

Lipase Production from Free Cells and Immobilized Whole Cells- A Comparative Approach

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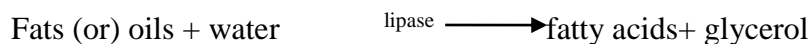
ABSTRACT

Lipases play a vital role in modern Biotechnology owing to their multifaceted properties. Their production and purification procedures need to be improved in order to enhance their stability and turn over. This work was done to study the effect of immobilization of whole cells of lactobacillus in the production of lipase in comparison with free cells of lactobacillus. Ammonium sulphate precipitation and High performance liquid chromatography were used as purification procedures. Activity of purified lipases were found to be 10 μ mol fatty acid released/ml of substrate for free cell fermentation and 40 μ mol fatty acid released/ml of substrate for immobilized cells respectively. Tolerance of lipase against organic solvents such as ethanol, acetone, benzene, and hexane were determined. Michaelis Menton parameters for the enzyme like V_{max} , K_m , β were found to be 1.295 μ g/ml/min, 0.924 μ g/ml, 1.0663 for free cell fermentation and 0.201 μ g/ml/min, 9.378 μ g/ml, 10.825 for immobilized whole cell fermentation.

INTRODUCTION

Lipases (triacylglycerol acyl hydrolases E.C.3.1.1.3) have been emerged as key enzymes in swiftly growing biotechnology owing to their multifaceted properties which find immense usage in wide array of industrial applications such as food technology, detergent, chemical industry and biomedical sciences (Pandey *et al.*, 1999). They catalyze both the hydrolysis of triglycerides and synthesis of esters from glycerol and long chain fatty acids. In addition, they

also serve as a biocatalyst for alcoholysis, acidolysis, esterification and aminolysis. (Jaeger *et al.*, 1998). The common mode of action of lipases as a biocatalyst is shown below.



Lipases are characterized by their ability to catalyze the hydrolysis of triglycerides at the interface between substrate and water, a phenomenon called interfacial activation. The catalytic triad is composed of Ser-Asp/Glu-his and usually also a consensus sequence.(Gly-X-Ser-X-Gly) is found around the active site serine. The three dimensional structures of lipases reveal the characteristic α/β -hydrolase fold. (Nardini & Dijkstra 1999). However these enzymes also display catalytic activity towards a large variety of alcohols and acids in ester synthesis reactions provided that the water activity is very low. Synthesis of esters mediated by lipases has been under scrutiny by numerous researchers in recent years as a wide variety of such compounds are important to us. Protein engineering of enzymes is an indispensable tool for improving the enzyme properties. The properties of lipases that need to be improved are stability and turnover under application conditions. Lipases need to be stable against proteolytic action; they need to be thermo stable and also stable against oxidative compounds and detergent ingredients, when lipases are used for fatty stain removal in laundry. A desire for kinetic changes is more likely in the food, chemical and pharmaceutical areas. More understanding is needed concerning the observed stereo preference of lipases acting on triacylglycerol lipases or of pharmaceutical compounds of interest. Lipases are indeed one of the most versatile enzymes. In the coming years there is going to be a large scale commercial exploitation of these enzymes.

MATERIALS AND METHODS

Isolation of Microorganism: Lactobacilli MRS Agar and Lactobacilli MRS Broth are recommended for use in the isolation, enumeration and cultivation of *Lactobacillus* species. Lactobacilli MRS Agar and Lactobacilli MRS Broth are based on the formulations of *deMan, Rogosa and Sharpe*. The ingredients were suspended in sterile distilled water and mixed thoroughly. The mixture is heated with frequent agitation and boiled for 1 minute until the powder was completely dissolved. Then autoclaved at 121°C for 15 minutes. Transferred Lactobacilli MRS Agar into sterile Petri dishes containing 1 ml volumes of diluted sample of commercially available curd. The medium is allowed to solidify on a flat surface for 5-10 minutes. Alternatively, plates of Lactobacilli MRS Agar used for direct recovery of organisms using the streak oculation technique and incubated at 35°C for 3 days.

Identification of Microorganisms: Identification of the selected isolates was carried out using morphological and biochemical methods. The studies included Catalase test, Gram's staining and *E.Coli* inhibition Test.

Determination of Growth Pattern: The different phases of growth of the *Lactobacillus* cells were observed by OD values using UV visible spectrometer. Different phases such as lag, log, stationary and decline phases can be viewed by plotting time in the X-axis and optical density value (Absorbance) in the Y-axis. At the end of log phase growth of cells are maximum.

Dry Cell Mass Determination: To determine the dry weight of the cells, the 20 hr cells were separated using centrifugation process at 10000 rpm, 4°C in 20mins and filtered through filter paper (Whatman No. 40). It was washed first with 0.1 mol/L HCl to remove CaCO₃, followed by washing with distilled water. The washed cells are dried at 60±1°C to constant mass. It was placed in the desiccators and then the mass was determined.

