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PURIFICATION AND CHARACTERIZATION OF LIPASE FROM *PSEUDOMONAS AERUGINOSA* SJ2

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ABSTRACT

The present study was aimed to purify and characterize extracellular lipase from a newly isolated Pseudomonas aeruginosa SJ2. An extracellular lipase with molecular weight of 32,768 Da was purified using salt precipitation, ion exchange chromatography and molecular exclusion chromatography. Purification resulted in 2,826 U/mg specific activity with 6.57-fold purification and 74.24% yield. SDS-PAGE and revealed it to be near to homogeneity showing a relative molecular weight of about 32,768 Da that was confirmed by mass spectrometry. Zymography confirmed the activity of the purified lipase. Further stability studies were performed on the purified lipase. The optimum temperature and pH for activity were found to be 40°C and pH 8.0. Ca^{2+} ions at 1mM stimulated lipase activity while other metals inhibited lipase activity. Lipase retained good residual activity in the presence of various organic solvents (methanol, iso-propanol, ethanol, toluene, n-hexane, DMSO, and acetone). Lipase was fairly stable in the presence of non-ionic surfactants while lost some of its activity in the presence of ionic surfactants. Lipase was fairly stable in the presence of inhibitors (EDTA, urea, PMSF, β-mercaptoethanol). For oxidizing agents lipase was fairly stable in the presence of NaOCl and H2O2 while some of its activity was lost in the presence of NaBO3. Lipase was fairly stable and retained 92.15 % and 76.67 % relative activity after 30 days of storage at 4°C and 30°C respectively. The Km and Vmax were 0.24 mM and 3.51 µmol/min/mg respectively towards pNPP as a substrate.

KEYWORDS: Lipase, *Pseudomonas aeruginosa* SJ2, Mass Spectrometry, SDS-PAGE, zymography, Km and Vmax.

1. INTRODUCTION

Lipases belong to the class serine hydrolases. Triacylglycerol acyl-hydrolases EC 3.1.1.3, also commonly called as lipases are a group of hydrolytic enzymes that are primarily involved in the hydrolysis of ester linkages of insoluble substrate at substrate-water interface.[1] Lipases are ubiquitous in nature and are produced by various animals, plants, fungi, bacteria and archaea. They catalyze the hydrolysis of triglycerides into diglycerides, monoglycerides, glycerol and fatty acids, and under certain conditions, the reverse reaction leads to esterification and formation of glycerides from glycerol and fatty acids. The hydrolysis is a reversible reaction and the potential of lipases for ester synthesis.[2,3,4,5] and for interesterification in organic media.[6] Studies have confirmed that lipases are highly potent in using large spectrum of available substrates, are highly stable even in the extreme temperature fluctuations and they do not require any cofactor for optimal operations.[7] Due to their inherent enantioselective and regioselective tendencies, lipases are used in many industrial set ups, like synthesis of coca butter constituents, fat modification, resolution of chiral drugs, synthesis of biofuels, production of personal care and flavor enhancers etc. Thus, due to their specific chemo, regio and enantioselective nature; lipases are the most preferred microbial enzymes in variety of scientific and/or industrial applications.[8] Among all lipases, extracellular bacterial lipases are of considerable commercial importance, because of their diverse substrate specificity, stereo-specificity, and tolerance against heat and various organic solvents.[9,10] Therefore, they are widely used in food technology, in the detergent and chemical industries, and in biomedical sciences.[11,12,13] At present, lipases originated from Pseudomonas and Burkholderia are the most commonly used lipases in household detergents and in the trans-esterification process in the fine chemical industry.[9,10]

For the industrial use of any enzyme its complete characterization must be done, so that we can make its maximum possible use.[1] Considering the above facts, in the present work we have made an attempt to purify and characterize lipase from a newly isolated bacterium Pseudomonas aeruginosa SJ2.[14]

2. MATERIAL AND METHODS

All the chemicals and reagents used in the present work were of analytical grade and purchased from HiMedia (Mumbai, India) unless otherwise mentioned. The standard protein markers (Phosphorylase b (97.4 kDa), Bovine se- rum albumin (66 kDa), Ovalbumin (43 kDa), Carbonic anhydrase (29 kDa), Soybean trypsin inhibitor (20.1 kDa), and Lysozyme (14.3 kDa)) were purchased from GeNei Laboratories Pvt. Ltd. (Bengaluru, India). DEAE-cellulose and Se- phadex G-75 were purchased from Sigma-Aldrich (St. Luis, USA).

2.1. Source of lipase

Lipase produced by the new isolated *Pseudomonas aeruginosa* SJ2 under optimized conditions was used for purification and characterization.[14]

2.2 Lipase activity and protein estimation

Lipase activity was determined as described by Winkler and Stuckman (1979) using p-nitrophenyl palmitate (pNPP) as substrate with some modifications.[15] The substrate solution was prepared by mixing solution A (30 mg of pNPP in 10 mL of 2-propanol) with solution B (0.1 g of gum Arabic in 90 mL of 50 mM Tris–HCl buffer (pH 7.5) while stirring until all was dissolved. 2,700 µl of substrate solution and 300 µl of enzyme solution were pre-incubated at 40^0C for 5 min and then were mixed and incubated at 40^0C for 10 min and after 10 min, 50 µl Triton-X 100 was added to dissolve the released free fatty acids. Tubes were immediately kept on ice until the absorbance was measured at 410 nm. Blank was also prepared in the similar way except enzyme was added after the incubation period. Molar extinction coefficient (ε) for p-nitrophenol in the given reaction system was found to $5.3*10^3$ M^{-1} cm^{-1}. One unit of enzyme activity was expressed as one nanomole of p-nitrophenol released per minute under the assay conditions.[16] Protein was estimated by Lowry et al., (1951), method using bovine serum albumin as a standard protein.[17]

2.3. Purification of Lipase

After lipase production under optimized conditions the culture broth was centrifuged (10,000 g for 10 min at 4^0C) to recover the supernatant. Supernatant was used as a crude enzyme source. The crude lipase was further purified by ammonium salt precipitation, ion exchange chromatography followed by molecular exclusion chromatography.

2.3.1. Salt precipitation

Supernatant was used for salt precipitation, by mixing with different concentrations of ammonium sulphate (20, 30, 40, 50, 60, 70, 80, and 90%); under ice cold condition with continuous stirring at 300 rpm. The suspension was kept overnight, after which it was subjected to centrifugation again at 10,000 g for 10 mins at 4^0C. The pellet obtained was dissolved in minimum volume of 50 mM Tris-HCl buffer (pH 7.2) and was used for estimation of lipase activity and protein content. Fractions showing the maximum lipase activity and protein content was used for further purification process. Dialysis of the precipitate was done using cellulose tubing (molecular weight cutoff 12-14kDa). For dialysis 50 mM Tris HCl buffer (pH 7.2) was used.

