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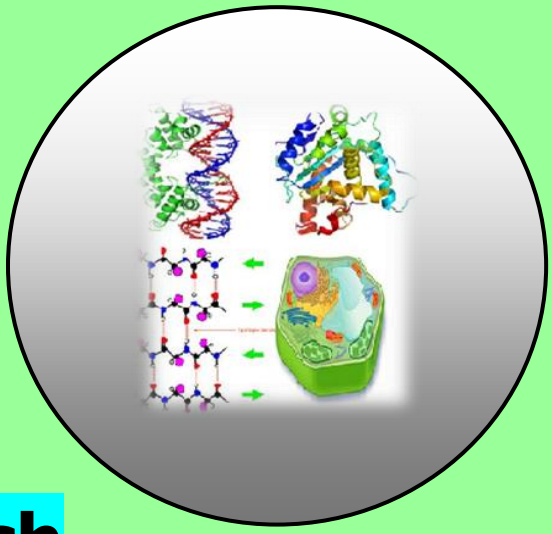

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RESEARCH PAPER

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Purification and Characterization of Lipase Production from *Bacillus subtilis* PCSIR-NI39

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ABSTRACT

The current trend in lipase research is to focus on microbial lipases rather than lipases derived from animals and plants, as lipases from micro-organisms have many advantages. In the present study, extracellular lipase producers were isolated from local environment. A microorganism, Bacillus subtilis showed extracellular lipase activity and high growth rate at optimum conditions was selected. The excreted lipase of bacillus subtilis was purified to homogeneity by ammonium sulfate precipitation, dialysis and ion exchange chromatography. The relative molecular mass of the lipase was determined on sodium dodecyl sulfate polyacrylamide gel electrophoresis.

The purified enzyme exhibited maximum activity at 70°C (124.00 U/ml) and pH 8 (174.06U/ml). The enzyme activity was highly promoted in the presence of Mg^{2+} (159.67U/ml) and Na^{+} (149.33U/ml). The enzyme activity also enhanced in the presence of almond oil as a substrate (139.50 U/ml).Molecular weight was found to be 41kDa.

Lipases produced from Bacillus subtilis can be used as biodetergent and for bioremediation of waste water.

Key words: Microbial lipases, Enzyme purification, Enzyme characterization, Bacillus subtilis and Ammonium sulfate purification.

INTRODUCTION

Lipases like, EC 3.1.1.3 triacyl glycerol acyl hydrolases are water soluble which hydrolyse triacylglycerol into fatty acid and glycerol and because of an opposite polarity between enzyme and their substrate. The reactions of lipase are oil water interface (Reis *et al.,* 2008).Lipases play an efficient role in certain reaction like aminolysis in organic solvents, transesterification and esterification. According to Tan, *et al.,* 2015, the world enzyme industry is worth $5.8 billion US dollars in 2010, and is forecasted to rise 6.8% annually to $8 billion US dollars in 2015

Lipases can be obtained from animals, plants and microbes. The most important source of lipases is microorganisms that are produced by fermentation of different bacteria. As mentioned by Vakhlu and Kour (2006) a more stable form of lipases is microbial lipases which are more stable when compare to the enzymes extracted from animal and plants. Microbial Lipases are easy to produce and cost of production is low. Bacteria like *Pseudomonas* spp. *Staphylococcus* spp., *Chromobacterium* spp*., Alcaligenes* spp*., Achramobacter* spp*.* and *Bacillus* are commercially used for lipase production (Vishwe *et al.,* 2014 & Reza *et al.,* 2015). Other major advantages are that microbial lipases are: (i) structurally stable in organic solvents; (ii) independent of cofactors; (iii) catalyze reactions utilizing a wide range of substrates; and (iv) have high enantio-selectivity.

Purification of lipases has great commercial importance. Different lipases obtained from fungal, bacterial, plant and animal sources have already been purified to the level of homogeneity. For purpose of purifying various fungal liapses different strategies are in use (Saxena*et al.,* 2003). As mentioned by Kumar *et al.,* (2005) DEAE-Sepharose column chromatography and ammonium sulfate precipitation can be used for purification (40-fold) of Bacillus coagulans. Usually employed ion exchangers are the carboxymethyl (CM) in cation exchange (20%) and the diethylaminoethyl (DEAE) group in anion exchange (58%). Strong ion exchangers based on Q-Sepharose (Menge*et al.,* 1990) and triethylaminoethyl groups (Veeraragavan *et al.,* 1990) are frequently used for purification process for protein (Abbas, *et al.,* 2007; Tan, *et al.,* 2015).