Determination of Specific Growth Rate (μ) and Growth Yield ($Y_{X/S}$): To determine the specific growth rate (μ), natural log of biomass ($\ln X/X_0$) was plotted against time (t). The slope of the line at any moment gives the specific growth rate at that moment. Biomass growth yield was determined on the basis of lipid consumption. A graph was plotted between changes in dry weight and changes in the amount of the lipids consumed. The slope of the graph was $Y_{X/S}$.

Free Cell Fermentation: The free cell fermentation is carried out in shake flask using the MRS medium. The flask containing 50 ml fermentation medium is inoculated by 20 h old inoculums. The inoculums development and the fermentation are carried out at 35-37°C in an anaerobic environment. At the end of 20 hr crude lipase enzyme can be isolated.

Immobilized Whole Cell Fermentation: The *Lactobacillus* cells are harvested at 20 hr by centrifuging in a table top centrifuge at 12000 rpm for 15 mins. The cells are washed three times with saline and used for immobilization. The harvested *Lactobacillus* cells (10.0 g/l dry weight) are suspended in 30 ml of sterile 3% sodium alginate solution. The suspended cells are extruded with a help of syringe through a needle into 5% calcium chloride solution and the beads are kept in ice cold saline solution for 2 hrs. Then the beads are packed into a column at 37°C. Immobilized cells are loaded in a column and 50 ml of MRS fermentation medium is poured to it. The inoculums development and the fermentation are carried out at 35-37°C in an anaerobic environment. After the cells cross lag phase (at the end of 20 hr) crude lipase can be drained out of the column.

Screening of Lipase: A simple and reliable method for detecting lipase activity in lactobacillus is the use of surfactant Tween 80 in a solid medium to identify a lipolytic activity. The formation of precipitation around the well is an indication of lipase production by the organisms.

Purification by Ammonium Sulphate Precipitation: Solid Ammonium sulphate was added to the extract with stirring to bring the saturation of 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80% and after standing it for 4 h at 4°C, the precipitates were removed by centrifugation. Lipase activity both in precipitate and supernatant was determined. Additional ammonium sulphate was added to the supernatant to bring the saturation to 60% and the mixture was left overnight. The precipitates were collected and used as enzyme.

High Performance Liquid Chromatography: HPLC analysis was performed with a Hewlett–Packard 1050 Series pump and a Hewlett Packard 1050 Series variable wavelength detector. Normal phase HPLC separations were performed with a Chiracel OD column (250 mm × 4.6 mm, particle size 10 µm, Daicel) at ambient temperature. UV-visible detector was performed at 580 nm. Aceto nitrile was used as components of the mobile phase at a flow rate of 0.5 mL min⁻¹. Sample concentrations were 1% (w/v) in mobile phase as solvent and injection volume was 20 µL.

Lipase Assay-Determination of Activity: Lipase activity in the broth is determined titrimetrically on the basis of olive oil hydrolysis (Martinez *et al.*, 2000). One ml of sample solution is added to the assay substrate containing 1ml of 10% homogenized Olive oil and 1 ml of 0.1M Tris HCl buffer, pH8.5. The enzyme substrate mixture was incubated on orbital shaker with a shaking speed of 120 rpm at 30°C for 20min. To stop the reaction, by using 10 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.05N NaOH with Phenolphthalein as a indicator. Lipase activity is expressed as units per ml of the broth. One ‘lipase unit’ (U) is defined as the amount of the enzyme that release one mole fatty acid per min.

Measurement of Protein Concentration: The Lowry protein assay is a biochemical assay for determining the total level of protein in a solution. The total protein concentration is exhibited by a color change of the sample (free cell & immobilized cell fermentation) solution in proportion to protein concentration, which can then be measured using colorimetric technique in the 1940s.

Optimization of lipase-Effect of Temperature and pH: For the effect of temperature, the purified enzyme was incubated at 30°C, 35°C, 40°C, 45°C, and 50°C for a period of 15min

using Olive oil as substrate. After incubation the reaction stop by using acetone added to the mixture. The residual lipase activity was determined. To study the effect of pH, the lipase activity was measured at pH from 5, 5.5, 6.5, 7.5, 8.5 and 9.5. The pH of the reaction mixture was varied using Phosphate buffer buffer. 150 μ l purified lipase was incubated in different pH buffers for 15min at 37°C and residual specific activity was determined.

Tolerance Test: The effect of organic solvents on the enzyme from free cell fermentation and immobilized whole cell fermentation were performed. The organic solvents Ethanol, Acetone, Benzene, Hexane were investigated at different concentration 0.1%, 1%, 5%. Enzymes were mixed with different concentration of different organic solvent and inoculated in modified agar medium.

Determination of V_{max} , K_m and β : The purified lipase was incubated with various concentrations of olive oil. The Michaelis constant (K_m) was calculated by plotting $1/V$ against $1/[S]$ in Lineweaver and Burk plot. The slope of the plot is K_m/V_{max} and the intercept is V_{max}^{-1} . $\beta = C_{s0}/K_m$ is the dimensionless Michaelis constant.

