morphology. (Spilker *et al.,* 2004).

Optimal temperature and pH for the production of bacteria

The bacterial suspension was cultured once at constant temperature but different pH (5.5, 7 and 9) and once at constant pH but different temperature (32, 35 and 37°C) and measures the absorbance at 556 nm (Spilker *et al.,* 2004).

Extraction of enzyme

Taken 230ml of production broth (the broth that contain the bacterial cells in which the extracellular lipase was found) and transported it into centrifuge tubes, at 10000 rpm the bacterial cells were centrifuged for 15 minutes at 4°C. Supernatant consuming extracellular protein was taking and the pellet having bacterial cells was discarding.

Determination of Protein Concentration

Protein concentration was determined according to the method of Bradford (1976), the protein concentration determined. A 20µl of GTF crude was mixed with 50µl of 1 M NaOH with shaking for 2-3 minutes then 1 ml of Bradford solution, was added with shaking. The absorbance was measured at 595 nm by spectrophotometer (Baron *et al.,* 2013).

Measurement of crude enzyme activity

Lipase activity was determined titrimetrically using olive oil hydrolysis (Gombert *et al.,* 1999), 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 (Mariam *et al.,* 2015), 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 indication. 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 (Whitaker *et al.,* 2015).

Ammonium sulfate precipitation

The ammonium sulfate was added in more than soaking ratio (20% to 80%), to achieve the best ratio of ammonium sulfate by adding gradually the amount of salt to each 10ml enzyme solution in ice bath and magnetic stirrer for 1 hour, centrifuge the solution 10000 rpm/min for 15 min., dropped the supernatant and take the precipitate and dissolved it in 25ml Phosphate buffer Saline pH 7.2 and calculated the activity and protein concentration (Small *et al.,* 2015).

Separation of enzyme through ion exchange resin (DEAE Cellulose)

The DEAE-Cellulose was accomplished along with the method suggested by (Wang *et al.,* 1975). Twenty gram from ion exchange resin were put off in 1 liter distilled water, remain in graduated cylinder to decay, after that the supernatant was discard, this step was done continuously, until the supernatant became pure, the ion exchange resin was washed away all contaminate manner by using Buchner's funnel under vacuum (without drying the ion exchange resin), then the resin was stimulated in 250 ml from buffer which contain 0.25M sodium hydroxide- 0.25M sodium chloride for 30 minutes, the resin was repeated the filtration and washed under vacuum using distilled water, then the resin was postponed in 250ml hydrochloride acid 0.25M with agitation for 30 minutes, after that, the resin was cleaned with distilled water under vacuum, then the resin was postponed in phosphate buffer pH=7.2) and the ion exchange resin was removal all gases by using vacuum pump, the resin was boxed softly in glass column (2.5×16cm) and the equilibration was achieved by the same phosphate buffer (Buccolo *et al.,* 2013).

Enzyme separation through Sephacryal S-200 column

The preparation of gel was achieved as recommended

by supplied company, 20g of gel Sephacryl S-200 powder was put off in 500 ml distilled water and place in water bath at 90°C for 3 hours for activation, then wash double with phosphate buffer (pH=7.2), after that, the gel was postponed in amount of the similar buffer, then was remove the gases by using vacuum pump, the gel was boxed softly in glass column with dimensions (1.5×10) cm (Baron *et al.,*1973).

Measurement of triglycerides in blood with the use of the enzyme Lipase, prepared and purified from *P. aeruginosa:*

The ratio of triglycerides in serum was estimated by Young and his group (Høiby *et al.,* 2007). The ratio of triglycerides in serum was determined by the enzymatic degradation of Lipase based on the quinonimine concentration of hydrogen peroxide, 4 aminopitherapine and 4 chlorophenol under the influence of pyroxidase enzyme as in the following equations:

$$\text{Triglycerides} \xrightarrow{\text{Lipase}} \text{Glycerol + Fatty acid}$$

$$\text{Glycerol + ATP} \xrightarrow{\text{Glycerokinase}} \text{Glycerol-3- Phosphate + ADP}$$

$$\text{Glycerol-3-Phosphat} + O_2 \xrightarrow{\text{oxidase}} \text{Dihydroxy acetonphosphate} + H_2O_2$$

