Research Article

COMPARATIVE EVALUATION OF SPECIFIC ACTIVITY OF CRUDE AND PARTIALLY PURIFIED LIPASE PREPARATION FROM LIPOLYTIC BACTERIA

Dr. Kanika Sharma and Madhu Rathore*

Address for Correspondence

Microbial Research Laboratory, Department of Botany, University College of Science, M.L.S. University Udaipur, Rajasthan – 313001, India

E-mail: madhurathore25@gmail.com

ABSTRACT

Most of the medical and commercial applications demands a certain degree of purity in lipase preparations for efficient and successful use hence, lipase from twelve bacterial strains was partially purified by ammonium sulphate precipitation and dialysis method. Agar well diffusion technique as a qualitative method and titrimetric assay as a quantitative method of lipase activity was used. Highest qualitative and quantitative lipolytic activity was observed for crude as well as partially purified lipase from MRL S and MRL 05. Whereas least but significant activity of lipase was observed for strains MRL b and MRL 01.Comparative study of crude and partially purified lipase preparations showed that lipase activity enhanced with concomitant increase in the purity level of lipase. Hence further physicochemical characterisation and purification of lipid degrading principal will be helpful for mass cultivation of these strains as well as for the development of formulations of medical and commercial applications.

KEY WORDS: Lipolytic activity, qualitative, quantitative, medical and commercial applications, lipid degrading principal

INTRODUCTION

Lipases are enzymes belonging to the group of serine hydrolases (E.C.3.1.1.3). Their natural substrates are triglycerides. Lipases are able to catalyze hydrolysis, esterification, transesterification and lactonization (intramolecular esterification) [(1)]. Depending on the source of isolation, lipase production from bacteria may be induced by adding fatty substrate to lipase induction media or constitutively produced in bacterial strains isolated from oily sources. In both cases lipase from bacteria not only have good turn over but also have capability to withstand varying range of temperature, pH and mixture of physical and chemical condition. This flexibility, associated with the possibility of different substrate specificity among the different lipases, gives these enzymes an enormous potential for applications [(2)]. Several workers studied the qualitative and quantitative lipolytic activity in bacterial isolates. Bhatnagar *et al*.[(3)] have studied the lipolytic activity in halobacteria by agar plate method and calorimetric method. Jaeger *et al*. [(4)] and Kim *et al*.[(5)] determined the lipolytic activity by using tributyrin-containing agar plates. Kamaly *et al*.[(6)] studied lipolytic activity in *Streptococcus lactis*, *Streptococcus cremoris* and their mutants by titrimetric method. Lee *et al*. [(7)] determined lipase activity in *Aeromonas* sp. LPB 4 by using p-nitrophenyl esters as a fatty substrate. Ren *et al*. [(8)] studied the lipase activity in *Pseudomonas fluorescence* 27 by agar well diffusion and titrimetric method. Reports are also available on partial purification of lipase from different bacteria. Lipase from *Pseudomonas fluroscence* Strain 2D was partially purified by ammonium sulphate precipitation technique [(9)]. Schuepp *et al*. [(10)] partially purified the lipase from *Pseudomonas fragi* CRDA 037. Lipases, triacylglycerol hydrolases, are an important group of biotechnologically relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries. Lipases are by and large produced from microbes and specifically bacterial lipases play a vital role in commercial ventures [(11)]. Lipases with new specificities are needed and the engineering of cloned enzymes as well as

the isolation of new lipases from natural sources and subsequent purification is therefore of increasing potential value. There are few reports available on isolation of lipolytic bacteria from food samples and subsequent partial purification of lipase. Hence in the present study attempt has been made to determine lipase activity of crude lipase from twelve bacterial isolates and further partial purification of crude lipase preparations to get better activity and improve their possibilities of commercialisation.

MATERIALS AND METHODS

Lipolytic Bacterial isolates: MRL01, MRL 04, MRL 06,MRL 06a, MRL 05, MRL 11b, MRL 12, MRL A, MRL B, MRL a, MRL b, MRL S isolated from food sources like milk, dairy products, fermenting vegetables, juices etc by enrichment [12] and selective culture technique [4] were used for the study. Crude lipase preparations from these isolates subjected to partial purification and subsequent assay for lipase activity. Standard lipase: *Pseudomonas lipase* (Sigma- aldrich) and Lipase type II from porcine pancreas (Hi Media) were used as positive control. Experiments were repeated thrice and three replicates were maintained.