RESULTS AND DISCUSSION

24 hrs curd was diluted in distilled water and inoculated in MRS agar, incubated at 35°C for 3 days. Organisms were grown on MRS agar. Samples were inoculated directly into Lactobacilli MRS Broth. Broth was incubated at 35°C for 3 days.



Fig. 1(a): Isolation of microorganism



Fig. 1(b): Broth Enrichment

The isolated organisms were Gram positive and Rod shape.

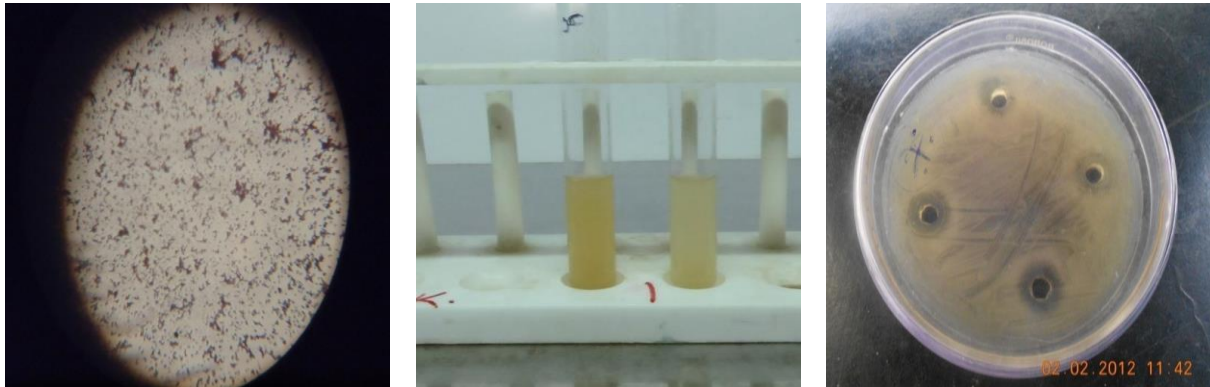


Fig. 2(a): Gram's staining Fig. 2(b): Catalase Test Fig. 2(c): E.Coli Test

A visible amount of bacterial growth was added to 3% of hydrogen peroxide solution in aseptic condition. There was no bubble formation. The Catalase test was negative which confirms the presence of *Lactobacillus* sp. *Lactobacillus* sp treated with E.coli on MRS agar. The opaque zones was formed around the colonies was an indication of *Lactobacillus* spp organisms.

The different phases of growth of the lactobacillus cells were determined by plotting time in the X-axis and optical density value (Absorbance) in the Y-axis. Specific growth rate of cells were determined by plotting $\ln(X/X_0)$ Vs T

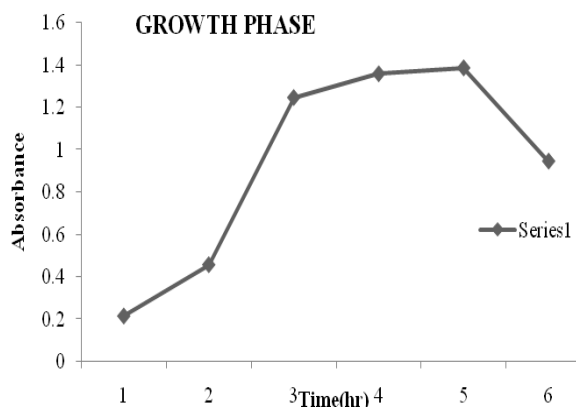


Fig.3: Determination of growth pattern

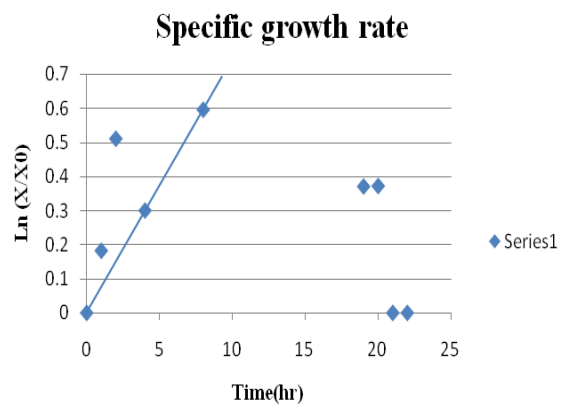


Fig.4: Determination of specific growth rate

The slope value of the plot of $\ln(X/X_0)$ Vs Time was found to be 0.004 hr^{-1} . A graph was plotted between changes in dry weight and changes in the amount of lipids consumed. From graph, the yield co-efficient $Y_{x/s}$ was $0.002 \text{g dry weight of cells/g of substrate}$.