The tubes were lightly shake solution and incubated for 5 minutes at a temperature of 37 m. The absorbance of both the sample and the standard solution was measured along at 505 nm W spectra by optical

Solution	Standard	Reagent	Sample
Reagent	1000 microliter	1000 microliter	1000 microliter
Standard	10 microliter	-	-
Sample	-	-	10 microliter

spectroscopy and use the same methods by change reagent with extract and purified lipase from *P. aeruginosa* and one more time use silver nanoparticle only and mix of lipase with silver nanoparticle for measure the triglycerides concentration.

$$\text{Concentration of triglycerides } \left(\frac{mg}{dl}\right) = \frac{\text{Absorption of sample}}{\text{Absorption of the standard solution}} \times 200$$

Characterization of lipase enzyme

Optimal pH for lipase activity

To determine the optimum pH for the lipase activity, sodium acetate $C_2H_3NaO_2$ was prepared with a concentration of 0.1 M and pH ranged between (4.5-5), Phosphate buffer saline with 0.1 M concentration and pH ranged from (6-7.5) and Tris-HCl 0.1 M concentration and pH ranged between (8-9). Then equal volumes of these buffers were mixed with the Substrate olive oil at 0.1 M concentrations was 1:1. After that, 0.1 ml of the purified enzyme was added to 0.9 ml substrate. Solution of substrate with pH values, then plotted the relationship between enzymatic activity and pH to determine the optimal pH of enzyme activity (Copeland *et al.,* 2000).

Optimal temperature for lipase activity

To determine the optimum temperature for lipase activity, 0.9 mL of the substrate solution with 0.1M concentration was added to 0.1 mL of purified enzyme solution and then incubated for 10 minute in a water bath at different temperatures (25, 30, 35, 40, 45°C) and then determined the enzyme activity for each temperature. Then plotted the relationship between enzymatic activity and temperature to determine the optimal temperature for enzyme activity (Bongaerts *et al.,* 1978).

Optimal pH for lipase stability

To determine the optimal pH for enzyme stability, equal volumes of pure enzyme (0.4) ml were mixed with each buffer with pH rang between (from 3 to 9) at 0.1M concentration and substrate. The solutions were incubated in a water bath at 37°C for 30 minute and then piped to ice bath. The absorption was then measured with the optical spectrometer at a wavelength of 440 nanometers, calculated and then the relationship was plotted percentage of residual activity and optimal pH for enzyme stability (Polacco and Winkler, 1984).

Optimal temperature for lipase stability

To determine the optimum temperature for enzyme stability, 0.4 ml of purified lipase was incubated in a water bath at different temperatures (10, 20, 30, 40, 50°C) for 30 minutes. The enzyme-containing tubes were then transferred directly to an ice bath. Estimation of residual activity, the relationship between temperature and percentage of residual activity was determined to determine the optimal temperature for enzyme stability (Kara *et al.,* 2006).

Results and Discussion

Identification of bacteria

Identification of bacteria began primarily by culturing the samples on Muller Hinton agar and incubates at 37°C overnight, finally biochemical test was done to ensure that the isolate is belong to *P. aeruginosa*. The result deal with (Abdel-Fattah *et al.,* 2005*)*. As shown in table 1.

Optimal pH and temperature for bacterial Growth

The bacterial suspension was incubated once at constant temperature but different pH (5, 5, 7 and 9) and once at constant pH but different temperature (32, 35 and 37°C) and measure the absorbance at 565nm, compare the absorbance with McFarland number. It was

Table1: Identification of P. aeruginosa.