- **Crude lipase preparation from lipolytic isolates**

Lipase production media was inoculated with 4x 10^6 CFU of respective lipolytic isolates and incubated in an orbital shaker at 220 rpm at 37 ^{0}C for 24h . The culture was subsequently centrifuged at 5000 rpm for 10 minutes to obtain clarified supernatant, which was filtered through 0.22 µm membrane filters to obtain cell free extract. Cell free extract was used as crude lipase preparation.

- **Partial purification of lipase**

Lipase from twelve bacterial strains was partially purified by ammonium sulphate precipitation and dialysis method. Ammonium sulphate precipitation was done by modified method of Fucinos *et al*. [13]. According to this, solid $(NH_4)_2SO_4$ was slowly added to the crude enzyme solution under continuous stirring at 0^0C until the required saturation percentage was reached (50–100%). Then, the solution was centrifuged (10,000 rpm, 20 min, 4^0C) and the precipitate obtained was re-dissolved in 0.02 M Tris HCl buffer (pH 7.5) and stored at 4^0 C. Precipitate obtained from ammonium sulphate precipitation was incorporated to dialysis using dialysis membrane-50 (Hi-media). For this purpose, precipitate was dissolved in minimum amount of Tris-HCl buffer (pH 7.5), containing $CaCl_2$ and dialyzed against the same buffer for 24 h. The dialyzate was collected in a sterile container and stored at 4°C as a partially purified lipase preparation.

- **Qualititative determination of lipolytic activity**

Cup well method [14] was used to determine lipolytic activity of crude lipase from these bacterial isolates. 100 µl of cell free extract was filled into 12 mm wells in tributyrin agar plates which were subsequently incubated at 37 ^{0} C for 24 to 48 h. A clear zone around the well indicated lipid hydrolysis. The size of clear zone was measured with the help of Hi-Antibiotic zone scale-C. Nutrient broth was used as negative control while porcine lipase (Hi-Media) and Pseudomonas lipase III (Sigma-Aldrich) as were used as positive control.

- **Quantitative estimation of lipolytic activity**

Titration method of Deeth and Touch [15] was used to measure the amount of FFA released by the activity of crude lipase. The reaction mixture containing crude lipase preparation, tributyrin and phosphate buffer (0.2 M, pH 7.2) in 1:1:2 ratio respectively was placed in a glass stoppered test tube and incubated for 24h at 37 ^{0} C temperature. After specified time of incubation, 2 ml of reaction mixture was mixed with 10 ml of extraction mixture (40:10:1, vol/vol/vol, isopropanol: petroleum ether: 4N H_2SO_4), 6 ml of petroleum ether and 4ml of water. The mixture was shaken

vigorously. The layers were allowed to separate for 10 min. at 25^0 C and 2 ml of the upper layer were then titrated with 0.02 N methanolic KOH using 50 µl of methanolic phenolphthalein as an indicator. Each time FFA content was determined using the formula $(TN/PV)x10^3$, where T is the net titration volume, N is the normality of the methanolic KOH , P is the proportion of the upper layer titrated, and V is the volume (in millilitres) of sample. One activity unit was defined as the amount of enzyme that produced 1 µmol butyric acid per hour under standard assay conditions. Lipase activity was expressed in U /mg.

- **Determination of protein content**

Protein content of cell free extract was assayed by the Bradford method [16] using 5 ml of CBB dye and 1ml of 0.1 M phosphate buffer (pH 7.5). Bovine serum albumin was used as a standard.

RESULTS AND OBSERVATIONS

Isolation of crude and partially purified preparations from bacterial isolates

Total 200 bacterial and fungal isolates were obtained from 31 samples screened. Among these 200 isolates, 12 bacterial strains named as, MRL 01, MRL 04, MRL 06, MRL 06a, MRL 05, MRL 11b, MRL 12, MRL A, MRL B, MRL a, MRL b and MRL S, showing significant lipolysis were purified from different food samples. Clarified centrifuged overnight culture of these isolates was used as crude lipase preparations while further precipitation and dialysis of crude lipase preparations, give certain level of purity, and was used as partially purified lipase.

