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Production of Lipase By using *Pseudomonas* sp. L1 Isolated from Alkaline Meteorite Crater Lake Lonar, India

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Production of lipase in nutrient broth pH 9.5 was studied at laboratory level using *Pseudomonas* sp. L1 isolated from Lonar meteorite crater lake. The *Pseudomonas* sp. L1 was confirmed by partial 16s r-RNA sequencing & by growth on *Pseudomonas* isolation agar containing triclosan and by using biochemical tests according to Bergeys manual of Systematic Bacteriology. The confirmation of lipase production was done by clear zone on Tween 80 medium and Egg yolk medium. Production of lipase was carried out using different oils like olive oil, coconut oil, groundnut oil, soyabean oil, Sunflower oil, corn oil and effects of different Physical & chemical factors like incubation period, pH, temperature, metal ions, different organic solvents on activity of lipase was studied. The maximum production was obtained at 27°C and at pH 9.5 & the enzyme showed maximum of 28 U/ml/min activity at temperature 40ºC, 43 U/ml/min activity at pH 8.5, 47 U/ml/min activity in Na+(1mM NaCl) & 32 U/ml/min activity in organic solvent methanol. The enzyme was partially purified by organic solvent precipitation using different (v/v) concentrations of acetone. The activity was checked spectrophotometrically by using ρ-Nitrophenyl acetate ester. 34 U/ml/min activity was observed in 60% (v/v) acetone precipitate & it was further purified by using DEAE Cellulose column affinity chromatography with retention time of 1ml/4min. in which fraction 21 showed maximum activity of 25 U/ml/min.

Key words: *Pseudomonas* sp., Lonar lake, Lipase, Alkaliphilic, DEAE Cellulose.

Microbes are ubiquitus in nature on earth. *Pseudomonas* species are one of the bacterial species found on earth. The habitats for growth of *Pseudomonas* sp. include normal soil, water, air. But apart from these, *Pseudomonas* sp. are also inhabitant of some extreme environments on earth[3] like hypersaline lakes[3,10] dead sea & volcanic acid lakes. One of these extreme habitats is Lonar crater soda lake. The pH of the Lonar soda lake is alkaline having approximate range in between pH 9- 14. The pH is higher during summer season. These *Pseudomonas* sp. are capable of producing different industrially important enzymes. Lipases (E.C.3.1.1.3) are one of them that are currently used in different industries like detergent industries, leather industries, chemical industries, pharmaceutical industries, etc. If the microbial species is isolated from extreme environment the enzymes produced by that species may be more stable & may remain active at different reaction stages of varying physiological conditions during industrial processes[7].

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MATERIALS AND METHODS

Sample collection

The soil & water samples are collected from Lonar crater lake[9,10]. The soil samples are collected in sterile plastic zipper (polyethylene) bags by digging the Lake shore 5-10 cm deep from different sites around the lake & rhizospheric soil of some inhabitant plants also collected with sterile

spatula[5]. The pH & temperature of the lake water was recorded in March 28, 2011. The pH was 9.5 and temperature was 27°C. Water sample I & II are collected in sterile water Sampling bottles from different sites around the shore. Both soil & water samples are kept in an icepack cabinet maintained at temperature below 10°C.

Media used

The different media used for isolation & identification were Nutrient broth & agar, Horikoshi media I & II broth & agar[3] , cetrimide broth & agar and selective media include *Pseudomonas* isolation agar base, *Pseudomonas* agar for fluorescein, some of which compositions & specifications are given below:

Horikoshi I & II media[3] (For isolation of alkaliphilic microbes).

Isolation of Bacterial species

The collected water samples were added in Nutrient broth of pH 7, 9.5 & 11 within 6 hrs. of sampling & enriched by incubating them at optimum temperature for 18 to 24 hrs in a rotary Shaking incubator at 120 rpm at 27°C at Microbiology lab, Dept. of Microbiology, Yeshwant College, Nanded, Maharashtra, India. After incubation the enriched mixed culture from water samples were streaked on to Nutrient agar plates, Cetrimide agar plates, *Pseudomonas* isolation agar plates of pH 7 , 9.5 & 11 , Horikoshi I & II agar plates and incubated at optimum temperature for 18 to 24 hrs. The isolated colonies observed after incubation & colony characters were recorded were then sub cultured on to respective media slants of respective pH. The soil samples from lake shore & from rhizosphere of some inhabitant plants were serially diluted by using sterile D/W & higher dilutions were spreaded on to Nutrient agar plates, Cetrimide agar plates, *Pseudomonas* isolation agar plates of pH 7 , 9.5 & 11 , Horikoshi I & II agar plates and incubated at optimum temperature for 18 to 24 hrs. The isolated colonies observed after incubation & colony characters were recorded were then sub cultured on to respective media slants of respective pH.

