ISOLATION, OPTIMISATION AND PARTIAL PURIFICATION OF LIPASE ENZYME

LEAD AUTHOR

KALAPATAPU VISHNU PRIYA MSc, PGDEM, PGDCA, MPhil, MBA, FISCA

DOCTORAL CANDIDATE- BIOCHEMISTRY, GITAM UNIVERSITY, VISAKHAPATNAM

MOBILE PHONE # 09963880450 EMAIL ADDRESS kalapatapu.priya@gmail.com

CO-AUTHOR

PROF. I. BHASKAR REDDY MSc, PhD

HEAD OF DEPARTMENT- BIOCHEMISTRY, GITAM UNIVERSITY, VISAKHAPATNAM

MOBILE PHONE # 09440119739 EMAIL ADDRESS hod@biochemistry.gitam.edu

ABSTRACT

*Screening and isolation of lipase producing strains of bacteria was carried out from eleven different soil samples collected from various places in Andhra Pradesh and Hyderabad. The isolates were positive on tribuytrin agar media and thus are selected as lipase producing strain. The strain was identified and characterised by the microscopic and biochemical tests as bacillus.*sps*, a lipase producing organism. The optimisation of various cultural conditions was carried out by which the lipase production was enhanced with the optimal parameters being incubation period of 48 hours (24.1U/mL), palm oil as carbon source (24.3 U/ml, peptone as nitrogen source (24.5 u/ml), initial pH of 7.0 and incubation temperature of 36ºc (25.0 U/mL). The optimum agitation speed of 160 rpm produced lipase having 25.9 U/ml activity. Finally, the enzyme lipase was purified by ammonium sulphate fractionation, dialysis and column chromatography. The ammonium sulphate precipitation and dialysis showed an increased specific activity of 1.71 U/ml and 6.17 U/ml when compared to crude enzyme which showed specific activity of 0.45 U/mg. Further purification was carried out by ion-exchange chromatography using DEAE column. The purified enzyme showed higher specific activity (15.24 U/mg) with a purification fold of 33. The molecular mass of purified lipase was estimated to be approximately 40.14 kDa by SDS-PAGE. This result showed that*

Bacillus sps *under study is a good producer of lipase, which can be beneficial for industries.*

Key words: *bacillus sps,* lipase, chromatography, dialysis.

1. Introduction

Lipases catalyse the hydrolysis of triglycerols releasing fatty acids, an alternate source of energy other than carbohydrates in all organisms universally. These are mostly helpful in food and drug industry. Lipases blood serum can be used as a diagnostic tool for detecting conditions such as acute pancreatitis and pancreatic injury. A relatively smaller number of bacterial lipases have been well studied if compared to plant and fungal lipases. Most of the bacterial lipases reported so far are constitutive and are nonspecific in their substrate specificity, and a few bacterial lipases are thermostable.Among bacteria, Achromobacter sp, Alcaligenes sp, Arthrobacter sp, Pseudomonas sp., Staphylococcus sp., and chromo bacterium sp have been exploited for the production of lipases. Microbial enzymes have a great number of uses in food, pharmaceutical, textile, paper, leather and other industries (Hasan, et al. 2006).

2. Material and methods

2.1 Microorganisms

Eleven different soil samples were taken for isolation of lipase producing organisms under laboratory condition. Sources are: Bakery, Automobile industry, Groundnut field, Sunflower field, Hussain Sagar effluent, Oil sediment, Diary industry oil cake, Marine sediment, Coconut oil mill, and Vegetable crop soil. The labelled samples were spread on to the isolated media and were incubated at 37 degrees centigrade for 48 hours after serial dilution of 10_1 to 10_5 times.

2.2 Isolation and screening of Lipase producing Strains

The isolated bacteria were identified based on cellular morphology, growth condition, gram staining, endospore staining, capsule staining and biochemical tests (Sneath and Halt; 1986).

Tribuytrin Media:

The media used for optimum production of lipase consisted of Ammonium sulphate 0.5,K2HPO4 -0.5%, MGSO4.7H20-0.3%, yeast extract-0.03%, CaCO- 0.05%, Olive oil -1% at ph 7.

2.3 Optimisation of various cultural conditions for enhanced production of lipase:

Selection of a suitable carbon source is done. Selection of a suitable nitrogen source, effect of ph, effect of incubation temperature, effect of incubation period, effect of agitation speed (rpm) were all taken into account. In all the studies, experiments were carried out in triplicate and the average values are presented. The optimised parameters in each step were employed in subsequent experiments. The newly isolated bacterial species isolate L-3 that produces lipase was employed in the present study. The isolate was subcultured onto nutrient agar slants and incubated at 37 degree centigrade for 24 hours. These slants were subcultured at monthly intervals and stored at 4^0c in the refrigerator.

2.4 Effect of Incubation Period on Lipase Activity:

L2 was cultured in Tribuytrin broth containing yeast extract, NaCl, peptone and 1% (w/v) olive oil at 36°C in an inorbital shaker at agitation speed of 150rpm. The culture broth was harvested at 8 hour intervals by centrifugation at 10,000 g, 30 min, 4°C. The supernatant collected was used as crude enzyme solution and was assayed for enzyme activity.