Qualitative estimation of lipase activity of crude and partially purified lipase

Table no.1 and 2, showed the results of qualitative lipolytic activity of crude and partially purified lipase from bacterial isolates. Lipase preparations from all the bacterial isolates possess significant lipolytic activity. As the level of purity increase improvement in lipolytic activity was also observed. Among the crude as well as partially purified preparations, lipase from MRL S showed maximum lipolytic activity followed by MRL 05, MRL 11b, MRL 12, MRL 06, MRL 04, MRL A, MRL 06a, MRL B, MRL a and MRL b. Lipolytic activity of crude and partially purified preparations from MRL 04, MRL A and MRL 06a found to be almost similar to maintained positive controls. Whereas these preparations from bacterial isolates MRL S, MRL 05, MRL 11b, MRL 12 and MRL 06 found to be possess lipolytic activity higher than maintained control.

Quantitative estimation of lipase activity of crude and partially purified lipase

Results of specific activity of crude and partially purified lipase are given in table no 3 and 4. After 72 h incubation of reaction mixture, maximum lipase activity was observed for MRL S whereas least activity was observed for crude lipase of MRL 01 and MRL b. Specific activity of crude lipase from MRL 06, MRL 05, MRL 11b, MRL 12, and MRL S were observed to be greater than maintained controls Whereas lipase activity of MRL 04A, MRL06 a and MRL A found to be comparable with maintained controls. Lipase activity from MRL B, MRL a and MRL b was observed to be lesser than positive controls. Specific activity of partially purified lipase was observed to be superior over crude lipase. Partial purification leads 28.37 % increase in lipase activity of MRL S over crude lipase. Maximum percent increase in lipase activity was observed for MRL b followed by MRL B, MRL A, MRL 01, MRL 04, MRL a, MRL 06a, MRL 06, MRL 12, MRL 11b, MRL 05 and MRL S. Maximum Lipase activity noticed for MRL S while least was observed for MRL 01. Specific lipase activity of partially purified preparations from all the bacterial isolates was found to be higher than maintained controls.

Table 1: Qualitative lipase activity from crude lipase preparations

S.No.	Isolates	Zone of hydrolysis (in mm)			Mean± S.D.
		R1	R2	R3	
1	MRL 01	2.0	2.1	1.9	2.0±0.08
2	MRL 04	7.0	6.9	7.1	7.0±0.08
3	MRL 06	9.1	9.0	8.9	9.0±0.08
4	MRL 06a	6.0	6.1	5.9	6.0±0.08
5	MRL 05	11.9	12.0	12.1	12.0±0.08
6	MRL 11B	9.9	10.0	10.1	10.0±0.08
7	MRL 12	9.1	9.0	8.9	9.0±0.08
8	MRL A	5.0	4.9	5.1	5.0±0.08
9	MRL B	4.0	4.1	3.9	4.0±0.08
10	MRL a	3.0	3.1	2.9	3.0±0.08
11	MRL b	2.1	1.9	2.0	2.0±0.08
12	MRL S	13.1	13.0	12.9	13.0±0.08
13	Pseudomonas lipase	9.5	9.0	9.5	9.3
14	Pancreatic Porcine lipase	8.0	7.5	8.0	7.8

Table no.2: Qualitative lipase activity from partially purified preparations

S.No.	Isolates	Zone of hydrolysis (in mm)			Mean± S.D.
		R1	R2	R3	
1	MRL 01	5.0	5.1	4.9	5.0±0.08
2	MRL 04	9	8.9	9.1	9.0±0.08
3	MRL 06	11.1	11.0	10.9	11.0±0.08
4	MRL 06a	7.0	7.1	6.9	7.0±0.08
5	MRL 05	13.9	14.0	14.1	14.0±0.08
6	MRL 11B	11.9	12.0	12.1	12.0±0.08
7	MRL 12	11.1	11.0	10.9	11.0±0.08
8	MRL A	7.0	6.9	7.1	7.0±0.08
9	MRL B	6.0	6.1	5.9	6.0±0.08
10	MRL a	5.0	5.1	4.9	5.0±0.08
11	MRL b	5.1	4.9	5.0	5.0±0.08
12	MRL S	15.1	15.0	14.9	15.0±0.08
13.	Pseudomonas Lipase	9.5	9.0	9.5	9.3
14.	Pancreatic porcine lipase	8.0	7.5	8.0	7.8