Identification of *Pseudomonas* species

The *Pseudomonas* species were identified by performing different biochemical tests on the isolated cultures & were confirmed by using Bergeys manual of determinative bacteriology[1] (5th edition).

Screening of Selected Cultures for Production of Lipase on Tween-80 & Egg yolk agar medium

After recording biochemical test results most of the cultures were identified as *Pseudomonas* species. Three cultures were showing highest zone of Tween 80 hydrolysis[2] & highest zone on Egg yolk medium respectively. These were chosen & one out of these that was no. 23 culture (PSL1) was again confirmed by 16s RNA sequencing and bioinformatic analysis[9] as *Pseudomonas* species L1. The 16s sequence was submitted to NCBI Gen bank having accession number JX292018.

Production of Lipase

The production of lipase[8] was studied using *Pseudomonas* sp. L1 in nutrient broth containing 1% sodium carbonate, 2% culture inoculum (prepared in same medium one day before), 0.2% Triton X 100, 0.4% oil, 1% soya meal, 1.5% peptone, 0.5% yeast extract, 0.3% potassium dihydrogen phosphate, 0.04% magnesium sulphate seven hydrated using olive oil as substrate. The temperature & pH was adjusted at 27°C & 9.5 respectively kept in orbital shaking incubator at 120 rpm with 100ml volume in 250ml flasks.

The O.D. of production media flasks was taken at A^{600} (McFarland standards) by setting respective blank using Shimadzu 1601 U.V. -Visible spectrophotometer in quartz cuvettes after every 30 minutes during first day for 5 hrs. for determination of generation time of culture in production medium. It was found to be ~2 hr.30 min. The enzyme activity was checked every three hour by removing 5ml of production media and centrifuging it at 6000 rpm in cooling centrifuge at 5°C for 20 min. at A^{410} using Á-Nitro phenyl acetate ester of both pellet (sonicated) & cell free supernatant. Activity was found more in supernatant, this clears that the enzyme produced was extra cellular one.

Assay of Lipase

Assay of produced lipase was carried out by slightly modified combination of Huggins & Lapides[4] (1947) and Winkler & Stuckman method[12] (1989) using Á-Nitro phenyl acetate ester. The assay substrate solution A was prepared by dissolving 63 mg of Á-Nitro phenyl acetate (Himedia) in 10 ml of Isopropanol (Qualigens) can be stored in refrigerator for 1 to 4 days. Solution B was 90 ml of double distilled water containing 0.4 gm

of Triton X 100, 0.1 gm of gum arabic. The complete substrate solution for assay was prepared by addition of 2 ml of solution A to 18 ml of solution B. The assay mixture contained 0.2 ml of cell free supernatant as crude enzyme, 1 ml of 50 mM Tris-HCl of pH 8.5 & 2 ml of complete substrate with incubation time 10 min. for each assay sample. In blank the crude enzyme is replaced with double distilled water. The reaction was stopped using 0.4 ml of 1:1 solution of Acetone & isopropanol. The enzyme causes release of ρ-nitro phenol as product which can be read at A^{410} against respective blank using spectrophotometer. The assay was carried out and repeated thrice for each step and the mean values are shown.

One unit of enzyme activity was expressed as the amount of enzyme which released 1 nanomole of ρ-nitro phenol / ml / minute under the experimental conditions. For this std. solutions of commercially available ρ-nitro phenol (Qualigen) ranging from 0.000001M to 0.000020 M were prepared and graphs were plotted by taking O.D. at A^{410}.

Purification of Lipase

Filtration

Purification[8] of lipase was carried out step by step and repeated thrice. The first step was filtration at 5°C inside a cooling incubator using whatmann paper no. 41 & activity of crude lipase was checked after each step.