2.3.2. Ion exchange chromatography

Dialyzed sample was applied to DEAE cellulose column (Borosil, 0.5 cm x 20 cm); which was pre-equilibrated with 50 mM Tris HCl buffer (pH 7.2). Post incubation, washing was done with wash buffer to remove unbound fractions (10 mL at a flow rate of 1 mL/min). The elution buffer was prepared with 0.05 to 0.5 M linear gradient NaCl. All the fractions were collected at a flow rate of 1mL/min and were analyzed for enzyme activity and protein content. Fractions showing highest lipase activity were pooled together.

2.3.3. Size exclusion chromatography

Sephadex G-75 resin was prepared by overnight incubation of 4 g resin in 200 mL 50 mM Tris HCl buffer at pH 7.2. The resin was poured gradually into the column (Borosil, 1.5 cm x 40 cm) and allowed to bind. Washing was done with 50 mL of buffer, after which samples were added slowly. The elution buffer used was 50 mM Tris HCl (pH 7.2), fractions of 2 mL were collected at a flow rate of 0.5 mL/min. Fractions showing highest lipase activity were pooled together.

2.4. Characterization of lipase

2.4.1. Molecular mass determination by SDS-PAGE

The purity and molecular mass of the purified lipase was determined by SDS-PAGE using 12% polyacrylamide gel.[18,19]

2.4.2. Native PAGE and Zymography

Purity and homogeneity of lipase was further confirmed by Native PAGE and activity was confirmed by zymography. The solution of resolving gel (8%) was poured into a vertical slab and a few drops of distilled water were layered on top of the gel solution for getting a uniform gel surface and to exclude oxygen. After polymerization of the resolving gel. The stacking gel solution (4.5%) was poured and immediately the comb was fixed at the top to make the wells for sample application. After this, pre-electrophoresis was carried

out at 10 mA for 15 min. Standard protein markers and purified lipase (20 µl) dissolved in sample buffer were loaded into the wells. Electrophoresis was run at 10mA. After the electrophoresis, the gel was stained with Coomassie brilliant blue R-250 staining solution. After staining, the staining solution was removed and the gel was transferred to the destaining solution. The gel was destained with gentle shaking on a gel rocker by changing the destaining solution several times till the gel background was clear.

Zymographic analysis was done following Native PAGE. The molten chromogenic substrate was overlaid on the gel, and was then incubated at 30°C for 15-20 min. After incubation, activity was observed as a yellow band. The chromogenic substrate was prepared using 2% agar, 0.01% phenol red, 1% olive oil, and 10 mM $CaCl_2$; pH was adjusted to around 7.3–7.4.[20]

2.4.3. Molecular mass determination by MALDI-TOF MS

MALDI-TOF mass spectroscopy (Bruker Daltonics, Ultraflex II, Germany) was used to determine the extent of purification and the molecular mass of lipase. It was equipped with a pulsed Neodymium yttrium-aluminum garnet smart beam solid-state laser (337 nm), in reflection positive-ion mode, and a 19 kV acceleration voltage. The matrix was prepared in 50 % acetonitrile, using 100 µl of 0.1% trifluoroacetic acid (TFA) and 10 mg of sinapinic acid. The mixture was sonicated in a water bath for 15 min and was then centrifuged for 10 min at 15,000 rpm. Thereafter, 50 µl of supernatant was diluted with 100 µl of 0.1% TFA in 50% acetonitrile, which served as a matrix. On the MALDI plate, 2 µl of the purified lipase sample was spotted along with 2 µl of freshly prepared sinapinic acid matrix. This mixture was then allowed to air dry before analysis. For the calibration purpose, proteins of known molecular masses were used.

2.4.4. Peptide Mass Fingerprinting (PMF) by MALDI-TOF MS

The amino acid sequence of the purified lipase was determined by MALDI-TOF MS analysis of the tryptic digested peptides using MALDI-TOF MS (Bruker Daltonics model Ultraflex-II Spectrometer, Germany). Further analysis was carried out with Flex Analysis software to produce the peptide mass fingerprint (PMF); the masses obtained were then submitted to the MASCOT database for protein identification.

2.4.5. Effect of pH on lipase activity and stability

Optimum pH for the enzyme activity was determined across the range of 4-11 by using 50 mM citrate phosphate buffer (pH 4-6), 50 mM Tris-HCl buffer (pH7-9) and 50 mM glycine-NaOH buffer (pH 10-11). Tris HCl buffer used in standard assay was replaced with these buffer solutions and enzyme reaction was carried out. After this, relative enzyme activities were measured under given assay conditions considering maximum activity as 100%.

Enzyme stability was studied by keeping the enzyme for 1 h at 1:1 volume at 30^{o}C with different buffer solutions (pH 4-11). After incubation enzyme samples were dialyzed to remove the buffer solution and then were subjected to en- zyme assay under standard assay conditions. Control sample was not pre-treated with any buffer. Relative enzyme activities were then measured considering activity in control as 100%. Negative control consisted of heat inactivated enzyme.

2.4.6. Effect of temperature on lipase activity and stability

The temperature optima for lipase activity was determined by conducting the enzyme assay at various temperatures ranging from 20 to 70°C, and the activities were evaluated under given assay conditions, considering maximum activity to be 100%.

Thermal stability of the enzyme was studied by incubating the enzyme at different temperatures (20-60°C) for 2 h. After heat treatment samples were immediately cooled on ice bath. After this, relative enzyme activities were measured under standard assay conditions considering highest activity as 100%.

2.4.7. Effect of metal ions

The effect of metal ions (K, Na, Mg, Ca, Mn, Fe, Hg, Co) on lipase activity was determined at two different concentrations (1 mM and 10 mM). Lipase was pre-treated with the metal ions for 1 h at 30°C. The relative enzyme activities were evaluated after pre-treatment under standard assay conditions for all the ions considering the untreated enzyme activity to be 100%.

2.4.8. Effect of organic solvents

The effect of organic solvents (methanol, iso-propanol, ethanol, toluene, hexane, DMSO, and acetone) on the lipase activity was analyzed by incubating the enzyme with organic solvents (25%) at 30^{0}C. The control contained no organic solvent. After this, relative enzyme activities were measured after 1 h and 2 h of incubation under standard assay conditions considering activity in control as 100%.

2.4.9. Effect of surfactants

The effect of following surfactants Triton-X-100, sodium dodecyl sulfate (SDS), sodium thioglycolate, Tween-40, Tween-60, and Tween-80 was studied at 0.1 and 1% (w/v). Lipase activity was determined by incubating the enzyme with different surfactants for 1 h at 30 C. The control

contained no surfactant. After this, relative enzyme activities were measured under standard assay conditions considering activity in control as 100%.