Table 3: Quantitative estimation of lipase Activity from Crude preparations (After 72 h)

S.No.	Isolates	µmole of FFAs released/ h (U)				Specific activity (Total activity/mg)
		R1	R2	R3	Mean±S.D.	
1	MRL 01	0.500	0.500	0.458	0.486±0.019	4.811
2	MRL 04	1.041	1.000	1.041	1.027±0.019	13.166
3	MRL 06	1.000	1.083	1.041	1.041±0.033	14.260
4	MRL 06a	0.875	0.833	0.875	0.861±0.019	10.762
5	MRL 05	1.791	1.750	1.791	1.777±0.019	71.08
6	MRL 11b	1.416	1.458	1.500	1.458±0.034	31.021
7	MRL 12	1.208	1.166	1.208	1.194±0.019	19.573
8	MRL A	0.875	0.833	0.875	0.861±0.019	10.373
9	MRL B	0.750	0.708	0.791	0.749±0.033	7.721
10	MRL a	0.708	0.666	0.708	0.694±0.019	7.543
11	MRL b	0.541	0.500	0.583	0.541±0.033	4.873
12	MRL S	2.041	2.000	1.958	1.999±0.033	90.863
13	PC 1	1.083	1.041	1.083	1.069	13.362
14	PC 2	0.833	0.958	0.833	0.874	10.925

Table 4: Quantitative estimation of lipase Activity from partially purified preparations (After 72 h)

S.No.	Isolates	µmole of FFAs released/ h (U)				Specific activity (Total activity/mg)	Fold Purification
		R1	R2	R3	Mean±S.D.		
1	MRL 01	1.208	1.166	1.208	1.194±0.01	11.821	59.30
2	MRL 04a	2.333	2.291	2.333	2.319±0.01	31.958	58.83
3	MRL 06	2.458	2.416	2.125	2.333±0.14	29.758	52.07
4	MRL 06a	1.958	1.916	2.000	1.958±0.03	24.475	56.02
5	MRL 05	2.625	2.666	2.625	2.638±0.01	105.52	32.63
6	MRL 11b	2.541	2.500	2.541	2.527±0.01	53.765	42.30
7	MRL 12	2.458	2.416	2.458	2.444±0.01	40.065	51.14
8	MRL A	2.166	2.125	2.083	2.124±0.03	25.590	59.46
9	MRL B	1.958	1.916	1.958	1.944±0.01	20.041	61.47
10	MRL a	1.625	1.583	1.625	1.611±0.01	17.510	56.92
11	MRL b	1.583	1.625	1.583	1.597±0.01	14.387	63.90
12	MRL S	2.791	2.750	2.833	2.791±0.03	126.863	28.37
13	PC 1	1.083	1.041	1.083	1.069	13.362	-
14	PC 2	0.833	0.958	0.833	0.874	10.925	-