Centrifugation

The filtrate from first step was centrifuged in cooling centrifuge (Remi) at 5ºC at 6000 rpm for 20 min. to get cell free supernatant. To this cell free supernatant $CaCl_2$ was added[8] giving final concentration of 5 mM. This solution was kept at 5ºC in cooling incubator (Remi) till preparation of next step.

Organic solvent precipitation

The purification was carried out by precipitation using different (v/v) acetone concentrations[8]. The precipitates were collected separately by centrifugation at 5ºC at 5000 rpm and were dissolved in minimal amount of 50 mM Tris-HCl buffer of pH 8.5 in separate tubes and stored at 5ºC in cooling incubator. The acetone[8] concentration 60 % (v/v) gave highest activity 34 U/ml/min.

Affinity DEAE Cellulose column chromatography

The next step was affinity column chromatography[6] using DEAE Cellulose column. The resin DEAE Cellulose (anion exchanger) for column chromatography (Himedia) 5 gm was slowly added to 0.1M NaOH 250 ml with gentle stirring for 30 min. till pH reached to 13, then NaOH was discarded and resin washed with double distilled water till pH reached 8. Then the solution is replaced with 0.1 M HCl 250 ml gentle stirring for 30 min. till pH reached 1. The resin was then washed with double distilled water till pH reached 3.The distilled water is then discarded and to the resin 10X buffer 500 mM Tris-HCl pH 8.5 was added with gentle stirring for 30 min. till pH reached 8.5. Then the 10X buffer was discarded & the resin is equilibrated with 50mM Tris-HCl pH 8.5 and degassed before the DEAE Cellulose was transferred to glass column 30 × 1cm in size. The resin was transferred to column to reach 28 cm height, then equilibrated with 50mM Tris-HCl pH 8.5 at 5ºC. The flow rate of the column was adjusted to 1 ml/ 4 min. 1 ml of crude lipase solution obtained after dialysis containing ~5 mg of protein were applied to the DEAE Cellulose column. The enzyme was eluted with 100 ml of 50mM Tris-HCl pH 8.5 and followed with 100 ml of linear gradient of 0.1 M NaCl in 50mM Tris-HCl pH 8.5. The fractions were collected in 1 ml volume & checked for activity at A^{410} also monitored at A^{280} for total protein using Shimadzu U. V.- Visible spectrophotometer 1601. The fraction no. 21showed highest acivity 25 U/ml/min.

RESULTS AND DISCUSSION

The meteorite crater lake is situated at Lonar, Dist. Buldhana, Maharashtra state, India having coordinates 19.9767º N, 76.5083º E having mean depth of ~150 meters. Thirty seven *Pseudomonas* sp. cultures were isolated out of which four showed highest lipase production. One out of these is 16 s sequenced and confirmed as *Pseudomonas aeruginosa* Sp.L1. Effects of various factors on production & activity of lipase

Table 1. Effect of incubation period

S. No.	Day/ Hrs. of Incubation	Lipase activity (U/ml/min) at A^{410}
1	First day (24 hrs)	32
2	Second day (48 hrs)	08
3	Third day (72 hrs)	02
4	Fourth day (96 hrs)	0.1
5	Fifth day (120 hrs)	-01

by *Pseudomonas aeruginosa* Sp.L1 was studied. The values mentioned are mean values of each and every step repeated thrice.

Effect of Incubation period on production and activity of lipase

The effect of incubation period on production of lipase was studied for 5 days at an interval of 24 hrs. The substrate used in media was olive oil 2% (v/v) at pH 9.5. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation. Every day 5ml of production media was

Table 2. Effect of pH

Lipase activity (U/ml/min) at A^{410}						
at pH 2	at pH 4	at pH 6	at pH 7	at pH 8.5	at pH 9	at pH 10.5
9	15	17	22	43	23	11

Table 3. Effect of temperature

Lipase activity (U/ml/min) at A^{410}						
at 5ºC	at 10ºC	at 20ºC	at 30ºC	at 40ºC	at 50ºC	at 60ºC
12	19	22	25	28	20	01