Lipase released from microbes has advantage to remove these lipid contents and it is environment friendly treatment of waste water. Cold adopted lipases have great potential in the field of waste water treatment (Buchon *et al*., 2000).Cosmetics industry is growing industry. The commercial products for personal care are used in everyday life. For the production of aroma compound, lipases also play an important role. Lipase is an active ingredient in so called functional cosmetics.

The present work has been designed for the purpose of lipase production in cost free medium. Main focus is on purification and characterization of lipase from locally isolated bacillus.

MATERIALS AND METHODS

Isolation and Screening of Lipolytic bacteria

Collection & Processing of Soil Samples

Different sites were visited to collect the samples of soil and water. These sites include oil industry, automobile garages and household wastages. The samples are named as Sample-1, Sample-2 and Sample-3. These were stored in the refrigerator at 4^{o}C in sealed sterilized containers before use.

Isolation and screening of Lipolytic bacterial strain

Serial Dilution Technique was used in order to isolate the lipase producing bacterial strains from collected samples. Tributyrin agar base medium was used for screening lipase producing bacteria. Medium (Liquid) for lipase production contained glucose 5gm/l, peptone 5gm/l, yeast extract 5gm/l, Olive oil 5%, $MgSO_4 7H_2O$ 0.5gm/l and NaCl 3gm/l autoclaved at 120ºC for 20 min at 15 lbs pressure.

Physical, biochemical and genetic characterization of isolate

Procedure for the characterization of isolates included Colony morphology, Gram staining, Oxidase test, Catalase test, Coagulase test, Urease test, Tyrosine decomposition, Starch hydrolysis, Sporulation test, Methyl red test, Citrate utilization, Nitrate reduction, Voges-Proskauer (V.P.) test, Indole test, Motility test, Growth in different temperatures, Growth in different pH and Acid release from sugars method.

16S rRNA gene sequencing: Overnight culture was inoculated into LB Broth (g/L: trypton, 10g, yeast extract 5g and Nacl5gpH 7.0), in shaker incubator at 37°C for 24 hours. DNA was isolated using Phenol/Chloroform method (Sambrook et al, 1989). A set of universal primer was used to amplify 16S rRNA gene. The sequence for 16S rRNA gene of the isolate was submitted in Gene Bank.

Lipase activity assay

For assaying lipase Olive oil was used as a substrate using standard titrimetric method. Olive oil (10%v/v) was emulsified with gum Arabic (5%w/v) in 5 ml of phosphate buffer PH 7.0 and 2.0 ml of 0.6% $CaCl_2$ solution. During emulsion 500µl aliquot was added to it. Rotary shaker was used to incubate the substrate mixture at 150 rpm and 30°C for 15 minutes. After incubation process, 3ml of acetone: ethanl (1:1) solution was used for termination of reaction and withdrawal of fatty acids. For estimation of fatty acid liberation Phenophathelin was used as an indicator during titration of fatty acids with 0.1NaOH (at pH10.5). One unit of enzyme is defined as the amount of enzyme required to hydrolyze 1 µmol of fatty acids from triglycerides under the assay conditions.

Purification of lipase enzyme Crude lipase enzyme preparation

Culture broth of *Bacillus subtilis* was centrifuged for 20min at 10,000 rpm in order to obtain culture supernatant which contains crude lipase enzymes. Using set methods the lipase assay and protein estimation was done.

Ammonium sulfate precipitation

For ammonium sulfate precipitation solid ammonium sulfate was added to supernatant at 20% saturation and left for 4 hours. The process of centrifugation was used to separate the precipitate. The process was performed again with 40% saturation. The precipitate was then again refined using 60% and 80% saturation in supernatant. The finalized precipitates were added to small amount de-ionized water. All the concentrated fractions were subjected to protein and enzyme activity assay to choose the fraction containing maximum activity.