2.4.10. Effect of inhibitors

The effect of following surfactants Triton-X-100, sodium dodecyl sulfate (SDS), sodium thioglycolate, Tween-40, Tween-60, and Tween-80 was studied at 0.1 and 1% (w/v). Lipase activity was determined by incubating the enzyme with different surfactants for 1 h at 30 C. The control contained no surfactant. After this, relative enzyme activities were measured under standard assay conditions considering activity in control as 100%.

2.4.11. Effect of oxidizing agents

Effect of oxidizing agents (H_2O_2, sodium perborate, and sodium hypochlorite) was assessed by mixing the enzyme with 0.1 and 1% (w/v or v/v) solution of the oxidizing agents and was incubated at 30^{O}C for 1 h along with enzyme control. Control sample was not pretreated with any oxidizing agent. Relative enzyme activities were then measured considering activity in control as 100%.

2.4.12. Determination of kinetic parameters (Km and Vmax)

The Michaelis constant (Km) and maximum velocity (Vmax) were determined for lipase under standard assay conditions. Para-nitrophenyl palmitate (p-NPP) was used as a substrate. Lipase was incubated with varying concentrations of p-NPP (0.0 to 100 mM) and the lipase activity was measured under standard assay conditions.

2.4.13. Storage stability

Storage stability of lipase was studied by keeping the enzyme samples at refrigeration (4°C) and 30°C and periodically analyzing the enzyme activity on 2nd, 5th, 10th, 15th, 20th, 25th, and 30th day. After incubation, relative enzyme activities were measured under standard assay conditions considering activity in control (day 1) as 100%.

3. RESULTS

3.1. Purification of lipase

3.1.1. Salt precipitation

Crude lipase isolated from *Pseudomonas aeruginosa* SJ2 was first salt precipitated using ammonium sulphate. The enzyme activity and protein content were found to be maximum at about 80% of the saturation level; without any significant growth in the enzyme activity beyond 80%. At this saturation, the specific activity of enzyme was 650 U/mg, with 1.51 fold purification and 82.80% yield (**Table 1**).

3.1.2. Ion exchange chromatography

After salt precipitation, the enzyme solution was desalted by dialyzing it against 50 mM Tris HCl buffer (pH 7.2). The dialyzed enzyme sample was passed through a DEAE cellulose column. The bound lipase was eluted with increasing concentration gradient (0.05 M) of NaCl in Tris HCl buffer (50 mM) of pH 7.2. Fractions showing highest lipase activity were pooled together for further purification. Ion-exchange chromatography resulted in 2.47 fold purification with 78.81% yield and 1,062 U/mg specific activity (**Table 1**).

3.1.3. Gel filtration chromatography

Lipase was further purified by gel filtration chromatography using Sephadex-G 75 column. Fractions showing highest lipase activity were pooled together. Gel filtration chromatography resulted in 6.57 fold purification, with the recovery of 74.24% and specific activity 2,826 U/mg (**Table 1**). Results obtained from the purification were found to be in accordance with other studies.[21,22] At the same time, it should be noted that many other studies confirmed higher purification of lipase with limited rate of recovery, using different techniques such as ultra-filtration and gel exclusion chromatography etc.[23,24] As a matter of fact, many commercial applications of lipases do not require highly purified enzymes; as they may turn out to be expensive with reduced rate of recovery.[25]

Table 1: Purification of lipase produced by *Pseudomonas aeruginosa* SJ2.

Purification Step	Activity (U/mL)	Protein (mg/mL)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	1,751	4.07	430	1.0	100
Salt precipitation	1,450	2.23	650	1.51	82.80
Ion Exchange	1,380	1.3	1,062	2.47	78.81
Gel filtration	1,300	0.46	2,826	6.57	74.24

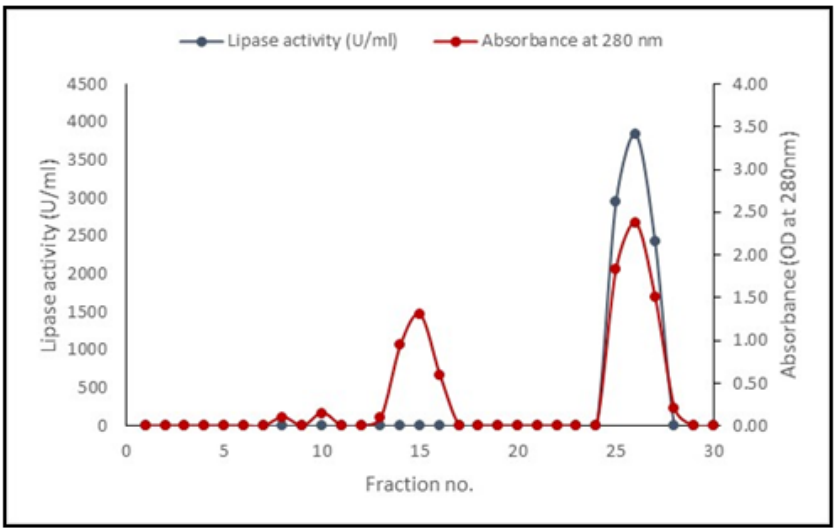

Figure 1. Elution profile of protein and lipase from diethyl aminoethyl (DEAE)-cellulose column.

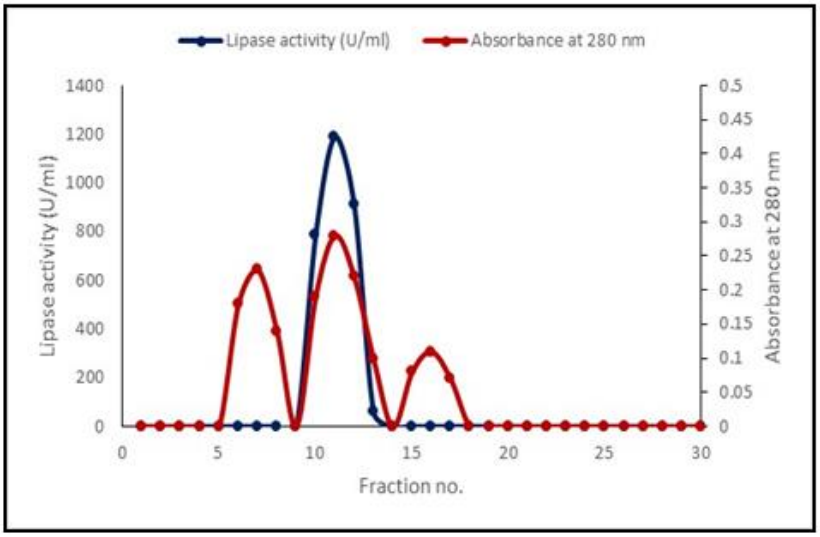

Figure 2. Elution profile of protein and lipase on Sephadex G-75 column.