Table 4. Effect of different substrates

S.	Oil	Lipase activity (U/ml/min) at A^{410}				
No.	Substrate	Day 1(24 hrs)	Day 2 (48 hrs)	Day 3 (72 hrs)	Day 4 (96 hrs)	Day 5 (120 hrs)
1	Olive oil	32	08	02	0.1	- 01
2	Sunflower oil	23	08	0.9	- 01	-02
3	Soya bean oil	28	09	01	- 01	- 03
4	Corn oil	27	07	0.7	- 02	- 06
5	Coconut oil	26	06	0.1	- 01	- 05
6	Ground nut oil	25	10	0.1	- 04	- 07

Table 5. Effect of carbon sources

S.	Carbon	Lipase activity (U/ml/min) at A^{410}				
No.	sources	Day 1(24 hrs)	Day 2 (48 hrs)	Day 3 (72 hrs)	Day 4 (96 hrs)	Day 5 (120 hrs)
1	Cellulose	19	17	08	02	01
2	Starch	30	20	03	07	03
3	β-Cyclodextrin	20	20	11	05	02
4	Sucrose	29	22	10	03	01
5	Lactose	01	37	48	44	32
6	Fructose	35	46	46	40	37
7	Maltose	54	29	06	22	07
8	Mannitol	03	46	41	34	27
9	Glucose	30	58	06	02	04
10	D-Galactose	27	19	16	11	06
11	D(+) Raffinose	31	24	12	07	05
12	D(-) Arabinose	27	21	19	13	11

removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme by taking O.D. at A^{410}. Maximum activity was observed as 32 U/ml/min during first 24 hrs.

The values obtained were as shown in graph

Effect of pH on activity of lipase

Effect of pH was studied on production and activity of lipase by inoculating *Pseudomonas aeruginosa* Sp.L1 to the same media with olive oil 2% (v/v) of pH 9.5 used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for 24 hrs. After 24 hrs. 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme and incubating with buffer of different pH as 2, 4, 6, 7, 8.5, 9, 10.5 by taking O.D. at A^{410} read against respective blank for each pH. Maximum activity was observed as 43 U/ml/min at pH 8.5 as shown in graph.

Effect of Temperature on activity of lipase

Effect of temperature was studied on activity of lipase. *Pseudomonas aeruginosa* Sp.L1 was inoculated to the same media containing olive oil in 2% (v/v) concentration at pH 9.5. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for 24 hrs. After 24 hrs 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min assay was carried out using the cell free supernatant as crude enzyme and incubating the assay mixture at different temperatures as at 5ºC ,10ºC, 20ºC, 30ºC, 40ºC, 50ºC, 60°C for 15 min. & by taking O.D. at A^{410} read against respective blank for each temperature. Maximum activity was observed as 28 U/ml/min at 40ºC temperature as shown in graph.

Effect of different oil substrates on production and activity of lipase

Effect of different substrate oils was studied on production and activity of lipase by inoculating *Pseudomonas aeruginosa* Sp.L1 to the same media without soya meal at pH 9.5 but with different oils as olive oil, soyabean oil, sunflower oil, ground nut oil, coconut oil, corn oil

Table 6. Effect of nitrogen sources

S. No.	Nitrogen sources	Lipase activity (U/ml/min) at A^{410}				
		Day 1(24 hrs)	Day 2 (48 hrs)	Day 3 (72 hrs)	Day 4 (96 hrs)	Day 5 (120 hrs)
1	Peptone	11	10	20	01	01
2	Soya bean meal	03	07	21	11	12
3	Ammonium nitrate	14	05	16	07	07
4	Ammonium sulphate	21	22	13	13	04
5	Ammonium oxalate	30	25	22	16	04

Table 7. Effect of metal ions

Lipase activity (U/ml/min) at A^{410}						
inBa^{++}	ink^{+}	in Mg^{++}	inCa^{++}	inZn^{++}	in Mn^{++}	inNa^{+}
02	15	16	11	02	10	47

Table 8. Effect of organic solvents

Lipase activity (U/ml/min) at A^{410}							
in Benzene	in Isopropanol	in Methanol	in Isooctane	in Acetone	in Diethyl ether	in Toluene	in Xylene
12	02	32	07	- 02	11	04	11