Desalting via Dialysis

Protein solution was put into a dialysis tube(50 with diameter of 14.3 mm, flat width of 24.26 mm, and capacity 1 mL/cm), tightened at the ends and it was placed in a large amount of de-ionized water and stirred for 16 to 24 hours. After repetition of equilibrium process several times the level of salt concentration was reached to minimum level. This dialyzed sample was assayed and subjected to column chromatography.

Column chromatography

The dialyzed solution containing both soluble and insoluble material was put together on cellulose powder column (2.5 × 15 cm) equilibrated with 10 mM phosphate buffer, pH 7.5. The column was washed with four bed volumes of the same buffer. The sample was eluted with a linear NaCl gradient (2 L), from 0 to 0.8M, in the same buffer. The flow rate was adjusted to 0.5 mL/min (fraction volume: 5 mL). The lipase assay and protein estimation was performed to each fraction. The lipase rich fractions were pooled and stored at 4°C.

Determination of molecular weight by SDS PAGE

Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis (SDS PAGE) was carried out using Thermo Scientific Electrophoreses device to determine the molecular weight and purity of the lipase purified from column (Laemmli, 1980).

For 5ml resolving gels (12%) polyacrylamide gel,2 ml of 30 % acrylamide mixture, 1.67 ml of water, 50 µl of 10 % SDS, 50 µl of 10 % ammonium per sulfate, 1.25 ml of 1.5 M Tris-HCl (pH 8.8) and lastly 5 µl of TEMED was mixed. The process of polymerization started instantly after adding TEMED in mixture. The mixture was poured between thin gaps of two glass plates (2/3 length) and allowed to polymerize for 10 minutes. The gel was covered by distilled water to avoid drying.

For 5ml stacking gels (4%), 665µl of 30 % acrylamide mixture,2.98 ml of water, 50 µl of 10 % SDS, 1.25 ml of 0.5 M Tris-HCl (pH 6.8), 50 µl of 10 % ammonium per sulfate and 5 µl of TEMED was mixed. It was poured over the polymerized resolving gel and comb was inserted to prepare wells for sample loading.

The prepared gel was placed in a buffer tank and the tank was filled upto the filling line level with 1X Tris-Glycine-SDS (TGS) running buffer. The sample was mixed with sample buffer (SDS reducing buffer) in the ratio of 1:4(v/v). Then sample was heated at 95^{o}C in water bath for 4 minutes and placed immediately in the ice.. Electrophoresis gel was run at constant voltage at 65 volts for 30 minutes and followed by constant voltage at 100 volts for 2 hours until the blue dye reached the bottom of the gel. After electrophoresis finished, the gel was taken out from two glass plates with the help of the distilled water and placed into the shallow staining tank. Coomassie colloidal blue staining solution applied on gel for 20 minutes with slow shaking. After staining process, the gel was washed with water and then treated with neutralization buffer (0.1 M of Tris-phosphate, pH 6.5) for three minutes. Then destaining solution (25 %, v/v, methanol solution) applied on gel less than one minutes. Lastly, gel was place on fixation solution (20 %, w/v, of ammonium sulfate) for one day. After each process, gel was washed with water.

Characterization of Enzyme

Effect of pH on the activity and stability of lipase

Various levels of pH value were attempted to study the lipase activity ranges from 3.5- 9.0 using the following buffer solutions, 0.1M citrate phosphate buffer (pH 3.5 - 6.0), 0.1 M sodium phosphate buffer (pH6.5 - 7.5), 0.1M Tris-HCl buffer (pH 8.0 - 8.5) and 0.1 M carbonate bicarbonate buffer (pH 9.0) with olive oil as substrate. For checking the effect of pH on lipase activity the lipase was incubated in desired buffer for 20, 40, 50, 60, 70 80 and 90 minutes respectively. The lipase activity was assayed after incubation period using 100µL aliquots of the buffered enzymes.

Effect of temperature on the activity and stability of lipase

Different range of temperature was used to observe the lipase activity with olive oil as substrate. The temperature range was started from 20°C and increase with a difference of 10°C until it reached 90°C. The lipase was incubated on above mentioned temperatures for 30 minutes and instantly cooled after heating. The lipase activity was then assayed.

Determination of substrate specificities

Triacylglycerol substrate effect on activity of crude lipase was measured at pH value of 7.0 and 40°C. For this purpose different oils such as castor oil, almond oil, sunflower oil and coconut oil were used. Emulsification of triole in was made in gum Arabic solutions just before use as mentioned by Abdelkafi *et al*. (2009).