2.5 Effect of Different Oils as Carbon Source on Lipase Activity:

Olive oil present in the growth media was replaced with different oils like palm oil, ghee, coconut oil, groundnut oil, sunflower oil and mustard oil at a final concentration of 1% (w/v).

2.6 Effect of Different Nitrogen Sources on Lipase Activity:

Different nitrogen sources like yeast extract, soya bean meal, NaNO, tryptone and peptone were added to the broth at a final concentration of 1% (w/v).

2.7 Effect of Agitation Speed on Lipase Activity:

To determine the optimal agitation speed for peak enzyme activity, the L2 was cultured in an orbital shaking incubator at 36°C at varying agitation speed from 120-200 rpm.

2.8 Effect of Temperature on Lipase Activity:

For selection of optimum temperature for the production of lipases, the temperatures varying from 21 to 42°C were selected.

2.9 Effect of pH on Lipase Activity:

The optimum pH for enzyme production was selected by varying the pH of the tributyrin broth from 5 to 9.

Lipase Assay by Titration Method

Mostly bacteria species secrete extracellular, inducible, alkalophilic lipase to hydrolyse fats and oils or lipids. Lipases act on lipids releasing fatty acids. These released fatty acids can be measured by titration with 50 mM NaOH solutions. Olive oil emulsion was prepared by mixing 2.5 ml of olive oil with 7.5ml of 1% gum Arabic solution. It was homogenised for 2 minutes.

Reaction mixture was prepared by adding 2.5 ml of olive oil emulsion, 2ml of 50mMTris-buffer (pH-8.0), 0.5ml of 110Mm $CaCl_2$ and 0.5ml of enzyme extract. This reaction mixture was incubated at 50^0 C for about 1hour under orbital shaking at 160 rpm. The reaction was immediately stopped after the incubation period by addition of 2ml of Acetone: ethanol (1:1 v/v) mixture. Two-three drops of Phenolphthalein indicator was added to it. The released fatty acids were titrated with 50mM NaOH. Sodium hydroxide was standardized with 0.01N oxalic acid. One lipase activity unit was defined as the amount that released 1 µmol of fatty acid per minute.

Calculation of lipase activity: Lipase activity (Units/ml) $=N_2\times (V_2-V_1) \times 1000/T$

N_2=Normality of NaOH (0.0)

V_2-V_1= Difference between the volume of alkali solution consumed for the test and the control

T= Incubation period of 60 minutes.

One unit of lipase activity was defined as amount of lipase capable of releasing one micromole of free fatty acid per ml per minute under the assay conditions and reported as U/ml.

2.10 Purification methods used:

Ammonium sulphate fractionation.

Dialysis

Column chromatography

SDS-PAGE

3.0 Results and discussion

3.1 Isolation and screening of lipase producing bacteria:

A total of 158 colonies were selected and isolated from the 11 samples. The lipase enzyme producing microbial colonies were identified by the clearing zones around the colonies. The selected isolates were transferred onto nutrient agar slants and incubated for 24 hours. The number of isolates from each sample and the zone of hydrolysis are analysed.

Out of 158 isolates, 28 were selected based on their macroscopic characters, eliminating those that appeared close to each other and zone of clearance greater than 1.0 cm. The results indicated that the isolate L-26 showed maximum lipolytic activity (A/B=2.8) followed by the isolates L-28 (A/B=2.7) and L-27(A/B=2.6).

3.2 Secondary screening:

The results indicated that the isolate L-26 exhibited maximum lipase activity (4.4 U/ml) followed by the isolates L-27(4.2 U/ml) and L-28 (4.2 U/ml). Hence, further studies were focused on the isolate L-26. To perform further investigation, the selected L-26 strain

was grown on Tributrin medium and incubated at 37ºC for 18 hours and stored until use at 4ºC in refrigeration.

3.3 Identification and characterisation of the isolate L-26 isolate

***Colony morphology*:** Colonies are large, opaque, irregular, producing green colour pigment. Based on the results of morphological studies and biochemical tests conducted, the isolated organism L-3 was identified as Bacillus species.

3.4 Optimisation of various cultural conditions for enhanced production of lipase

Effect of Incubation Period on Lipase Activity: The L26 isolate was inoculated in Tributyrin broth and was harvested at 24 hours interval. Maximum enzyme activity was observed at 72 hours at the early stationary phase. The activity of the enzyme gradually decreased after 72 hours. (figure 1).