Characteristic of P. aerugionsa	Test
Oxidase (+), Catalase (+)	Biochemical
Forms round colonies with florescent greenish color by procaine stain, fruity order, 2-3mm in diameter, smooth with irregular surface	Morphology
Pink rod gram stain	Gram stain

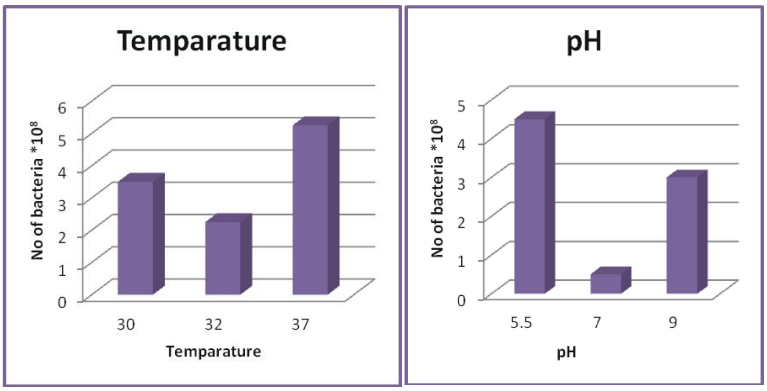

Fig. 1: show optimal pH and Temperature of *P.aeruginosa*

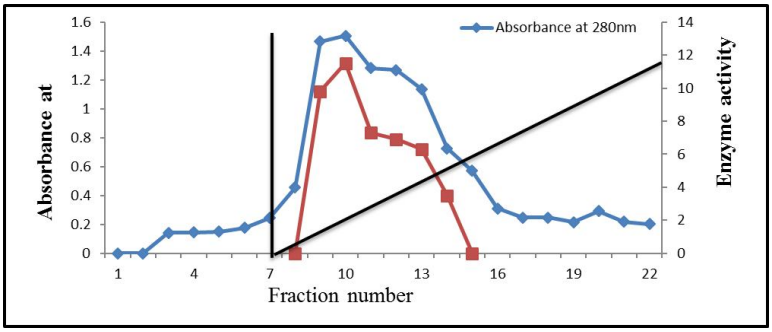

Fig. 2: Ion exchanger chromatography of Lipase produced by the mutant isolated *P. aeruginosa* using DEAE-Cellulose column (1.5×31 cm) with a flow rate of 36 ml/hour.

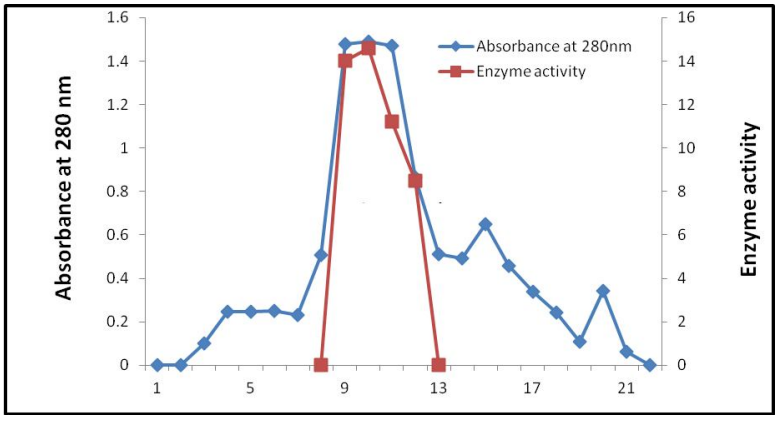

Fig. 3: Gel filtration for the purification of Lipase-enzyme produced from local isolation *P. aeruginosa* using S-200 Sephacryl gel column (1.5 × 57 cm). volume was 5 ml at flow rate of 36 ml/hours.

establish that the best conditions for the creation of bacteria at pH 5.5 with (4.5×10^8 cells) as compare with pH (9, 7) with (3×10^8) (0.5×10^8 cells) respectively and temperature 37°C with bacteria number (5.25×10^8 cells), as compare with (32 and 35°C) with bacterial number (2.25×10^8, 3.5×10^8 cells) respectively and this agree with the results of (Kojima and Shimizu, 2003) and (Gilbert *et al.,* 1991). As in the (fig. 1).