RESULTS

ISOLATION AND SCREENING OF LIPOLYTIC BACTERIAL STRAINS

Screening of Lipase producing Bacterial Isolates

Samples collected from different sources were examined after serial dilutions. Lipase producing strains were isolated on tributyrin agar plates on the basis of lipolytic activity, 13 bacterial isolates had potential for lipase production were screened for their quality of lipase production using different qualitative and quantitative methods of screening . The isolate PCSIRNL-39;showed clear zone of hydrolysis on tributyrin agar (Table 1) and maximum lipase production in the production medium, was selected as best lipase producer in the study.

Identification of Selected Isolate

Identification was carried out by morphological, biochemical characterization (Table 2) and rDNA sequencing. It was identified as *Bacillus subtilis* on the basis of the sequence homology with the existing Gen Bank sequences. Sequence for the distinct rDNA fragment was submitted to Gen Bank and accession number assigned is KT374117.

PURIFICATION OF EXTRACELLULAR LIPASES

Purification of lipase produced from *Bacillus subtilis*

The extracellular lipase of PCSIRNL-39 was purified employing ammonium sulfate precipitation method followed by dialysis and ion exchange chromatography.

Ammonium Sulfate Precipitation

The enzyme produced after optimization of the cultural conditions was subjected to ammonium sulfate precipitation for salting out the proteins. Table 3 shows that 80% ammonium sulphate saturation of the supernatant precipitated the highest amount of lipase i.e 59.8 U/mL by *Bacillus subtilis*. It revealed 8.6% recovery yield with 3.45purification fold. Ammonium sulfate precipitation was followed by ion exchange chromatography.

Ion Exchange Chromatography and SDS-PAGE

Desalted enzyme was subjected to DEAE-cellulose column for ion exchange chromatography. Out of total fractions taken during elution procedure; fraction no. 13 contained the maximum lipase activity. The purified lipase of PCSIR-NL39 exhibited a final specific activity of 446 U/ml with 8.38 fold of purification and 1.6% recovery yield (Table 4). Low yield of purified enzyme was due to loss during ammonium sulfate precipitation. After the purification of extracellular lipase from B*acillus subtilis* its molecular weight was determined through SDS PAGE which was 41kDa (Figure 1).

CHARACTERIZATION OF LIPASE

pH Optima of Extracellular Lipases

The effect of pH on the activity of lipases was shown in Figure 2. The effect of different pH values on the lipolytic activity of enzyme was studied by incubating. After one hour incubation of the enzyme in various buffers with pH range of 4.0 to 10.0 the maximum residual activity by *Bacillus subtilis* was 174.06U/mlat pH 8.0. Enzyme activity was decreased at low and high pH extremes.

Table 1. Diameter of zones of hydrolysis by crude enzyme on tributyrin agar plate after 24 hours.

Sr. No.	Isolates #	Diameter of zone of hydrolysis (mm)
1	PCSIRNL39	1.67 ± 0.12
2	PCSIRNL40	0.6 ± 0.1
3	PCSIRNL41	0.13 ± 0.05
4	PCSIRNL42	0.27 ± 0.05
5	PCSIRNL43	0.1± 0.01
6	PCSIRNL44	0.53 ± 0.06
7	PCSIRNL45	0.13 ± 0.05
8	PCSIRNL46	0.23 ± 0.04
9	PCSIRNL47	0.6 ± 0.01
10	PCSIRNL48	0.6 ± 0.17
11	PCSIRNL49	0.7 ± 0
12	PCSIRNL50	0.33 ± 0.02
13	PCSIRNL51	0.35 ± 0.01

Temperature Optima of Extracellular Lipases

Figure 3 showed the effect of temperature on the activity of purified lipases was observed by incubating the enzyme at different temperatures *i.e.,* from 10-90°C for one hour. The results showed that lipases retained 90% of its activity at 70^{o}C by *Bacillus subtilis*.

Effect of Metal Ions on the Activity

The residual activities were determined after incubation of the purified lipases with 1mM and 10mM of different monovalent and divalent metal ions. Figure 4 and 5 showed that sodium, calcium, potassium and magnesium ions had enhanced the lipase activity while for magnesium ion increase in ion concentration decreased the activity. Manganese, zinc, iron, cobalt, copper ions had adverse effect on the enzyme activity.