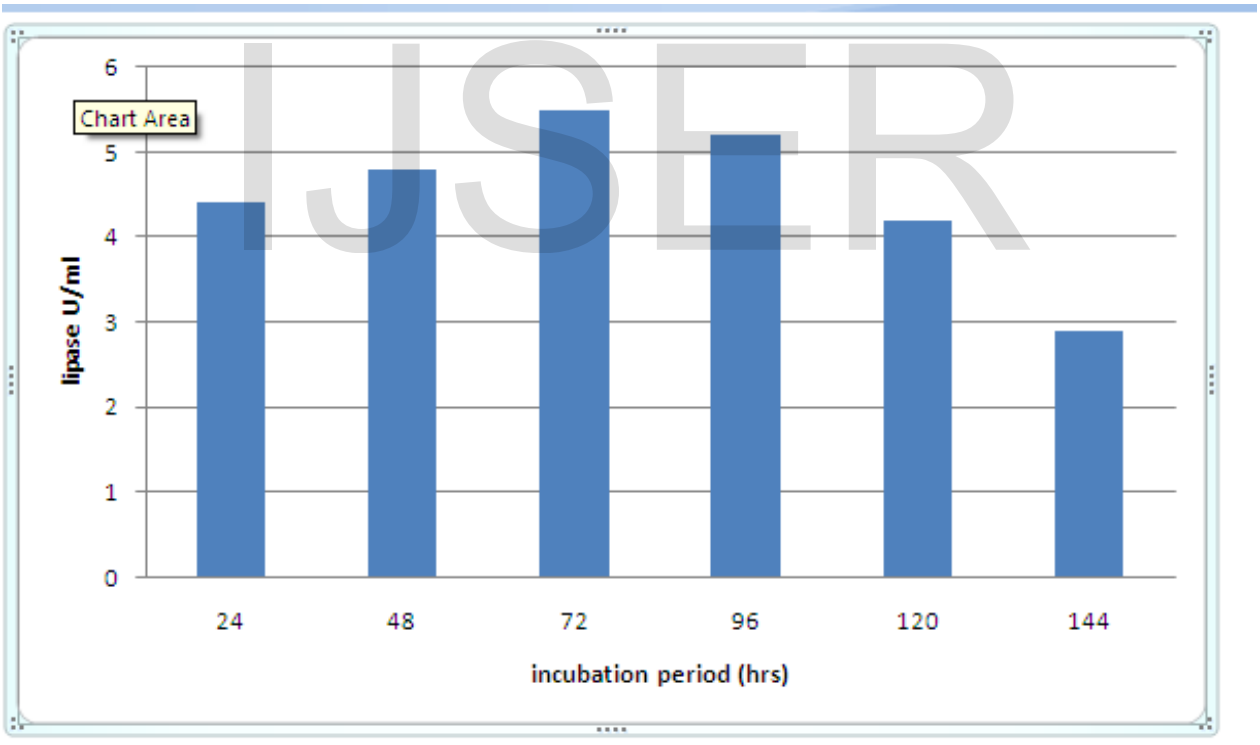
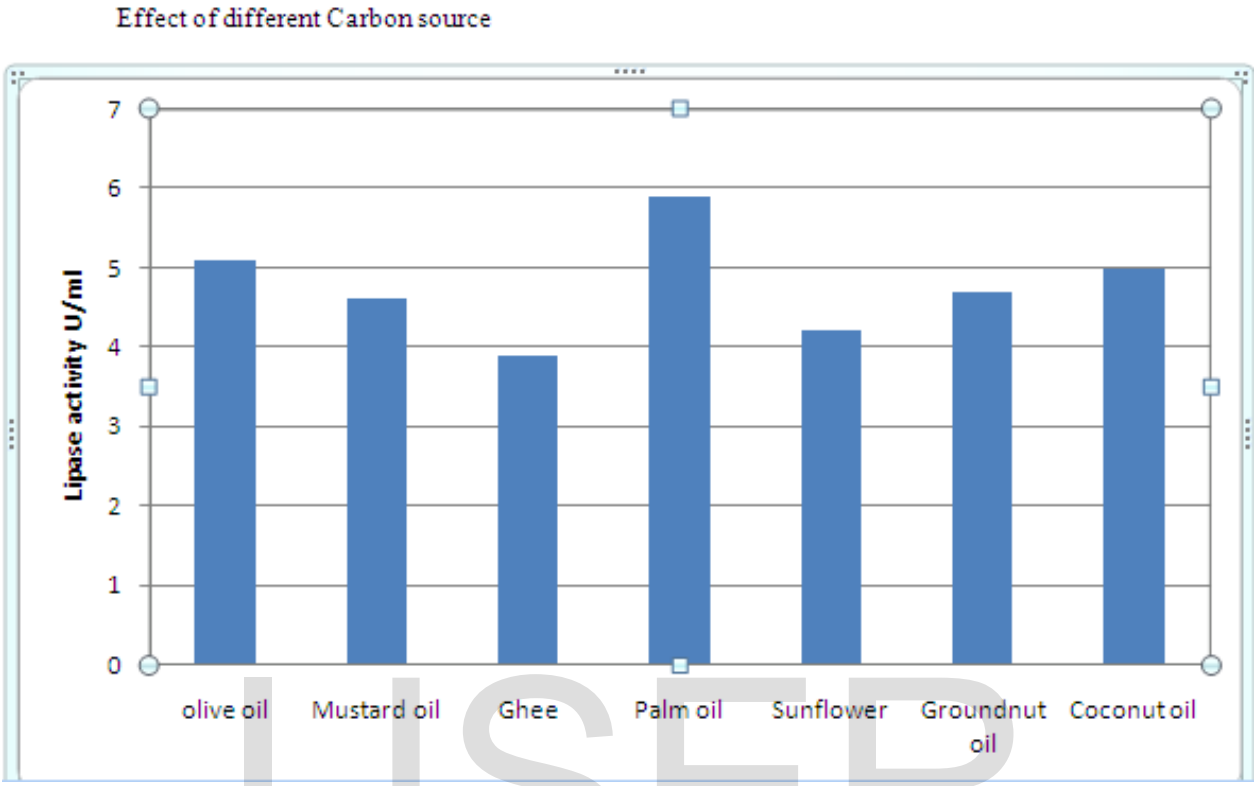