3.2. Characterization of the purified lipase

3.2.1. SDS-PAGE, Native PAGE and Zymography

Apparent homogeneity of the purified lipase from Pseudomonas aeruginosa SJ2 was demonstrated by the detection of a single protein band with an apparent molecular mass of 32 kDa after SDS-PAGE after comparing it with standard protein markers (Figure: 3 A). Further its purity and activity was confirmed by Native PAGE (Figure: 3 C) and zymography. In zymographic analysis a yellow spot was observed due to lipolysis of olive oil which resulted in the reduction of pH and color change from pink to yellow (Figure: 3 B). A wide range of molecular weights has been reported for Pseudomonas spp. lipase ranging from 29 to 62 kDa. For instance, P. aeruginosa LX1 produced an extracellular lipase of molecular weight 56 kDa.[26] The molecular weight of purified lipase produced by P.aeruginosa AAU2 was found approximately 81.7 kDa.[27] Gilbert et al. (1991) successfully purified P. aeruginosa EF2 lipase of 29 kDa molecular weight.[28] Lipases from Pseudomonas aeruginosa ATCC 21808, P. aeruginosa KKA-5, and P. mendocina 3121-1 with a molecular weight of 35, 30, and 62 kDa, were reported by Kordel et al. (1991), Sharon et al. (1998), and Surinenaite et al. (2002), respectively.[29,30,31] Muthusamy et al. (2018) reported lipase of 19.4 kDa from Bacillus subtilis.[32]

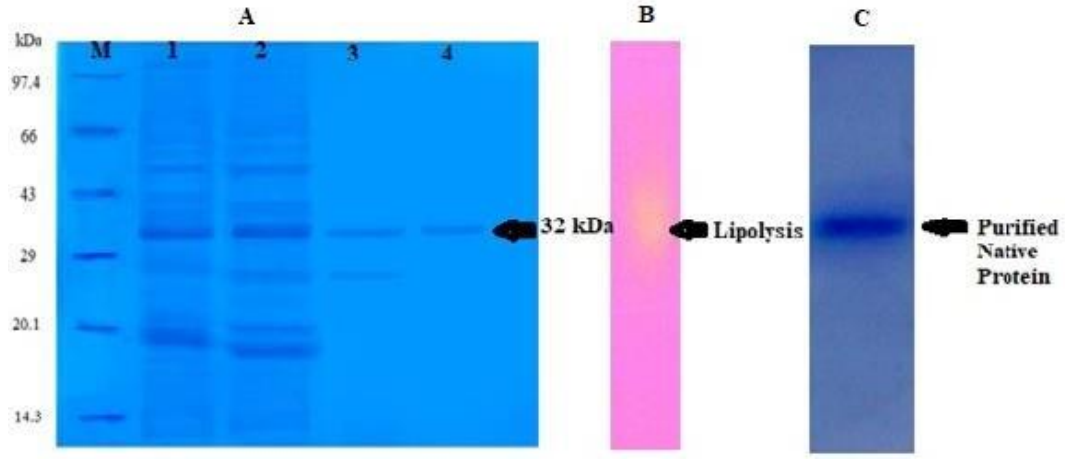

Figure 3. A: SDS-PAGE electrophoresis. Lane M: Molecular weight markers; lane 1: Crude enzyme, lane 2: Concentrated enzyme after salt precipitation; lane 3: Purified enzyme after ion exchange chromatography; lane 4: Purified enzyme after gel filtration chromatography.
B: Zymographic analysis showing yellow spot due to lipase activity**.**
C: Purified enzyme band after Native PAGE.

3.2.2. MALDI-TOF analysis of trypsin digested lipase and peptide sequencing

In peptide mass fingerprinting (PMF), lipase was digested by trypsin which generated numerous peptide fragments (**Figure: 4**). Out of the total trypsin digested peptide fragments of lipase only nine peptide sequences were hit in mass spectrometry protein sequencing database by Mascot search engine (https://www.matrixscience.com/). These nine peptides of lipase showed 38% sequence coverage with the known triacylglycerol lipase of *P. aeruginosa* (WP_134592233.1) (**Figure: 5**) when compared; exhibiting a significant mascot score of 103 and p-value of < 0.05. The ions of lipase peptides with masses 2898.689, 2252.058, 2095.986, 2066.156, 1457.745, 981.406, 2087.983, 1627.725 and 866.408 were the nine trypsin digested peptides corresponding to the known amino acid residues 5-33, 63-82, 64-82, 83-101, 102-115, 137-145, 196-215, 200-215, and 301-307 respectively of *P. aeruginosa* (WP_134592233.1). The amino acid composition of the 09 sequenced peptides is summarized in **Table 2**. About 153 amino acid residues were identified from these 09 peptides and designated as P1 to P9 respectively.

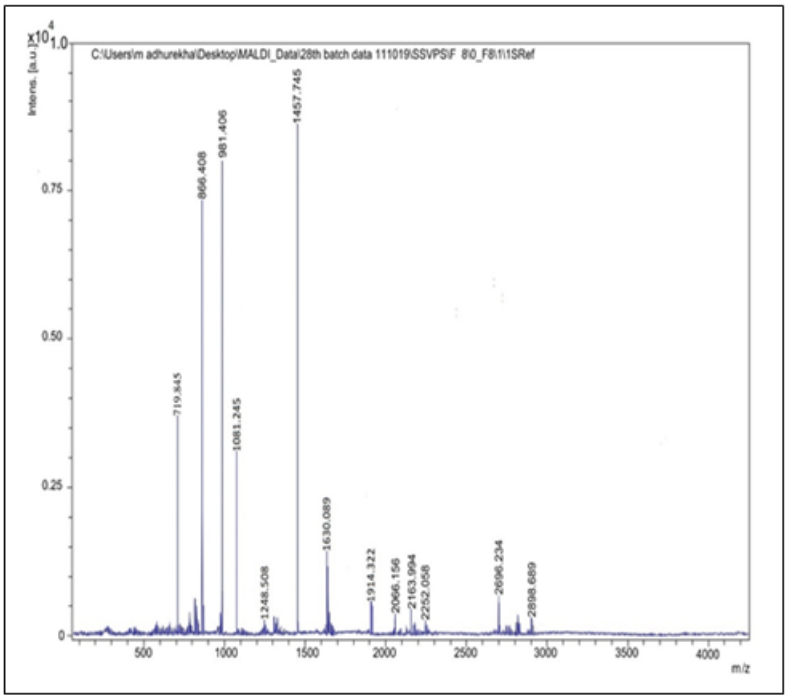

Figure 4: MALDI-TOF mass spectrum of trypsin digested peptide map of lipase.