Effect of substrate on enzymatic activity

Different substrates i.e. olive oil, mustard, almond, sunflower, castor and coconut oils were used for enzyme assay. Lipases obtained from *Bacillus subtilis* showed maximum activity at almond oil and less active at coconut oil (Figure 6).

Table 2. Morphological and biochemical characterization of bacterial isolate PCSIRNL-39.

Characteristics	**PCSIR-NL39**
Morphological	
Shape	Rods (Chain)
Gram Stain	G+ve
Motility	Motile
Spore formation	+ve
Growth	
Growth Temp.	15-50^{o}C
Growth pH	6-10
Biochemical Tests	
Oxidase	+ve
Catalase	+ve
VP	+ve
Indol test	-ve
Methyl red	-ve
Nitrate Reduction	+ve
Hydrolysis	
Casein	+ve
Starch	+ve
Gelatin	+ve
Growth in carbohydrates	
Glucose	+ve
Maltose	+ve
Sucrose	-ve
Fructose	-ve
Manitol	-ve

Table 3. Purification of lipase produced from *Bacillus subtilis* at different concentration of Ammonium Sulfate.

Ammonium Sulfate Saturation	**Fraction activity (U/ml)**	**Total activity (U)**	**Fraction dissolved protein (mg/ml)**	**Total protein**	**Specific activity (U/mg)**
0%	66.5	3990	1.25	75	53.2
20%	8.49	466.9	0.19	10.45	44.6
40%	45.7	2285	1.05	52.5	43.52
60%	50	2250	0.528	23.75	94.6
70%	23.3	815.5	0.5	17.5	46.6
80%	59.8	1196	0.325	6.5	184

Table 4. Purification of extracellular lipase from *Bacillus subtilis.*

Purification Step	Total activity (U)	Total dissolved protein (mg)	Specific activity (U/mg)	Recovery (%)	Purification (fold)
Cell free extract	3990	75	53.2	100%	1.0
Ammonium Sulfate	1196	27.6.56	184	8.6%	3.45
Dialysis	905	18.24.3	210	5.7%	3.4
Chromatography	536	11.51.2	446	1.6%	8.38

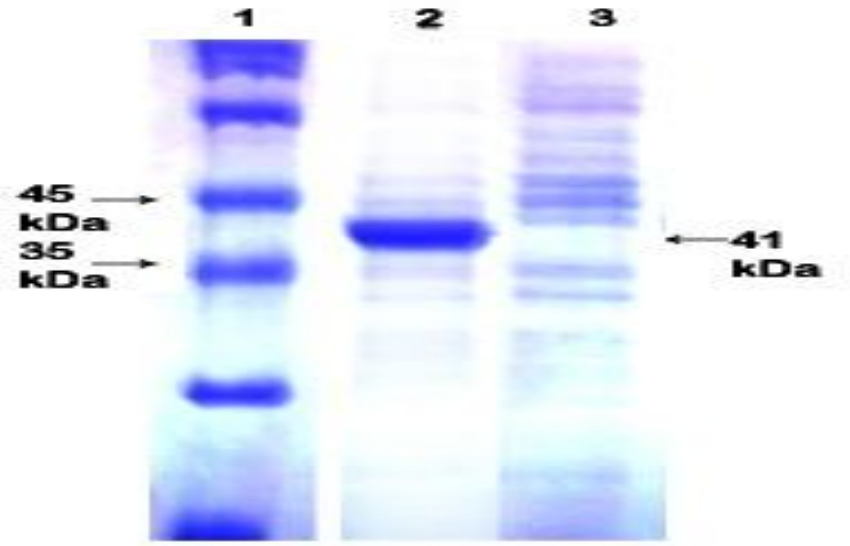

Figure 1. SDS PAGE showed molecular weight.

Lane 1. Molecular marker (fermentas), Lane 2; Purified Lipase, Lane 3; Lipase after ammonium sulfate precipitation.