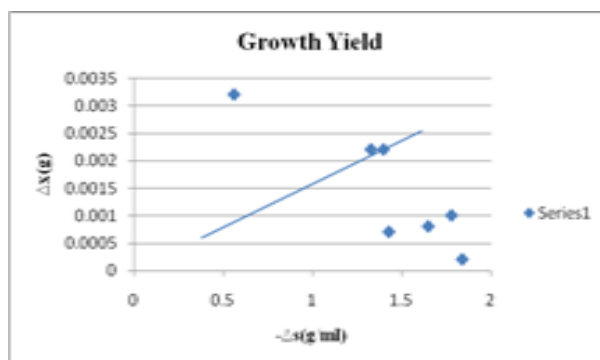


Fig.5: Growth Yield

The free cell fermentation was carried out in shake flask using the MRS medium. At the end of 20 hr crude lipase enzyme was extracted.



Fig. 6: Free cell Fermentation



Fig.7: Immobilized whole cell fermentation

Immobilized cells were loaded in a column and 50 ml of MRS fermentation medium was poured to it. The inoculum development and the fermentation were carried out at 35-37°C in anaerobic environment. After the cells cross lag phase (at the end of 20 hr) crude lipase can be drained out of the column. A simple and reliable method for detecting lipase activity in *lactobacillus spp* was the use of surfactant Tween 80 in a solid medium to identify a lipolytic activity. The precipitation lines were formed around the well.

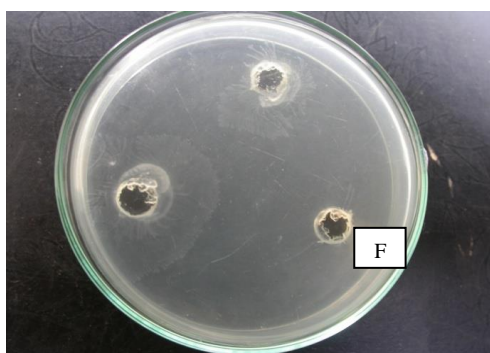


Fig 8 Screening of lipase, F-free cell lipase activity, I- Immobilized whole cell lipase activity

The enzyme obtained from free cell fermentation and immobilized whole cell fermentation was purified by ammonium sulphate precipitation. The Ammonium sulphate was optimized with std.BSA and protein concentration is estimated.