Extraction and purification of Lipase enzyme

In the present work lipase produced by *Pseudomonas aeruginosa* in the culture broth was subjected to a purification protocol. After that estimate the enzyme activity in crude supernatant. The enzyme activity 14.1 unit/ml and specific activity 0.7 unit/mg. The purification involved ammonium sulphate fractionation followed by ion exchange and Gel filtration.

Ammonium sulfate

The ammonium sulfate was applied in more than one saturation ratios (20, 40, 60 and 80)%, then the 60% ratio was choice as greatest ratio for precipitate the crude extract of enzyme, when the specific activity got to 1.1 U/mg, with purification fold 1.5 times and the yield 38.4%.

Ionic Exchange Chromatography

This is one of the most useful methods for protein purification. Depending on the surface molecule charge, the protein and the buffer conditions, the protein will have net a positive or negative charge. Lipase enzyme was obtained by using phosphate buffer solution (pH=7.2). Absorbance of eluted fractions were measured at 280 nm upon the arrival of absorbance to the line of zero (line base), then same buffer with the NaCl gradient (0.1-1M) used to elute the bounded protein. Ionic exchange chromatography patterns showed one protein peak in wash and one peaks in gradient elution, represented enzymic activity (tubes 8-13). Those fractions pooled and tested for specific activity (1.9 U/mg) a fold purification of (1.2 time) and enzymic yield of (14.7%) in parts. (fig. 2).

Gel filtration chromatograph

Purification carried out by a gel filtration using Sephacryl S-200. Enzymes fraction from DEAE cellulose were pooled and passed through gel filtration column. The fractionation yielded two protein peaks as

Table 2: The purification steps of lipase enzyme

Yield (%)	Fold	Total activity (Units)	Specific activity (U/mg)	Protein (mg/ml)	Enzyme activity (U/ml)	Volume (ml)	Step
100	1	3243	0.7	18.6	14.1	230	Crud extract
38.4	1.5	1248	1.1	18.3	20.8	60	Ammonium sulfate precipitation in 60% ratio
14.7	2.7	478	1.9	12.3	23.9	20	DEAE cellulose Ionic-exchange
11.1	5.7	363	4	6	24.2	15	Gel-filtration Sephacryl-S200

Table 3: Results of triglycerides concentration in the human serum using the standard kit and the purified lipase enzyme from *P. aeruginosa* bacteria, silver nanoparticle and the enzyme added to the silver nanoparticle.

Average error ± standard			
Concentrate of triglycerides using standard kit	**Concentration of triglycerides using lipase extracted from *P. aeruginosa***	**Concentrate of triglycerides using silver nanoparticle**	**Concentrate of triglycerides using lipase-silver Nano particle**
2.08 ± 416.00	0.94 ± 407.40	2.14 ± 375.76	0.79 ± 299.56
Value LSD : 5.279 ** ** (P<0.01).			

absorbance reading at 280nm (wave length), only one peak where appeared when reading absorbance at wavelength of 280 nm and when determined for enzyme activity in resulting parts enzyme activity recorded in (8-15), the specific activity reached (4 U/mg), fold of (5.7) and a yield (11.19%) as mentioned in table 4-5 and fig. 4. AL-Jumaily *et al*., (2009) who mentioned Mutants streptococci (serotype G) GTF has specific activity (0.752 U/mg protein).