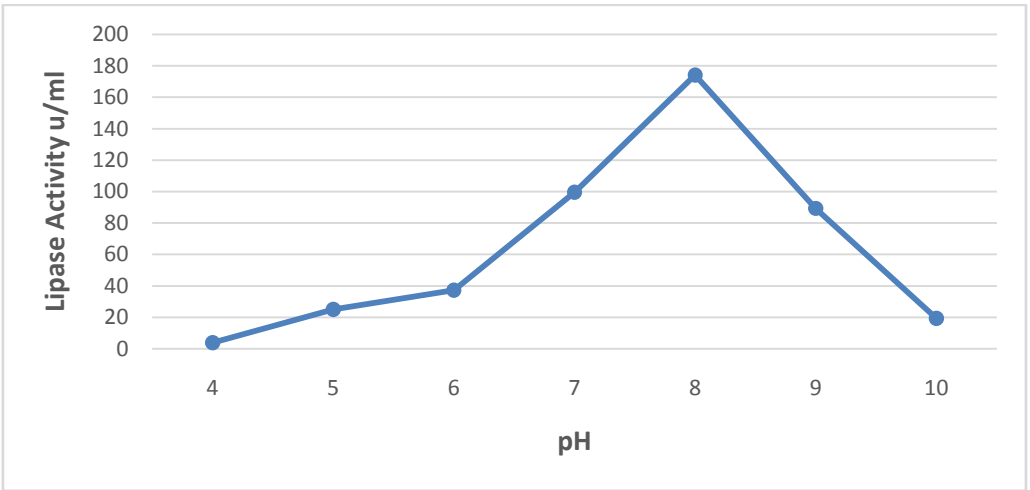

Figure 2. Lipase activity at different values of pH.

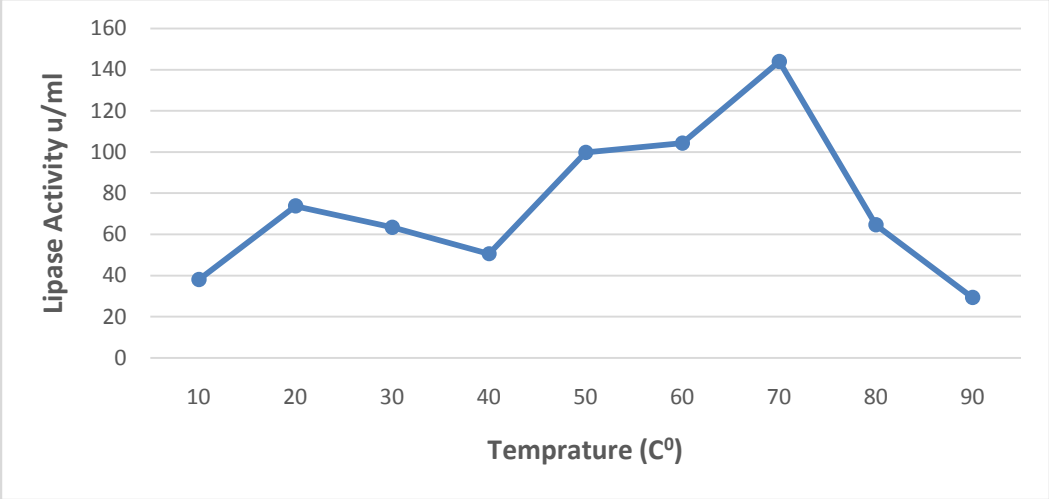

Figure 3. Lipase activity at different temperatures.