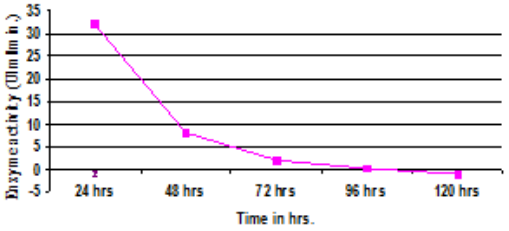

Fig. 1. Effect of incubation period on production and acitivity of lipase

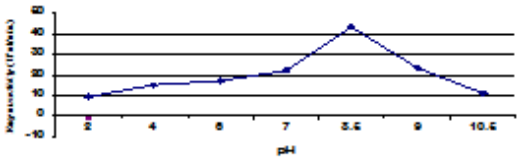

Fig. 2. Effect of pH on activity of lipase

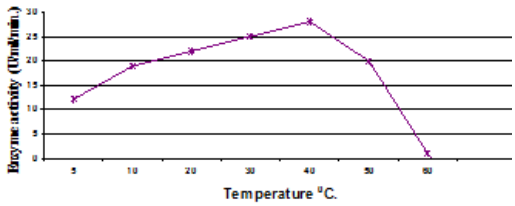

Fig. 3. Effect of temperature on activity of lipase

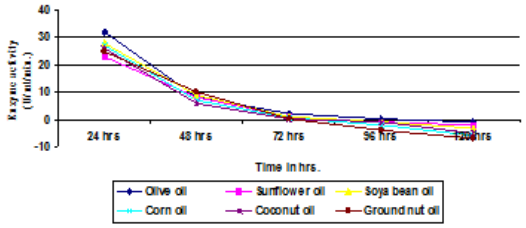

Fig. 4. Effect of different susbtrates on production and activity of lipase

in 2% (v/v) concentration. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for five days at an interval of 24 hrs. Every day 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme by taking O.D. at A^{410} read against blank. Maximum activity was observed as 32 U/ml after 24 hrs when olive oil is used as substrate as shown in graph.

Effect of different carbon sources on production and activity of lipase

Effect of different carbon sources was studied on production and activity of lipase by inoculating *Pseudomonas aeruginosa* Sp.L1 to the same media with olive oil 2% (v/v) concentration & different carbon sources in 1 % (w/v) concentration as cellulose, starch, β - cyclodextrin, sucrose, D- galactose, D(+) raffinose, D(-) arabinose, maltose, glucose, fructose, lactose & mannitol at pH 9.5. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for five days at an interval of 24 hrs. Every day 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme by taking O.D. at A^{410} read against a blank. Maximum activity was observed as 58 U/ml/min after 48 hrs when glucose was used as carbon source as shown in graph.

Effect of different nitrogen sources on production and activity of lipase

Effect of different nitrogen sources was studied on production and activity of lipase by inoculating *Pseudomonas aeruginosa* Sp.L1 to the same media only with single nitrogen source in each flask with olive oil 2% (v/v) concentration. The nitrogen sources used in 1 % (w/v) concentration as peptone, soya bean meal, ammonium nitrate, ammonium sulphate & ammonium oxalate at pH 9.5. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for five days at an interval of 24 hrs. Every day 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme by taking O.D. at A^{410} read against a blank. Maximum activity was observed as 30 U/ml/min after 24 hrs when ammonium oxalate was used as nitrogen source as shown in graph.

Effect of Metal ions on activity of lipase

Effect of various metal ions was studied on activity of lipase. *Pseudomonas aeruginosa* Sp.L1 was inoculated to the same media containing olive oil in 2% (v/v) concentration at pH 9.5. The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for 24 hrs. After 24 hrs. 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme and incubating the assay mixture with different metal ions like Ba^{++}, K^{+}, Mg^{++}, Ca^{++}, Zn^{++}, Mn^{++}, Na^{+} as $BaCl_2$, KCl, $MgCl_2$, $CaCl_2$, $ZnCl_2$, $MnCl_2$, NaCl at a final concentration of 1mM for 15 min. & by taking O.D. at A^{410} read against blank without metal ion. Maximum activity was observed as 47 U/ml/min in Na^{+} metal ion as shown in graph.