1 MKKKSLLPLG LAIGLASLAA SPLIQASTYT QTKYPIVLAH GMLGFDNILG
51 VDYWFGIPSA LRRDGAQVYV TEVSQLDTSE VRGEQLLQQV EEIVALSGQP
101 KVNLIGHSHG GPTIRYVAAV RPDLIASATS VGAPHKGSDT ADFLRQIPPG
151 SAGEAILSGL VNSLGALISF LSSGSTGTQN SLGSLESLNS EGAARFNAKY
201 PQGVPTSACG EGAYKVNGVS YYSWSGSSPL TNFLDPSDAF LGASSLTFKN
251 GTANDGLVGT CSSHLGMVIR DNYRMNHLDE VNQVFGLTSL FETSPVSVYR
301 QHANRLKNA

Figure 5: Homologous peptides (in bold) of purified lipase with known triacylglycerol lipase of *P. aeruginosa* (NCBI:WP-134592233.1).

Table 2: Calculated and observed peptide ions of trypsin digest of the enzyme lipase.

Peptide	Peak No.	Amino acid sequence of identified peptides			Intensity of identified peptide	$[M+H]^+$		No. of amino acids sequenced
		From	To	Peptide Sequence		Observed	Calculated	
P1	03	301	307	QHANRLK	7380.50	866.4082	865.4882	07
P2	15	137	145	GSDTADFLR	8012.00	981.4066	980.4563	09
P3	40	102	115	VNLIGHSHGGPTIR	8630.10	1457.7459	1456.7899	14
P4	53	200	215	YPQGVPTSACGEGAYK	218.36	1627.7252	1626.7348	16
P5	77	83	101	GEQLLQQVEEIVALSGQPK	410.27	2066.1568	2065.1055	19
P6	79	196	215	FNAKYPQGVPTSACGEGAYK	125.68	2087.9832	2086.9782	20
P7	80	64	82	DGAQVYVTEVSQLDTSEVR	130.58	2095.9860	2095.0070	19
P8	84	63	82	RDGAQVYVTEVSQLDTSEVR	304.25	2252.0583	2251.1081	20
P9	96	5	33	SLLPLGLAIGLASLAASPLIQAS TYTQTK	370.12	2898.6897	2897.6477	29

3.2.3. Molecular mass determination

The molecular mass and purity of lipase were determined by mass spectrometry (**Figure: 6**). It revealed a single peak corresponding to a molecular mass of 32,768.97-dalton which closely resembled the molecular mass obtained by SDS-PAGE (32,000-dalton).

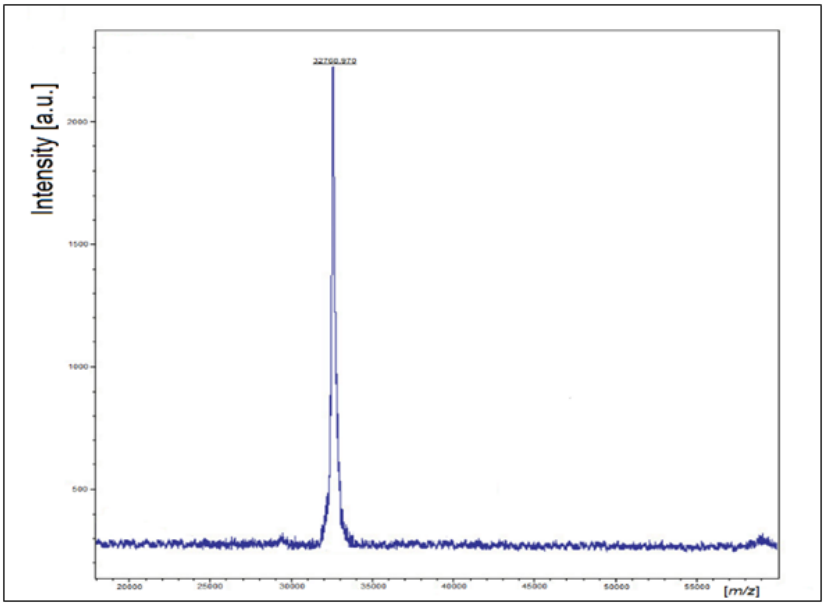

Figure 6. Molecular mass determination of lipase by MALDI-TOF MS.

3.2.4. Effect of pH on lipase acitivity and stability

The optimum pH for *P. aeruginosa* SJ2 lipase activity was assessed over a pH range of 4 to 11. The optimum pH for lipase activity was found to be 8.0 (**Figure 7a**). *P. aeruginosa* SJ2 lipase exhibited more than 70% activity in the pH ranges from 7.0-9.0. Enzyme retained 90.73% relative activity even at pH 9.0

which indicates its alkaline nature.

However, its activity was minimum at the extreme acidic and basic pH values of 4 and 11. In similar studies; Bisht et al. (2013) reported higher lipase activity for a mutant strain of P. aeruginosa at pH 8.0.[33] In another study; higher activity was reported by Sifour et al. (2010) for the lipase isolated from G. stearothermophilus.[34]

In the pH stability studies P. aeruginosa SJ2 lipase was found to be highly stable over the pH range of 7.0-9.0 for up-to 1 h of incubation at 30°C. Almost 100 % residual activity was retained over the pH range of 7.0-9.0. While more than 60% residual activity was retained over the pH range of 6.0-10 **(Figure 7b).** In similar studies; Bisht et al. (2013) reported that P. aeruginosa lipase was fairly stable within pH 7.0-9.0 for up-to 1 h of incubation, however, the activity was reduced at pH 10-11.[33] Lipase isolated from Pseudomonas sp. strain S5 was reported to be fairly stable over the pH range of 7.0-9.0; while the activity got reduced at alkaline pH range of 10-11 after 30 min of incubation by Rahman et al. (2005).[35] Similar findings for other strains of Pseudomonas have been reported by Bhatty (2011); Dharmsthiti & Kuhasuntisuk (1998); Chakraborty & Paulraj (2009).[36-38]

3.2.5. Effect of temperature on lipase activity and stability

p.aeruginosa SJ2 lipase activity was performed at different temperatures ranging from 20-60°C. Optimum temperature for the lipase activity was found to be 40°C. More than 80% relative activity was maintained between 30°C and 45°C. At 20°C 47.10% relative activity was found while at 60°C 19.94% relative activity was recorded (**Figure 7c**). Similar results have been reported by Bose et al. (2013), Bhatty (2011), Dharmsthiti & Kuhasuntisuk (1998), Kulkarni & Garde (2002), Kukreja & Bera (2005), Borkar et al. (2009).[24,27,36,37,39,40]