Figure-1- Effect of incubation period on lipase activity

Effect of different carbon sources



Effect of nitrogen sources

Fig-3 Effect of different carbon sources on lipase activity

Effect of different temperatures

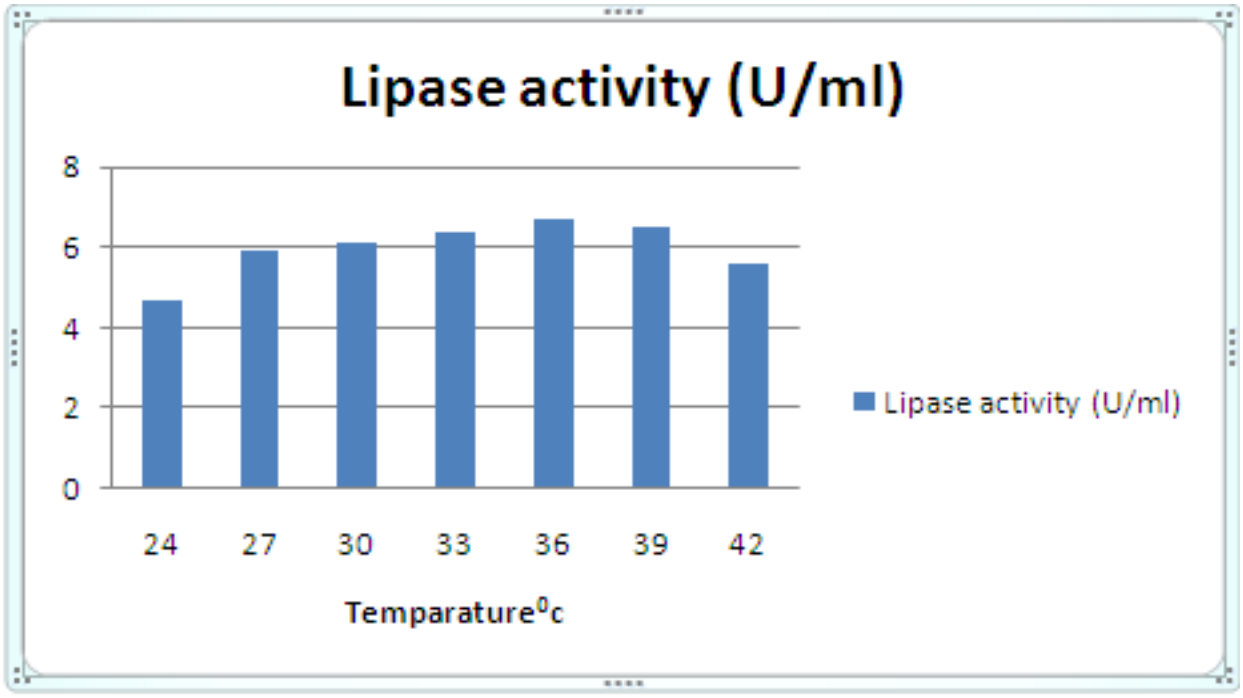


Fig-4 Effect of different temperatures on lipase activity

Effect of different Ph

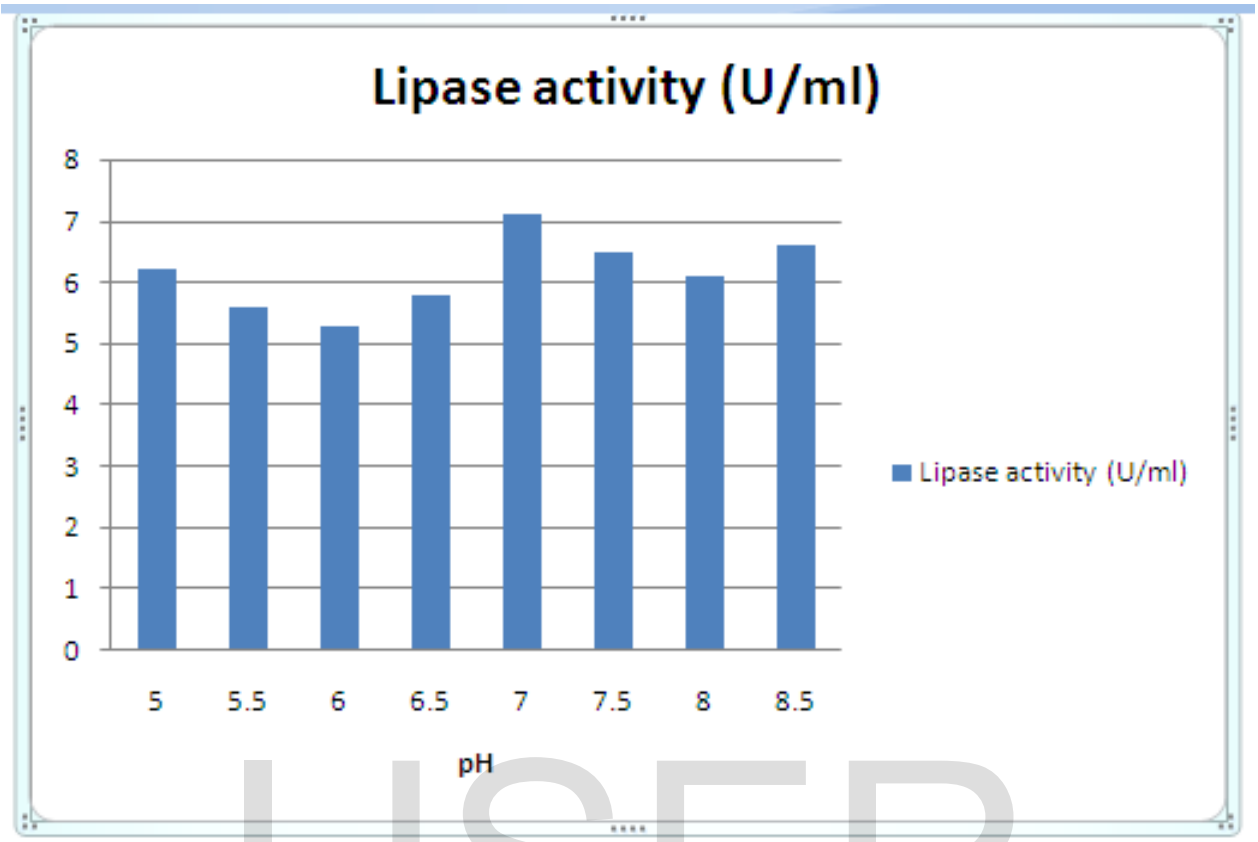


Fig-5 Effect of different pH on lipase activity

Effect of agitation speed

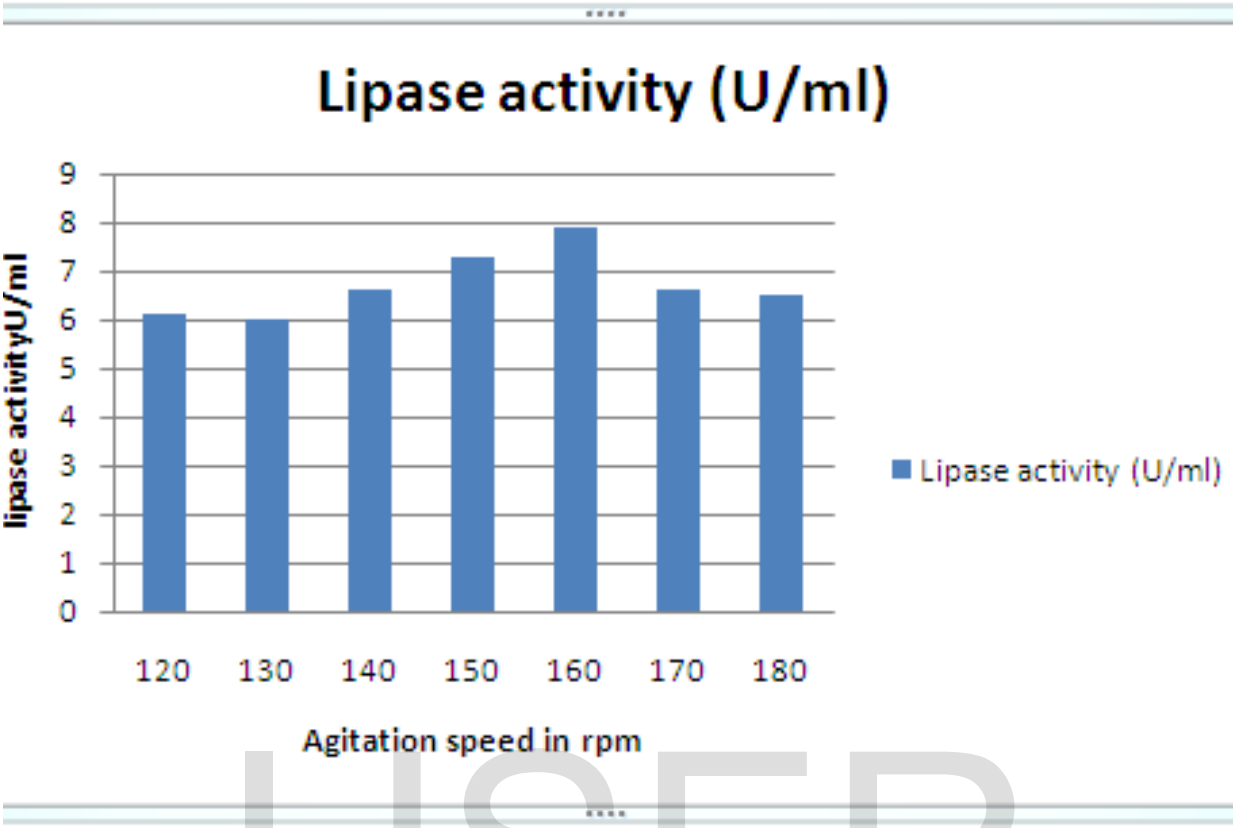


Fig-6 Effect of agitation speed on lipase activity

3.5 Effect of UV radiation on the survival of L-26 isolate