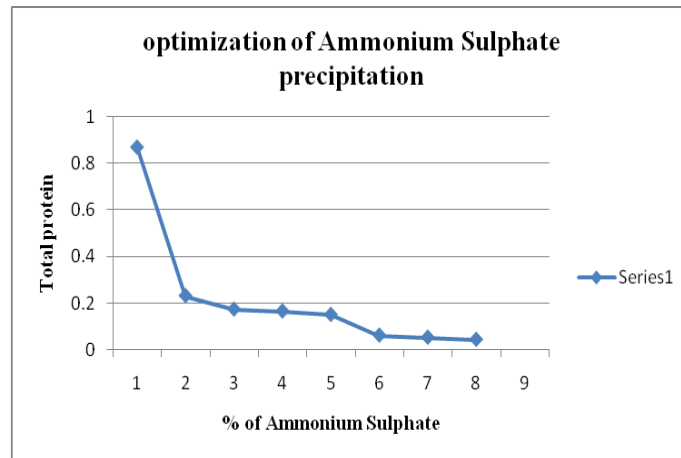


Fig. 9: Ammonium sulphate precipitation

High Performance Liquid Chromatography Report

HPLC ANALYSIS REPORT

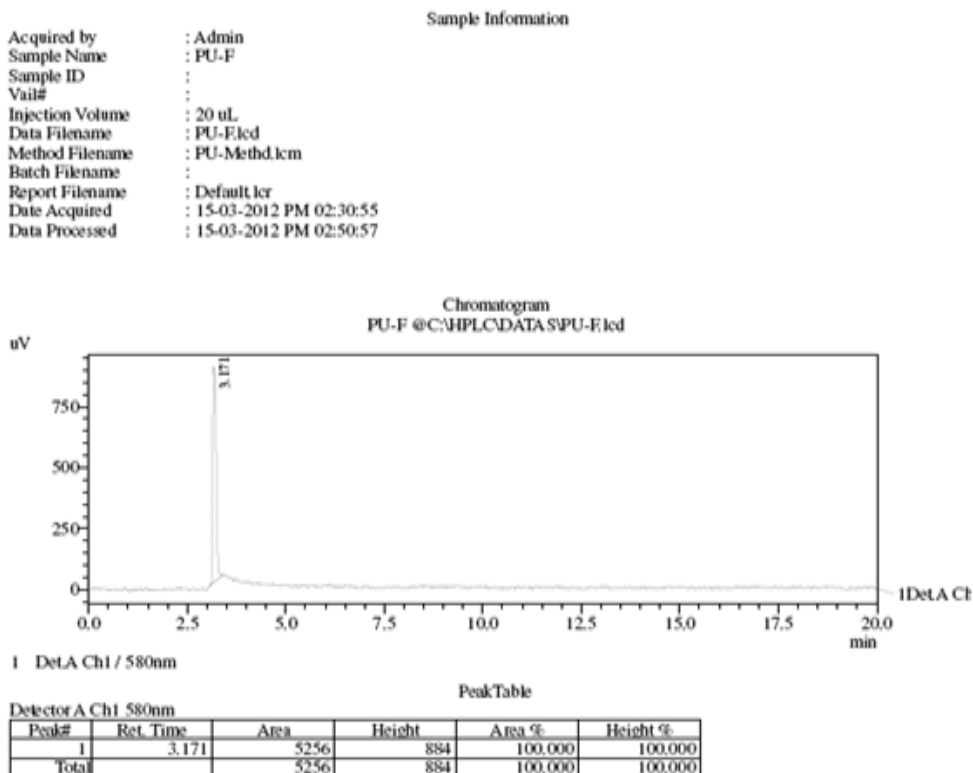


Fig. 10: HPLC analysis of lipase extracted from free cell fermentation

HPLC ANALYSIS REPORT

Sample Information
 Acquired by : Admin
 Sample Name : PU-IM
 Sample ID :
 Vial# :
 Injection Volume : 20 uL
 Data Filename : PU-IM.lcd
 Method Filename : PU-Methd.lcm
 Batch Filename :
 Report Filename : Default.lcr
 Date Acquired : 15-03-2012 PM 01:56:03
 Data Processed : 15-03-2012 PM 02:16:04

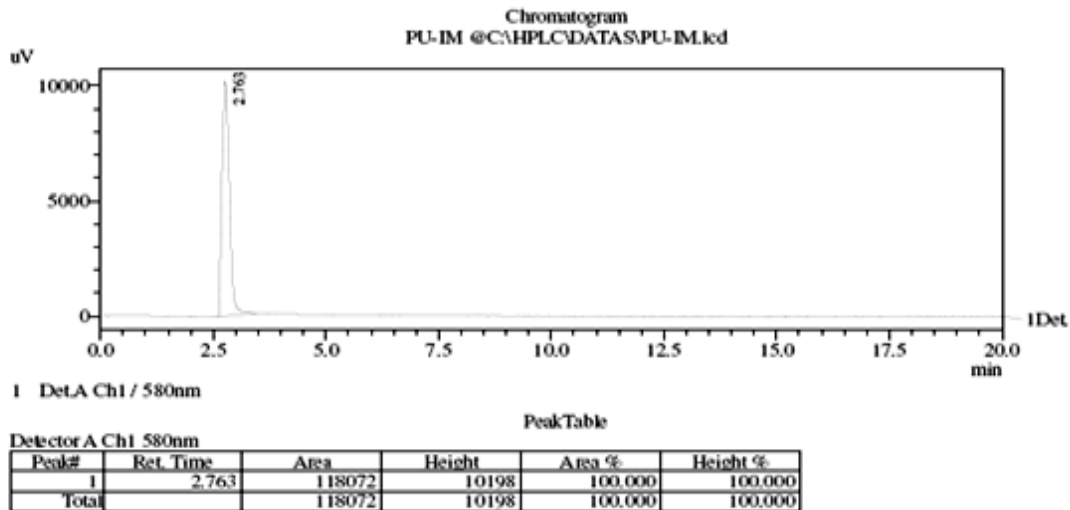


Fig. 11: HPLC analysis of lipase extracted from immobilized whole cell fermentation.

The total protein concentration was determined by Lowry's method.

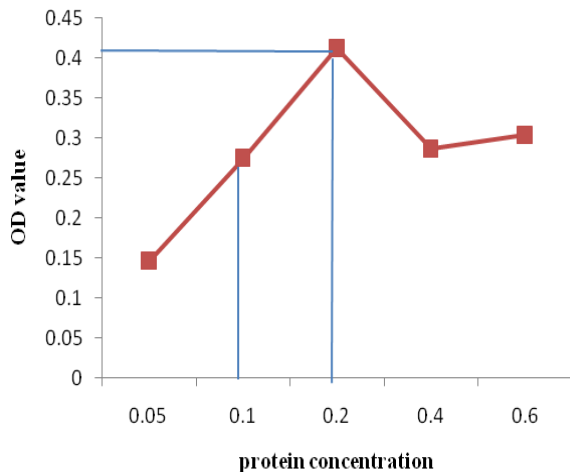


Fig. 12: measurement of protein concentration

Lipase activity in the broth was determined titrimetrically on the basis of olive oil hydrolysis (Martinez *et al.*, 2000). One ml sample solution (free cell& immobilized cell fermentation) was added separately to the assay substrate containing 1ml of 10% homogenized Olive oil and 1 ml of 0.1M Tris HCl buffer, pH8.5. The enzyme substrate mixture was incubated on

orbital shaker with a shaking speed of 120 rpm at 30°C for 20min. To stop the reaction, by using 10 ml ethanol acetone mixture (1:1) was added to the reaction mixture. Liberated fatty acids were titrated with 0.05N NaOH with Phenolphthalein as an indicator.

$$\text{Enzyme Activity} = \frac{(\text{ml of NaOH for sample} - \text{ml of NaOH for blank}) * N * 1000}{\text{ml of substrate}}$$

For Free cell fermentation

$$= \frac{(3.2-3) * 0.05 * 1000}{1}$$

$$= 10 \mu\text{mol fatty acid released/ml of substrate}$$

For Immobilized whole cell fermentation

$$= \frac{(3.8-3) * 0.005 * 1000}{1}$$

$$= 40 \mu\text{mol fatty acid released/ml of substrate}$$

The maximum Enzyme activity was reached at the temperature was 30 °c on free cell fermentation. The maximum Enzyme activity was reached at the temperature was 40°C on immobilized cell fermentation .