While studying the stability lipase retained 100% residual activity up-to 40°C when incubated for up-to 2 h. At and above 45°C lipase activity started reducing because of thermal denaturation at higher temperature. At lower temperature lipase showed lower activity which might be because of reduced frequency of collision between lipase molecules and substrate molecules (**Figure 7d**). Similar results were reported by Dharmsthiti and Kuhasuntisuk (1998) where *P. aeruginosa* LP 602 lipase retained 50% residual activity at 55°C after 2 h.[37] Lipase isolated from *P. fluorescens* NS2W retained more than 70% residual activity when incubated at 60°C for 2 h.[39] On the other hand, Rahman et al., (2005) have reported that *Pseudomonas* S5 lipase completely lost its activity at 55°C after 2 h.[35]

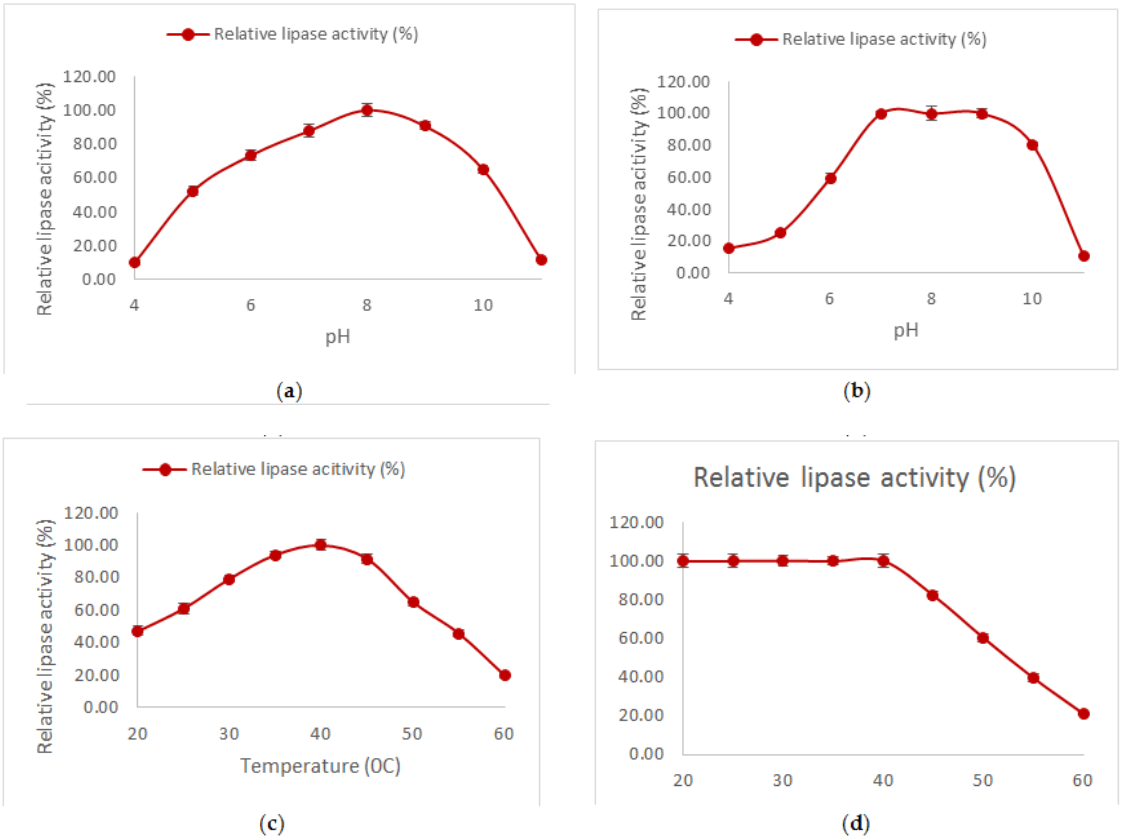

Figure 7. (a) Effect of pH on lipase activity. (b) Effect of pH on lipase stability. (c) Effect of temperature on lipase activity. (d) Effect of temperature on lipase stability.

3.2.6. Effect of metal ions on lipase activity

Among the metal ions studied, Ca^{2+} ions at 1 mM concentration enhanced the lipase activity to 112.73%. Lipase activity got inhibited by Ca^{2+} (at 10 mM), K^{+}, Na^{+}, Mg^{2+}, Mn^{2+}, Fe^{2+}, Hg^{2+}, and Co^{2+} at 10 mM as well as 1mM concentration (**Figure 8a**). According to Wills (1960) calcium ions may carry out three distinct roles in lipase action - removal of fatty acids as insoluble Ca^{2+} salts in certain cases, direct enzyme activation resulting from concentration at the fat-water interface and stabilizing effect on the enzyme (Wills, 1960). *Pseudomonas aeruginosa* SJ2 lipase got marginally inhibited by metal chelator EDTA indicating it's not a metalloenzyme. Hence, lipase activity got inhibited which may be due to the inhibitory effects and unfavorable ionic environment created by these metal ions at the catalytic site. Sharma et al. (2001) also reported that lipase activity was boosted in the presence of Ca^{2+} ions, whereas got inhibited in the presence Fe^{3+} and Zn^{2+} ions.[41]

3.2.7. Effect of organic solvents on lipase activity

P. aeruginosa SJ2 lipase retained good residual activity in the presence of various organic solvents (25%; methanol, iso-propanol, ethanol, toluene, n-hexane, DMSO, and acetone) when incubated for up-to 2 h. The highest residual activity of 105.67% was recorded for n-hexane when incubated for 1 h even after 2 h of incubation 104.29% residual activity was retained in the presence of n-hexane. Even after 2 h of incubation in the presence of DMSO lipase retained its complete activity (100.58%). The lowest residual activity of 54.33% was recorded for methanol after 2 h of incubation (**Figure 8b**).

Ogino et al. (2000) reported a similar effect on lipase produced by an organic solvent tolerant *P. aeruginosa* LST-03 in the presence of hydrophobic solvents.[42]

Doukyu and Ogino (2010) reported reduction in the activity of lipase in the presence of polar solvents which can be attributed to the stripping of the essential water required for maintaining enzyme in active conformation.[43] Rua et al. (1997) reported that the increase in lipase activity in the presence of nonpolar solvents can be attributed towards the change in lipase conformation leading in the opening of the lid that shields the enzyme's active site, keeping the lipase in its open conformation.[44] Volpato et al. (2008) reported an increase in the activity by 1.5-fold with 25% (v/v) *n*-hexane for *S. caseiolyticus* EX-17 lipase.[45]