UV irradiation of L-26 isolate and selection of mutants

The isolate L-26 was subjected to UV treatment for six different time intervals. The number of survivals from each exposure is given in Table-10. At 20 and 25 minutes exposure, more than 99% deaths were recorded, while 30 minutes exposure resulted in 100% death of the bacteria. The UV survival curve was plotted (Fig -23) and the plates having less than 1% survival rate (20 and 25 min) were selected for the isolation of mutants.

A total of 15 mutants were selected and tested for lipase production along with the control (Fig-24, 25 (a-d). The results are presented in Table-11. It is evident from the results that the mutants LUV-8, LUV-9 and LUV-10 showed higher lipase activity than the parent strain-26, with the mutant LUV-9 showing the maximum activity of 9.5 U/ml.

UV survival curve

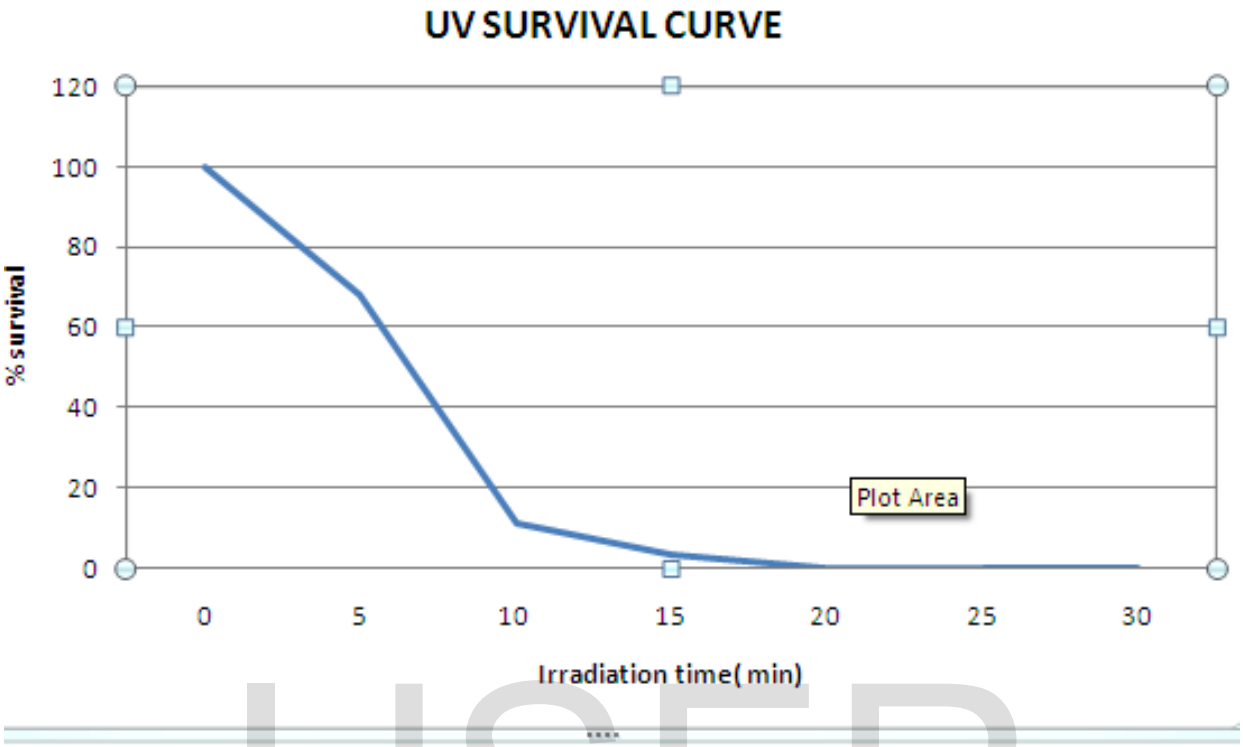


Fig –7 UV survival curve ofisolate L-26

3.6 Purification of Lipase:

Results:

Enzyme purification is done by ammonium sulphate fractionation, dialysis and column chromatography. The ammonium sulphate precipitation and dialysis showed an increased specific activity of 1.71 U/ml and 6.17 U/ml when compared to crude enzyme which showed specific activity of 0.45 U/mg.