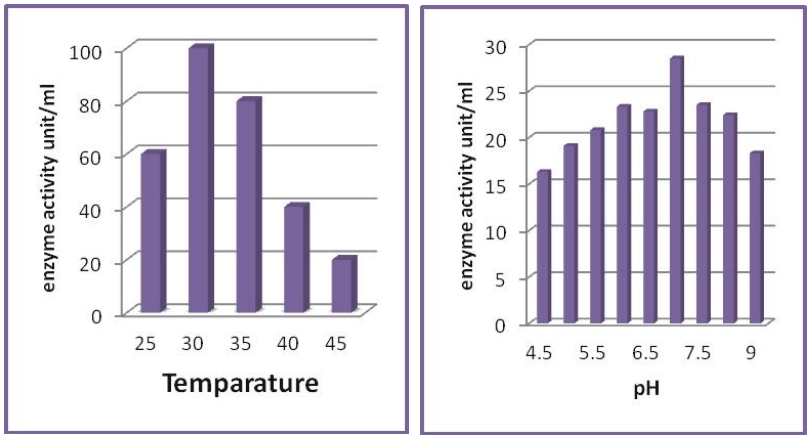

Fig. 4: Optimal activity of Temperature and pH

Determination of Lipid concentration in blood

The serum lipid concentration was measured using standard enzyme and using the extracted and purified lipase enzyme from *P. aeruginosa* with the use of $AgNO_3$. The results were compared between the fat content.(Table 3). There was no significant difference between the measured triglycerides using the enzyme of standard kit and the enzyme lipase extracted and purified from *P. aeruginosa*. While The results showed significant difference ($p<0.01$) between the concentration of triglycerides measured by the standard enzyme concentration and triglycerides concentration measured by the lipase by added $AgNo_3$ and the significant

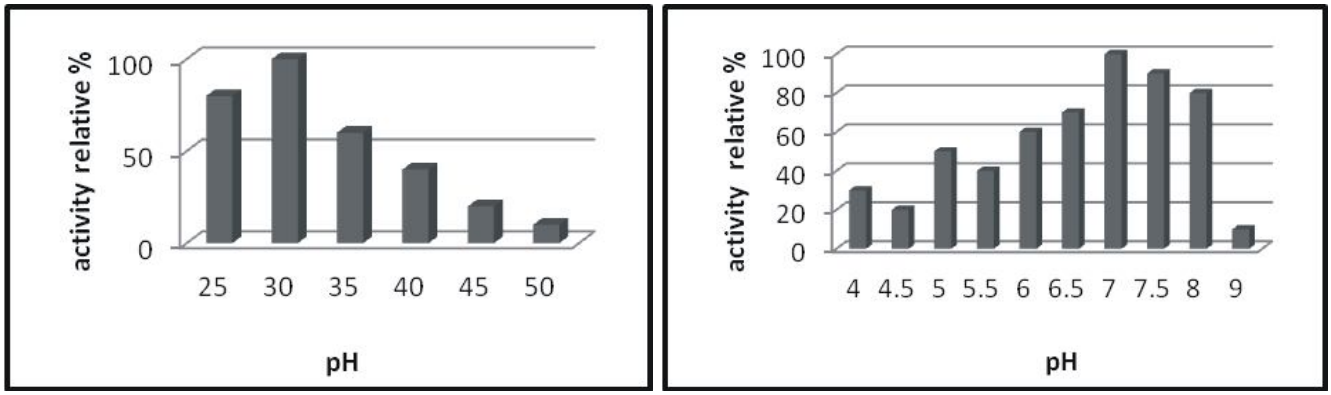

Fig. 5: Stability of Temperature and pH

difference ($p<0.01$) between the concentration of triglycerides using enzyme extracted and purified from *P. aeruginosa* and the concentration of triglycerides measured using the lipase enzyme added to the nanoparticles as shown in table 3. The difference is due to the decrease in the effectiveness of the enzyme after the addition of its nanoparticles.

Characterization of lipase enzyme

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 and reached the maximum lipase activity of 28.5 U/ml at pH 7. These data agree with (Abdel-Fattah *et al.,* 2005), 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. 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 (Kojima and Shimizu, 2003). (fig. 4).

pH and temperature stability

The pH stability of the lipase was determined by the activity retained at different pH from 3 to 9 after 30 min of incubation. The pH stability curve showed that the lipase was stable at pH 6 to 8 (fig. 5). 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. (Abdel-Fattah *et al.,* 2005) 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. The enzyme was found to be completely stable at 30°C after 30 min. At 35°C, the enzyme maintained 81% stability 1 h after the initial activity.

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