3.2.8. Effect of surfactants on lipase activity

P. aeruginosa SJ2 lipase retained a good amount of stability in the presence of non-ionic surfactants (Tween-40, Na-deoxycholate, Tween-60, Tween-80, Triton-X 100) after 1 h of incubation and retained almost 100% residual activity in the presence of 0.1 and 1.0% non-ionic surfactants, while it retained 70.45% residual activity in the presence of 0.1% ionic surfactant (SDS), and lost complete residual activity in the presence of 1.0% SDS (**Figure 8c**). The results are in agreement with Bhatty (2011), Sharma et al. (2002), Gulati et al. (2005), and Bose et al. (2013) for *Pseudomonas* lipases.[27,36,46,47] Non-ionic detergents appear to reduce the lipase's hydrophobic interactions, inducing disaggregation and hence retaining enzyme's activity, whereas the ionic detergent SDS acts on the disulphide bonds, inactivating the enzyme.[40]

3.2.9. Effect of inhibitors on lipase activity

Residual lipase activity was marginally reduced to 94.40% and 85.07% in the presence of EDTA at 2 mM and 5 mM concentration respectively. This indicates that the enzyme is independent of any metal cofactor. In similar studies Bisht et al. (2013), Saeed et al. (2005) and Lin & Ko (2005) reported that the activity of lipase was not affected by the chelating agent EDTA.[33,48,49] In the presence of β-mercaptoethanol (0.1%) a reducing agent the lipase activity increased to 108.33%; while lipase activity got reduced to 95.78 % at higher concentration. This may be due to the presence of very few sulfhydryl groups in lipases, which are required for the enzyme activity.[50] In the presence of PMSF at 2 mM and 5 mM concentration lipase activity got reduced to 93.52% and 90.98% respectively. This may be due to the presence of a hydrophobic lid at the active site of lipase.[51] In the presence of 0.2 mM urea lipase activity was slightly reduced to 98.33% and to 89.59% at 5 mM concentration. The urea can affect lipase activity and stability either directly by interfering with the lipase structure or indirectly by modifying the characteristics of the surrounding solvents[52] (**Figure 8d)**.

3.2.10. Effect of oxidizing agents on lipase activity

Among the various oxidizing agents studied the lipase activity increased to 107.72% in the presence of 0.1% sodium hypochlorite and 102.51% in the presence of 0.1% H_2O_2 respectively. On the other hand residual activity was reduced to 88.12% in the presence of 0.1% $NaBO_3$. Residual activity decreased to 98.63, 96.25, and 68.41% for H_2O_2, NaOCl, and $NaBO_3$ respectively at 1.0% concentration (**Figure 9a**). Bisht et al. (2013) also reported similar results for the lipase isolated from a mutant strain of *P. aeruginosa*.[33] Similarly, *B. cepacia* lipase when incubated for 1 h at 25°C in the presence of various oxidizing agents was reported to be very stable by Wang et al. (2009).[53] The lipase stability in the presence of detergents and oxidizing agents demonstrates its potential use

in detergent formulations, as these agents are active ingredients in household detergents.[54]

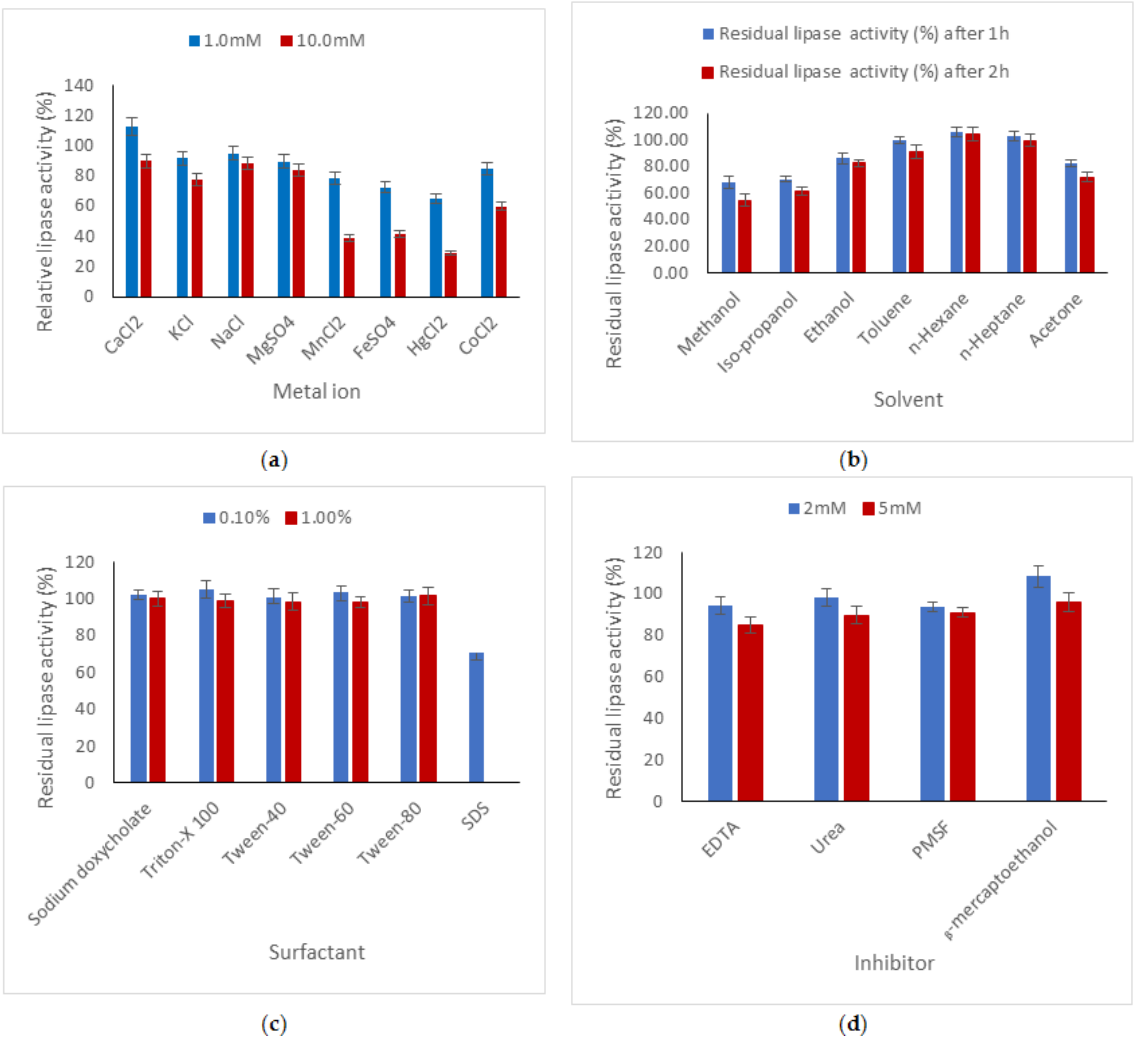

Figure 8. (a) Effect of metal ions on lipase activity. (b) Effect of organic solvents on lipase activity. (c) Effect of surfactants on lipase activity. (d) Effect of inhibitors on lipase activity.