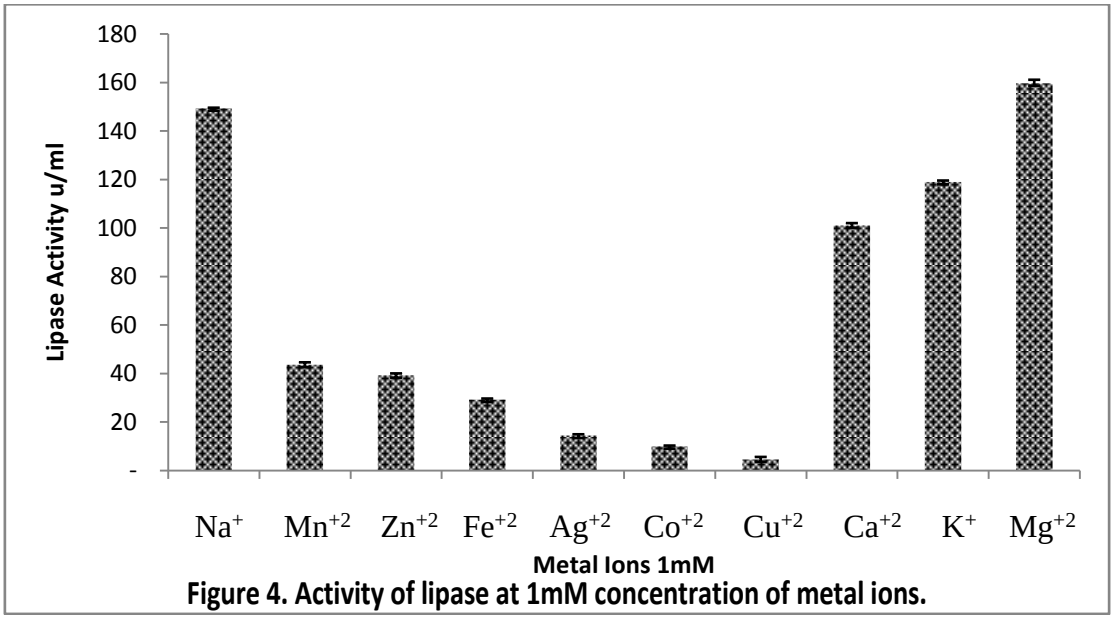

Figure 4. Activity of lipase at 1mM concentration of metal ions.

DISCUSSION

The current trend in lipase research is to focus on microbial lipases rather than lipases derived from animals and plants, as lipases from micro-organisms have the advantages including the ability to catalyse diverse reactions, produce high yields, and reduced production costs. In present study the lipase produced by *Bacillus subtilis* had maximum activity at pH 8. In support these results, many scientists reported the neutral pH lipases (Lee, *et al.,* 1999) or alkaline optimum pH (Kanwar and Goswami, 2002; Sunna, *et al.,* 2002).

Bacterial lipases are stable at wide range of temperature (Sugihara *et al*., 1991& Lesuisse *et al.*, 1993). In case of present experiments the lipases produced from *Bacillus subtilis* showed maximum residual activity at 70°C while lipase from *Bacillus* strain did not completely denatured even at 90°C. Almond oil as substrate showed high percentage activity whereas very little activity was shown in coconut oil substrate.

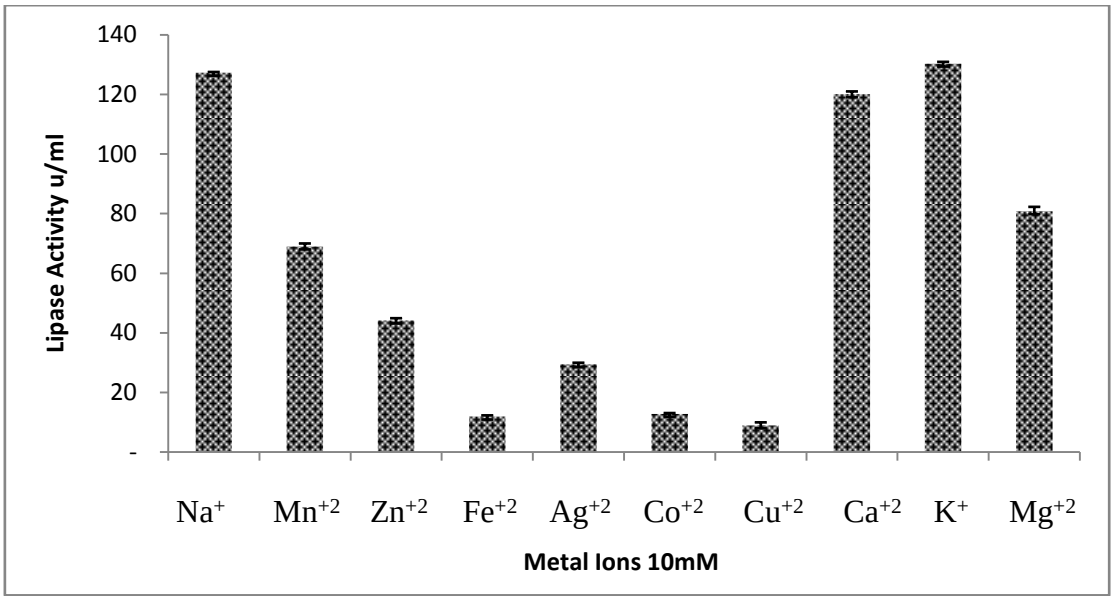

Figure 5. Activity of lipase at 10mM concentration of metal ions.