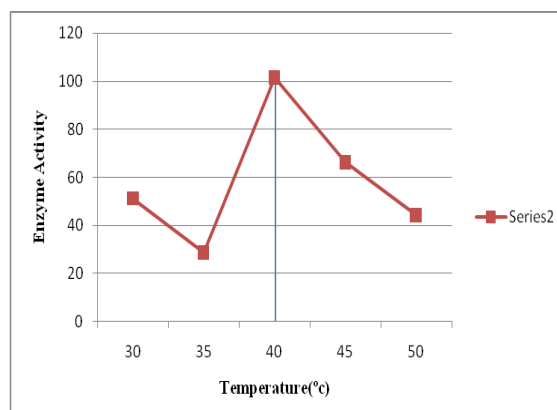
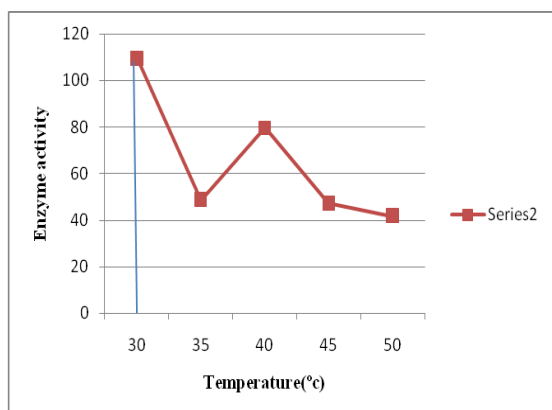


Fig. 13 (a): Effect of temperature on free cell fermentation

Fig. 13 (b): Effect of temperature on immobilized cell fermentation

The maximum Enzyme activity was reached at the pH was 5.5 on free cell fermentation. The maximum Enzyme activity was reached at the pH was 8.5 on immobilized whole cell fermentation .

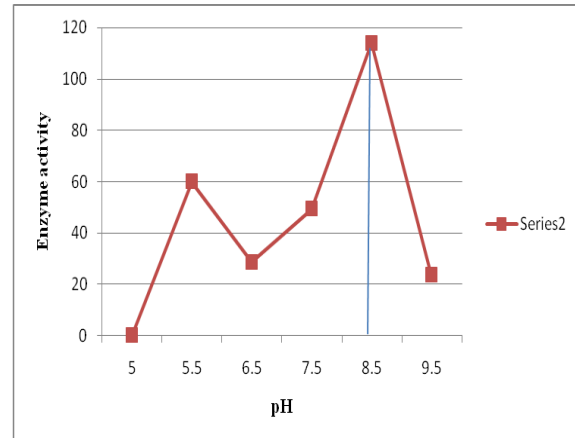
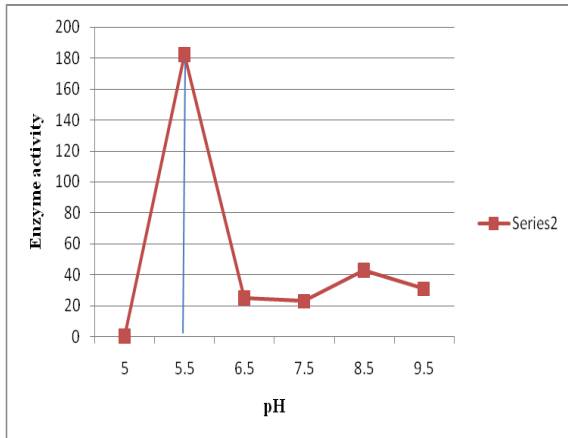


Fig. 14(a): Effect of pH on free cell fermentation

Fig. 14(b): Effect of pH on immobilized whole cell fermentation

The effect of organic solvents for free cell fermentation and immobilized whole cell fermentation were studied. Immobilized lipase was more tolerant against Ethanol, Acetone & Hexane when compared to free cell fermentation.