Further purification was carried out by ion-exchange chromatography using DEAE column. The purified enzyme showed higher specific activity (15.24 U/mg) with a purification fold of 33.8. The molwt of purified lipase was estimated to be 40.14kDa.

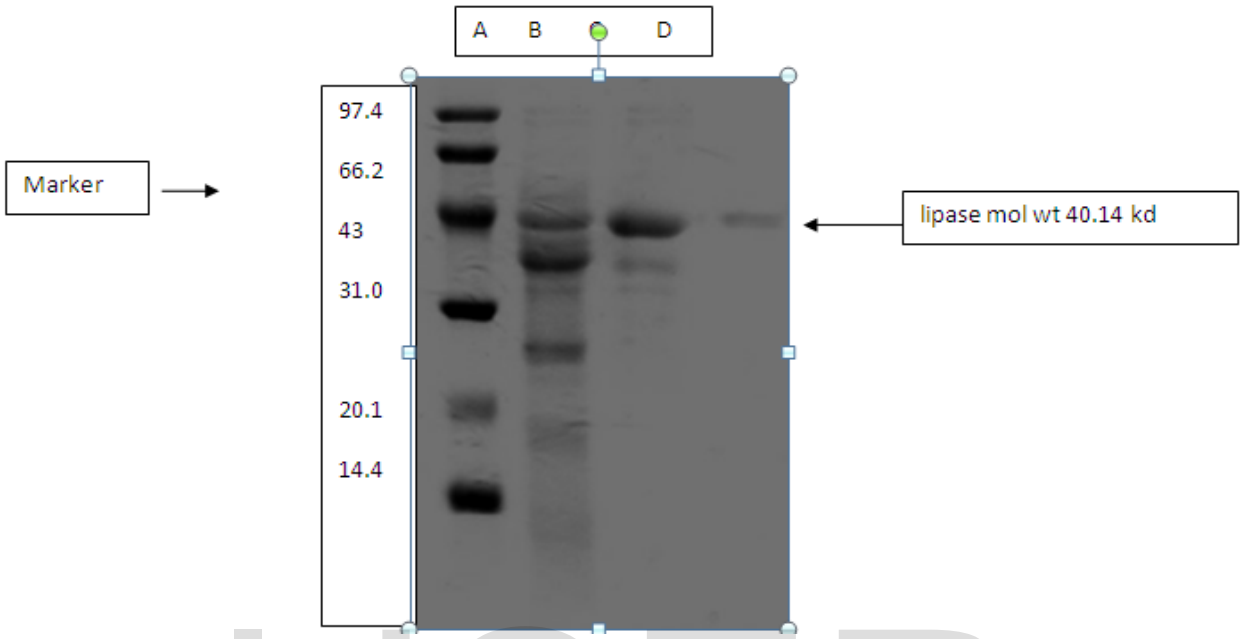
(Fig.1) picture taken- im gel doc

A–Marker

B-Ammoniumsulphate

C-Dialysis

D-Column 6 tube



3.7 Conclusion

Isolate L-26 is identified to be Bacillus sps. It could be a novel strain and further analysis is to be done by Maldi. It is evident from the results that the parameters found to be optimal for lipase production are:

- Incubation period of 72 hrs (24.5U/ml),
- incubation temperature 36^0c (25U/ml),
- pH 7.0 agitation speed of 160 rpm (25.9U/ml),
- Carbon source was palm oil (24.3U/ml),
- Nitrogen source was peptone (24.5U/ml),
- The optimisation of medium and physical conditions increased the enzyme production from 4.4 U/ml to 7.9 U/ml,
- UV mutant LUV-9 showing the maximum activity of 9.5 U/ml, more than the parent strain with 7.5U/ml. So the strain improvement by UV irradiation for high lipase production can be used for commercial purpose on a large scale.

ACKNOWLEDGEMENT

The authors acknowledged the support from Department of Biochemistry, College of Science, Gandhi Institute of Technology and Management, deemed to be University for providing the necessary research facilities.

REFERENCES

1. Isolation, Purification and Characterization of Lipase from *Microbacterium* sp. and its Application in Biodiesel Production by Ritu Tripathi' , Jyoti Singh, Randhir Kumar Bharti, Indu Shekhar Thakur 2014

2. Optimization And Purification Of Lipase Through Solid State Fermentation By Bacillus *Cereus* Msu As Isolated From The Gut Of A Marine Fish *Sardinella Longiceps* Suyambu Ananthi, Ramasamy Ramasubburayan, Arunachalam Palavesam, Grasian Immanuel* 2014.