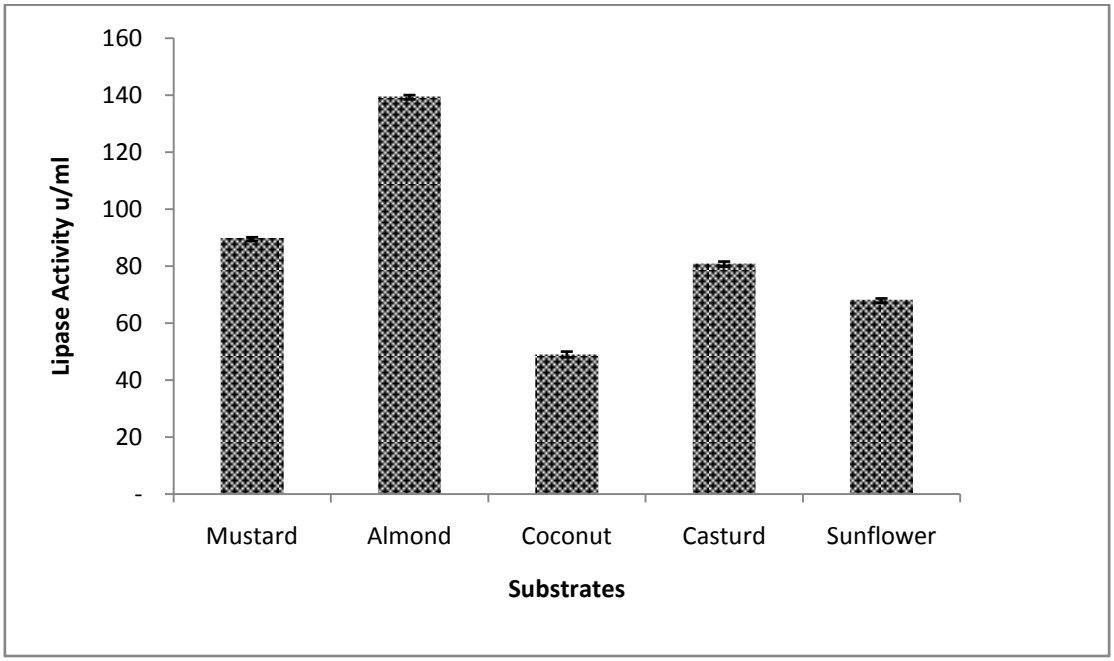

Figure 6. Effect of various substrates on lipase activity.

Industrialists and scientists are taking keen interest in lipases due to their enantio specific and chemo regio behavior. For final application of lipase in industries such as pharmaceuticals, cosmetics, food and chemical industries certain degree of purity is required. Various strategies used for the purification of lipase have been reported (Antonian, 1988; Taipa *et al*., 1992; Aires-Barros *et al*., 2003) showed clearly the optimal purification scheme for various microbial lipases.

In this observation the lipases produced by *Bacillus subtilis* was purified by ammonium sulfate precipitation proceeded by column chromatography yielded 446U/ml specific activity at 8.38 fold protein purification that were in accordance with the studies of Iftikhar *et al*., 2011. After purification the SDS-PAGE analysis showed the molecular weight of purified protein obtained from *Bacillus subtilis* was 41kDa.

CONCLUSION

Extracellular lipase producers were found from local environment. Bacterial strains, isolated from waste had potential of extracellular lipase production. Morphological, biochemical and genetic characterization of isolate PCSIRNL-39showed similarities with *Bacillus subtilis.* Extracellular lipases were produced under the optimum conditions. These were purified by ammonium sulfate precipitation, dialysis and column chromatography and its molecular weight was determined by SDS PAGE. Temperature, pH, and metal ions showed different effects on lipases activity. Lipases produced from *Bacillus subtilis* can be used as biodetergent and for bioremediation of waste water.

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