3.2.12. Enzyme kinetics

The kinetic parameters for *P. aeruginosa* SJ2 lipase were determined by Lineweaver-Burk plot. The Lineweaver-Burk plots were linear, suggesting that the lipase followed Michaelis-Menten kinetics in the hydrolysis of p-nitrophenyl palmitate (pNPP) as a substrate. The results showed that increasing the substrate concentration resulted in an exponential increase in lipase activity; however, after that, it remained constant, which could be due to the saturation of the active sites. The values of Km and Vmax were found to be 0.24 mM and 3.51 µmol/min/mg respectively towards pNPP as a substrate (**Figure 9c and 9d**). During the course of reaction initially the reaction follows first order kinetics where the rate of reaction is proportional to the substrate (pNPP) concentration. However, at higher substrate concentration, the reaction follows zero order kinetics where the rate of product formation becomes independent of the substrate concentration. Here, the active sites of the enzyme molecules are 100% saturated and thus the rate of product formation becomes independent of the substrate concentration and further increase in the substrate concentration has no effect on the rate of reaction. Low apparent Km indicates the higher affinity of the lipase towards pNPP. In similar studies Bose et al. (2012) reported Km value of 0.1 mM and Vmax value of 40 µmol/min/mg towards pNPP by partially purified lipase from *P. aeruginosa* AAU2.[27] In another study; Gaur et al., (2008) reported Km and Vmax values of 70.4 mM and 2.24 mmol/min/mg respectively towards pNPP.[22]

3.2.11. Storage stability

Result of percent relative activity of stored enzyme is depicted in **Figure 10b**. From the results it can be seen that the *P. aeruginosa* SJ2 lipase was stable at refrigeration (4°C) and room temperature (30°C)

with negligible reduction in lipase activity. At refrigeration (4°C) lipase was able to retain 92.15% relative activity and 76.67% relative activity at room temperature (30°C) after 30 days of storage. In similar studies *P. aeruginosa* BH-5 lipase was fairly stable at 4°C and 30°C up-to 42 days maintaining 90% and 73% residual activity at 4 °C and 30 °C respectively.[36]

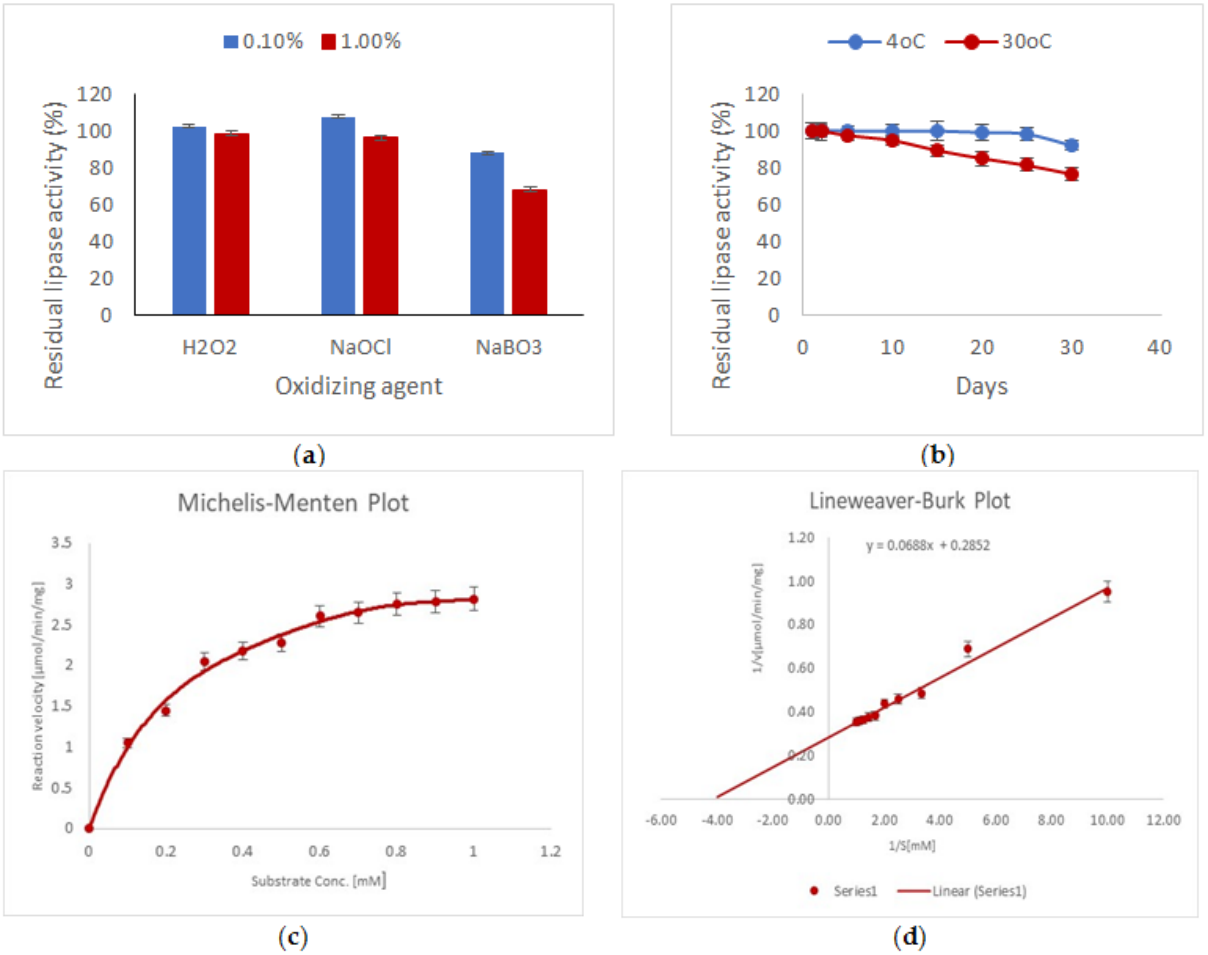

Figure 9. (a) Effect of oxidizing agents on lipase activity. (b) Storage stability of lipase. (c) Michalis-Menten plot of lipase using pNPP as a substrate. (d) Lineweaver-Burk plot of lipase using pNPP as a substrate.

4. CONCLUSION

The *Pseudomonas aeruginosa* SJ2 lipase has some promising characteristics which can lead to its further industrial use. The lipase has good stability at wide pH and temperature range. Also, it is fairly stable in the presence of oxidizing agents, surfactants, has good storage stability. Hence, these characteristics can make it a good candidate for industrial use in industries like detergent, petroleum, food, pharmaceutical, etc. Hence, further studies like enzyme 3-D structure determination, detailed studies of the catalytic site and mechanism of catalysis, enzyme immobilization etc. need to be done for conclusive results on its further industrial exploitation.

Conflicts of Interest: The authors declare that they have no conflict of interest.

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