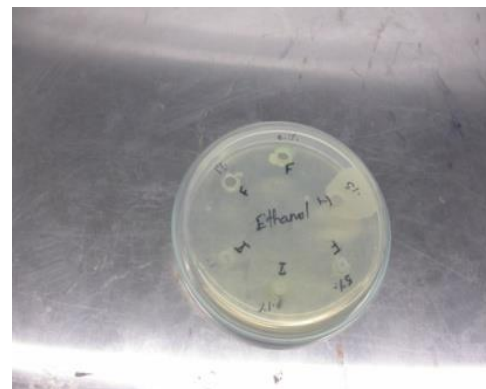
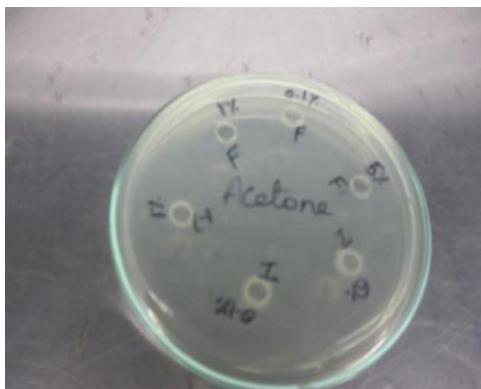


Fig. 14: Tolerance test results

V_{max} and K_m values are determined by plotting $1/V$ and $1/S$ for the lipase produced by free cell fermentation and immobilized whole cell fermentation. For free cell fermentation they are found to be $1.295 \mu\text{g/ml/min}$ and $9.378 \mu\text{g/ml}$. The values of V_{max} and K_m were found to

be $0.201\mu\text{g/ml/min}$ and $0.924\mu\text{g/ml}$ for immobilized fermentation. The dimensionless michaelis constant β , is found to be 1.0663 for free cell fermentation and 10.825 for immobilized whole cell fermentation

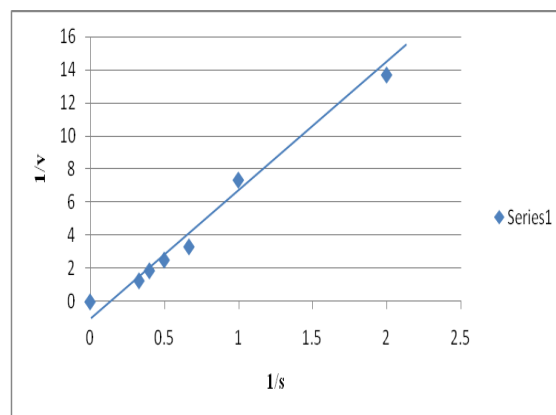
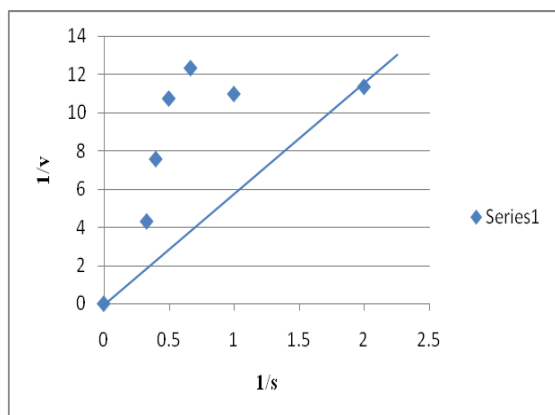


Fig. 15(a): Determination of v_{max} , k_m for Free cell fermentation

Fig. 15(b): for Immobilization Whole cell fermentation

These results showed that the immobilized cells performed better than the normal cells. The yield and activity of the enzyme obtained is also higher than that if free cell fermentation. This work present a better understanding of efficiency of immobilized cells which could be exploited in reactor level studies.

REFERENCES

1. Pandey A, Benjamin S, Soccol CR, Nigam P, Krieger N, Soccol UT (1999) The realm of microbial lipases in biotechnology. *Biotechnol Appl Biochem* 29: 119-131
2. Jaeger K-E, Reetz MT (1998) Microbial lipase from versatile tools for biotechnology. *Trends Biotechnology* vol.16 pp: 396-403
3. Nardini M, Dijkstra BW (1999) ' α/β Hydrolase fold enzymes: the family keeps growing'. *Curr Opin Struct Biol* vol.9 pp: 732-737
4. Martinez JM, Kok J, Sanders JW, Hernandez PE (2000) 'Heterologous coproduction of enterocin A and pediocin PA-1 by *Lactococcus lactis*: detection by specific peptide-directed antibodies.' *Appl Environ Microbiol*, vol.66 pp: 3543-3549.
5. Antonella Crapisi, Marco Paolo Nuti, Arturo Zamorani, and Paolo Spettoli (1987) 'Immobilized *Lactobacillus* sp. Cells for the Control of Malolactic Fermentation in Wine' *Am.J.Enol.Vitic.* vol.38:4pp:310-312
6. A.R.Bucky, P.R.Hayes and D.S.Robinson (1985) 'Lipase production by a strain of *Pseudomonas fluorescens* in Whole milk and skimmed milk', The University of Leeds, Department of Microbiology, Leeds, LS29JT, UK