3. Production and partial characterization of lipase by *bacillus sp* isolated from vellar estuary sediment K. Muthazhagan and M. Thangaraj* 2014

4. Screening, purification and properties of a thermophilic lipase from Bacillus thermocatenulatus.Schmidt-Dannert C[1], Sztajer H, Stöcklein W, Menge U, Schmid RD.2011

5. A Newly Isolated Thermostable Lipase from *Bacillus* sp.Fairolniza Mohd Shariff 1, Raja Noor Zaliha Raja Abd. Rahman 1,*, Mahiran Basri 2 and Abu Bakar Salleh 1 2011

6. Purification of a Novel Thermophilic Lipase from *B. licheniformis* MTCC-10498 Sharma Chander K. and Kanwar Shamsher 2012

7. Purification and characterization of Lipase from bacteria Akshatha K.N.1, Dr. S. Mahadeva Murthy2, Dr. N. Lakshmidevi3 2012

8. Screening, isolation and production of lipase/esterase producing *Bacillus* sp. strain DVL2 and its potential evaluation in esterification and resolution reactions Davender Kumar1a, Lalit Kumar1, Sushil Nagar1, Chand Raina2, Rajinder Parshad2, Vijay Kumar Gupta1* 2012.

9. Thermostable, alkaline tolerant lipase from *Bacillus licheniformis using* peanut oil cake as a substrate N. Annamalai*, S. Elayaraja, S. Vijayalakshmi and T. Balasubramanian 2012.

10. Production, optimization and purification of lipase from *Bacillus* sp. MPTK 912 isolated from oil mill effluent Mukesh Kumar DJ1*, Rejitha R2, Devika S2, Balakumaran MD1, A. Immaculate Nancy Rebecca1 and Kalaichelvan PT1 2012.

11. Achamman, T., Monoj, M.K., Valsa, A., Mohan, S., and Manjula.R. (2003) "Optimization of growth condition for the production of extra cellular lipase by *Bacillus mycoides"*, *Indian Journal of Microbiology*. Vol.43, 67 – 69.

12. Auria, S.D., Herman, P., Lakowicz, J.R., Tanfani, F., Bertoli. E. (2000 "The Esterase From the Thermophilic Eubacterium Bacillus acidocaldarius: Structural-Functional Relationship and Comparison With the Esterase From the Hyperthermophilic Archaeon Archaeoglobus fulgidus." *Proteins: Structure, Function, and Genetics*, vol.40, 473-481.

13. Bradford, M.M. (1976) "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye Binding". *Analytical Biochemistry* vol .72, 248–254.

14. Dordick, J.S. (1989), "Enzymatic catalysis in monophasic organic solvents".*Enzyme Microbial Technology*, vol.11, 194–211.

15. Hansan F., Shah A.A.and Hameed.A. (2006),"Industrial applications of microbial lipases", *Enzyme Microbial Technology*, vol.39, 235–51.

16. Hartdwood, J., (1989), "The versatility of lipases from industrial uses", *Trends in Biochemical Sciences*, vol.14, 125-128.

17. Henroette, C., S. Zinebi, M.F. Aumaitre, E. Petitdemange and H. Petitdemange.1993.Protease and lipase production by a strain of serratia marcescens. *J. Industrial Microbiol.*
12:129-135.

18. Hofelman, M., Hartmann., 1., and Schreier, P. (1985),"Isolation, purification., and characterization of lipase isoenzymes from a technical *Aspergillus niger* enzyme". *International Journal of Food Science*.vol. 50:1721-1726.

19. K.V.V.S.N. Bapiraju, Sujatha, P., Ellaiah P., and Ramana. T.(2004). "Mutation induced enhanced biosynthesis of lipase", *African Journal of Biotechnology*, Vol. 3, 618-621.

20. Kim, H.K., Park, S.Y. Lee J.K. and Oh. T.K. (1998), “Gene cloning and characterization of thermostable lipase from *Bacillus stearothermophilus* L1”. *Bioscience, Biotechnology and Biochemistry*.vol. 62, 66-71.

21. Laemmli, U.K., 1970. “Cleavage of structural proteins during assembly of head of bacteriophage T4”.*Nature (London),* vol. 227, 680-685.

22. Lee S.Y. and Rhee. J.S. (1993).”Production and purification of a lipase from Pseudomonas putida 35K”.*Enzyme and Microbial technology*.vol.15, 617-623.

23. Lee, D., Kim, H. Lee, K. Kim, B. hoe, E., and Lee. H. (2001). “characterization of two distinct thermostable lipases from the Gram-positive thermophilic bacterium *Bacillus thermoleovorans* ID-1”. *Enzyme and Microbial Technology*.29: 363-371.

24. Markossian, S., Becker, P. Marc, H. Antranikian. G. (2000), “Isolation and characterization of lipid-degrading *Bacillus thermoleovorans* IHI-91 from an icelandic hot spring”. *Extremophiles*.vol. 4, 365-371.

25. Motai.H., Ichishima E. and Yoshida. F.(1966).”Purification and properties of lipases from *Torudopsis”*. *Nature*.vol, 210. 308-309.