Effect of Organic solvents on activity of lipase

Effect of various organic solvents was studied on activity of lipase. *Pseudomonas aeruginosa* Sp.L1 was inoculated to the same media containing olive oil in 2% (v/v) concentration at pH 9.5.The production media used in 100 ml volume in 250 ml flask kept in rotary shaking incubator at 27ºC and at 120 rpm for incubation for 24 hrs. After 24 hrs. 5ml of production media was removed, filtered through Whatmann paper no. 41 and then centrifuged at 6000 rpm in cooling centrifuge at 5ºC for 20 min. assay was carried out using the cell free supernatant as crude enzyme and incubating the assay mixture with different organic solvents like benzene, isopropanol, methanol, isooctane, acetone, diethyl ether, toluene & xylene at a final concentration of 50% (v/v) for 15 min. & by taking O.D. at A^{410} read against blank without organic solvent. Maximum activity was observed as 32 U/ml/min in methanol as organic solvent as shown in graph.

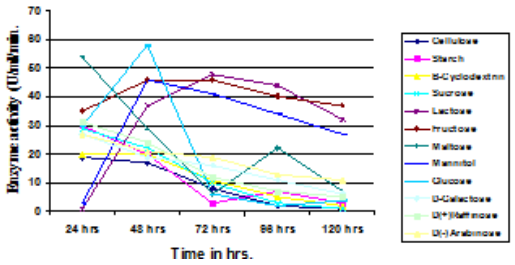

Fig. 5. Effect of different carbon sources on production and activity of lipase

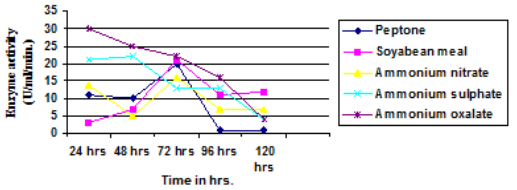

Fig. 6. Effect of different nitrogen sources on production and activity of lipase

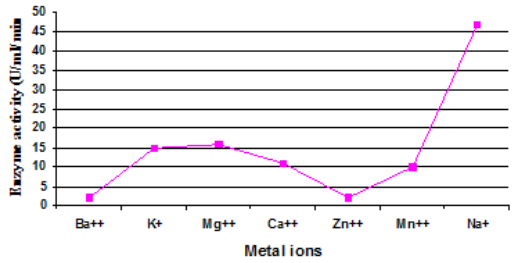

Fig. 7. Effect of different metals ions on activity of lipase

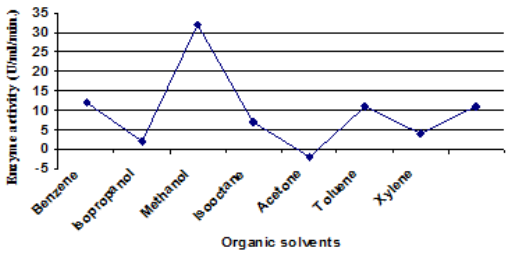

Fig. 8. Effect of different organic solvents on activity of lipase

CONCLUSION

From the results it can be concluded that the lipase produced by *Pseudomonas aeruginosa* Sp.L1 showed maximum production and activity of 32U/ml/min after incubation of 24 hrs. With 2%(v/v) olive oil, showed high level of activity 43 U/ml/min at pH 8.5, 28 U/ml/min at temperature 40ºC, 32 U/ml/min when olive oil used as substrate, 58 U/ml/min highest activity in presence of glucose as

carbon source after 48 hrs of incubation, showed highest activity of 30 U/ml/min in presence of ammonium oxalate as nitrogen source, showed 47 U/ml/min highest activity in presence of Na^+ metal ion, showed highest activity 32 U/ml/min in presence of methanol as organic solvent but acetone inhibited the activity completely in 50% (v/v) concentration.

From this it is clear that the enzyme is capable of catalysis in high alkaline pH, at temperatures up to 40ºC, presence of Na^+ metal ion, it can be concluded that it may be sodium ion dependant because the organism is isolated from the meteorite crater soda lake containing Na_2CO_3 as factor responsible for alkalinity[8] & is capable of catalysis in organic solvents specially in methanol, benzene, xylene & diethyl ether.

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