DISCUSSION

The main objective of this research was detection of activity of crude and partially purified lipase preparations from bacterial isolates. Different medical and industrial applications require different extent of purity level in enzymatic preparations. Hence it is very important to observe the significant lipolytic activity at different purity level of these enzymes. In the present study, significant lipase activity was observed from both preparations, crude as well as partially purified lipase further there was direct relation observed between enhanced lipase activity (qualitative as well as quantitative) and purity of preparations. As centrifuged and clarified overnight bacterial culture was used as crude lipase preparations hence in addition to lipase this preparation also contained other proteins, metabolites form bacteria. Whereas main content of partially purified preparations are lipase with small amount of other proteins. Partial purification of enzyme via ammonium sulphate precipitation based on neutralisation of unequal charges of proteins. Biswas and Biswas [17] stuidied that proteins remain in solution till the charges are unequal. Proteins are usually precipitate out at particular pH level i.e. at isoelectric point. Addition of more cations and anions in the form of salt solution also neutralize this charges and proteins got precipitated out [17]. Dialysis of precipitated protein is very important step to further concentrated enzyme with less impurity in available amount. As compared to crude lipase, improvement in activity was observed from partially purified lipase. Increase in lipase activity depend on the concentration of ammonium sulphate solution used [18]. Large quantities of material can be handled, and this step is less affected by interfering non-protein materials than chromatographic methods. In comparison to other techniques, which give lower yields (60–70%), precipitation methods often have high average yield (87%) [19]. Bacteria produce different classes of lipolytic enzymes, which are mostly extracellular; some of them have affinity to short-chain fatty acids, some have preference for unsaturated fatty acids and many others are non-specific [20]. Quantitative analysis is important to compare the lipolytic activities of various isolates. There is no single universal method of lipase assay. The choice of a particular method will depend on the user's own specific requirements. For the assay of any enzyme, the sensitivity, availability of substrates, and ease of the procedure have to be considered [21]. Free fatty acids released by lipase activity can be estimated by methods like titrimetric method [22], spectrophotometric method [3], HPLC [23], GC [24] etc. Among which most convenient and cheapest methods are titrimetric method and spectrophotometric analysis. In titrimetric method change in acidity of reaction mixture, due to released free fatty acids, are measured by acid base titration in which phenolphthalein used as an end point indicator. Various plate assay methods using selective media have been described for detection of lipolytic activity in microorganisms. These methods include incorporation of fluorogenic substrate like methyl umbeliferyl in plating media [25], incorporation of indicator dye like methyl red, phenol red, rhodamine B or Victoria blue B in

media [26], use of TBA media [14] etc. The basic principle behind these methods is the hydrolysis of lipid substrate present in the media by the microbial enzyme which can be observed as a clear zone around the bacterial colonies or wells containing cell free extract. The size of halo produce is directly proportional to lipase concentration [27]. Study suggested that purification of lipase from bacterial isolates improve specific lipase activity hence turn over number of enzyme. However ammonium sulphate precipitation leads certain level of purity but such enzyme preparations can be used for detergent formulations. However, for certain applications, such as synthetic reactions in pharmaceutical industry, further purification is needed.