7. Chetan J.Gadgil,K.V.Venkatesh (1997), Structured Model for Batch Culture Growth of Lactobacillus bulgaricus' , Department of Chemical Engineering, Indian Institute of Technology,Powai.
8. Carlos Raimundo Ferreira grossol;Carmen silvia favaro-trindade(2004) stability of free and immobilized lactobacillus acidophilus and Bifidobacterium lactis in acidified pp: 151-156
9. deMan,Rogos and Sharpe,(1960). J.Appl.Bacteriol.23;130.
10. A.R.Desai, B.Powell and P.Shah(2000) 'Compendium of methods for the microbiological examination of food' ,4th ed.American Public Health Association, Washington,D.C.
11. Deyang Huang, Jennifer G.Becker(2009) 'Determination of intrinsic monod kinetic parameters for two heterotrophic tetrachloroethene (PCE)-respiring strains nd insight into their application'.Biotech. Bioeng.vol:104pp: 301-311
12. el –Sawah MM, Sherief AA and Bayoumy SM (1995), Enzymatic properties of lipase and characteristics production by Lactobacillus delbrueckii sub sp. Bulgaricus' Antonie Van Leeuwenhoek.vol;67 (4) pp: 357-362
13. Fedor Malik,Vladimir Tefuca and Vladimir Bale (2004) determination of KINETIC parameters for immobilized whole cell'Journal of MOLECULAR Catalysis B: Enzymatic Volume 29, Issues 1-6 ,Page 81-87.
14. Frank Rosenau and Karl-Erich Jaeger (2000) 'Bacterial lipases from Pseudomonas: Regulation of gene expression and mechanisms of secretion'vol 3.pp115-7
15. Ghosh PK ,Saxena RK, Gupta R, Yadav RP, Davidson (1996) 'Microbial lipases: production and application': S.Sci Prog. Vol.79 pp:119-57
16. Guoqiang ; Rajni Kaul; Bo Mattiasson (1992) 'Immobilization of Lactobacillus casei cells to ceramic material pretreated with polyethylenimine ' Applied microbiology and biotechnology vol.37 pp305-310
17. Goran Molin (1983) 'Measurement of maximum specific growth rate using the method of wah-out' Biotechnology and bioengineering vol.04,pp:301-311
18. Jensen RG, Dejong FA, Clark RM (1983) 'Determination of lipase specificity' Lipid Mar:vol.8,pp:239-52
19. Kouwen TR, Nielsen AK, Denham EL, Dubois JY,Dorenbos R,Rasmussen MD, Quax WJ, Freudl R, Van Dijnl JM.(2009) 'Contributions of the pre-and pro-regions of a Staphylococcus hyicus lipase to secretion of a heterologous protein by Bacillus subtilis' Appl Environ Microbiol vol.30

20. Martine Papon, Regine Talon (1999) 'Factors affecting growth and lipase production by meat lactobacilli strains and *Brochothrix thermosphacta*' Station de Recherches sur la Viande, INRA, France
21. M.G. Murphy and S. Condon (1983) 'Dairy and Food Microbiology' University College, Cork and activity of Probiotic Lactobacilli in Skim Milk Containing Prebiotics
22. Mehta, AM Patel, KA and Dave, PJ (1983), 'Isolation and purification of an inhibitory protein from *Lactobacillus acidophilus*' VOL.37, PP : 37-43
23. Murray, Baron, Pfaller, Tenover and Tenover (ed.). (1999). 'Manual of clinical microbiology,' 7th ed. American Society for Microbiology, Washington, D.C
24. Nikolaos Agouridis, Argyro Bekatorou, Poonam Nigam and Maria Kanellaki (2005) 'Malolactic Fermentation in Wine with *Lactobacillus casei* Cells Immobilized on Delignified Cellulosic Material' J. Agric. Food Chem., vol.53, pp 2546-2551
25. Prabakaran G, Hoti SL (2008) 'Immobilization of alginate-encapsulated *Bacillus thuringiensis* var. israelensis containing different multivalent counterions for mosquito control' Curr Microbiol. Aug;57: 111-114
26. Prasad B, Mishra M (1995) 'On the kinetics and of immobilized whole-cell batch cultures' Bioresource technology ISSN 0960-8524, vol.53, pp. 269-275
27. S.V. Ramakrishna and R.S. Prakasham (2000) 'Microbial fermentations with immobilized cells' Biochemical and Environmental Engineering, Indian Institute of Chemical Technology, Hyderabad
28. Robledo-Ortiz JR, Ramirez-Arreola DE, Gomez C, Gonzalez -Reynoso O, Gonzalez-Nunez R. (2009) 'Bacterial immobilization by adhesion onto agave-fiber/polymer foamed composites'. Bioresour Technol. pp:1293-9
29. Subba Rao, R.S. Prakasham, Ch. Sri Lakshmi and A. Bhaskar Rao (2009) 'Effect of various immobilization matrices on *Lactobacillus delbrueckii* cells for optically pure L (+) lactic acid production', Current Trends in Biotechnology and Pharmacy, vol 3
30. Valeri Coeuret, Segolene Dubernet, Marion Bernardeau, Micheline Gueguen and Jean Paul Vernoux (2003), 'Isolation, Characterisation and identification of lactobacilli focusing mainly on cheeses and other dairy products' Electronic Journal of Biotechnology.
31. Wong H, Davis RC, Hill JS, Yang D, Schotz MC (1997), 'Lipase engineering: a window into structure-function relationships,' Lipid Research Laboratory, West Los Angeles VA Medical Center, California, vol 284: pp 171-84