REFERENCES

1. Jaeger, K. E. and Eggert, T.. 2002. Lipases for biotechnology. *Current Opinion in Biotechnology*, Oxford.13, 390- 397.
2. Gandhi, N. N., Patil, N. S., Swant, S. B. and Joshi, J. B.. 2000. Lipase-catalysed esterification. *Catalysis Reviews Science Engineers*, [S.l.]. 42(4), 439-480.
3. Bhatnagar, T., Boutaiba, S., Hacene, H., Cayol, J. , Fardeau, M., Ollivier, B. and Baratti, J. C.. 2005. Lipolytic activity from Halobacteria: Screening and hydrolase production. FEMS Microbiology Letters. 248, 133–140.
4. Jaeger, K.E., Ransac, S., Dijkstra, B. W., Colson, C., Vanheuvel, M. and Misset, O. 1994. Bacterial lipase. *FEMS Microbiol Rev*.15, 29–63.
5. Kim, E.K., Jang, W. H., Ko, J.H., Kang, J. S., Noh, M. J. and Yoo, O. J.. 2001. Lipase and its modulator from *Pseudomonas sp.* strain KFCC 10818: proline-to-glutamine substitution at position 112 induces formation of enzymatically active lipase in the absence of the modulator. *J Bacteriol*. 183(20), 5937–41.
6. Kamaly, K.M., Takayama, K. and Maath, E. H.. 1989. Acylglycerol Acylhydrolase (Lipase) activities of *Streptococcus lactis, Streptococcus cremoris,* and their mutants. *J Dairy Sci*. 73,280-290.
7. Lee, H., Ahn, M., Kwak, S., Song, W. and Jeong, B. 2003. Purification and characterization of cold active lipase from psychrotrophic *Aeromonas* sp. LPB 4. *The Journal of Microbiology*. 41 (1), 22-27.
8. Ren, T. J., Frank, J. F. and Christen, G. L. 1988. Characterization of Lipase of *Pseudomonas fluorescens* 27 based on Fatty Acid Profiles. *Journal of Dairy Science*. 71(6).
9. Makhzoum, A., Owusu-Apenten, R. K. and Knappb, J. S. 1996. Purification and properties of lipase from *Pseudomonas fluorescens* Strain 2D. *Dairy Journal*. 6, 459-412.
10. Schuepp, C., Kermasha, S., Michalski, M. and Morin, A. .1997. Production, partial purification and characterisation of lipases from *Pseudomonas fragi* CRDA 037. *Process Biochemstry*. 32 (3), 225-232.
11. Gupta, R., Gupta, N. and Rathi, P. 2004. Bacterial lipases: an overview of production, purification and biochemical properties. *Appl Microbiol* Biotechnol. 64, 763–81.
12. Vargas, V.A., Delgado, O. D., Hatti-Kaul, R. and Mattiasson, B. 2004. Lipase-producing microorganisms from a kenyan alkaline soda lake. *Biotechnology Letters*. 26, 81– 86.
13. Fucinos, P., Abad´ın, C.M., Sanrom´an, A., Longob, M. A., Pastrana, L., R´ua, M. L. 2005. Identification of extracellular lipases/esterases produced by *Thermus thermophilus* HB27: Partial purification and preliminary biochemical characterisation. *Journal of Biotechnology*. 117, 233–241.
14. Blake, M. R., Koka, R. and Weimer, B. C.. 1996. A semi automated reflectance colorimetric method for the determination of lipase activity in milk. *J Dairy Sci*. 79, 164-1171.
15. Deeth, H.C. and Touch, V. 2000. Methods for detecting lipase activity in milk and milk products. *Australian Journal of Dairy Technology*. 55, 153–168.
16. Bradford, M.M. 1976. A rapid and sensitive method for quantification of microgram quantity of protein utilizing the principles of protein dye binding. *Anal. Biochem*.72, 248-254.
17. Biswas, S. B. and Biswas, A. 1984. An introduction to viruses. (3rd edn).Vikas Publishing House. India.
18. Pabai, F., Kermasha, S. and Morin, A.. 1995. Lipase from *Pseudomonas fragi* CRDA 323: partial purification, characterization and interesterification of butter fat. *Appl. Microbiol. Biotechnol.* 43,42– 51.
19. Aires-Barros, M.R., Taipa, M. A. and Cabral, J.M.S. 1994. Isolation and purification of lipases, pp. 243– 270. In: Woolley, P., Petersen, S.B. (eds.), Lipases: Their Structure, Biochemistry and

Application.Cambridge Univ. Press, Cambridge..

20. Saxena, R.K., Ghosh, P. K., Gupta, R., Sheba Davidson, W., Bradoo, S., Gulati, R. 1999. Microbial lipases: potential biocatalysts for the future industry. *Curr Sci* .77, 101-115.
21. Hendrickson, H.S. 1994. Fluorescence-based assays of lipases, phospholipases, and other lipolytic enzymes. *Anal Biochem*. 219, 1–8.
22. Grbavcic, S. Z., Dimitrijevic- Brankovic, S. I., Bezbradica, D. I., Siler-Marinkovic, S. S. and Knezevic, Z. D. 2007. Effect of fermentation conditions on lipase production by *Candida utilis*. *J. Serb. Chem.Soc*. 72 (8-9), 757-765.
23. Maurich, V., Zacchigna, M. and Pitotti, A.. 1991. p-nitrophenyllaurate: a substrate for the highperformance liquid chromatographic determination of lipase activity. *J Chromatogr*.566(2), 453–9.
24. Patel, M. T., Nagarajan, R., and Kilara, A.. 1996. Lipase-catalyzed biochemical reactions in novel media: A review. *Chem. Eng. Commun*. 152-153, 365-404.
25. Kim, S. J. and Hoppe, H. G.. 1986. Microbial extracellular enzyme detection on agar plates by means of fluorogenic methylumbelliferyl- substrate. *Actes de Colloques*. 3, 175- 183.
26. Samad, M.Y. A., Razak, C. N. A., Salleh, A. B., Zin Wan Yunus, W. M., Ampon, K. and Basri, M. 1989. A plate assay for primary screening of lipase activity. *J.Microbiol.Methods*. 9(1), 51-56.
27. Thomson, C. A., Delaquis P. J. and Mazza. G.1999. Detection and measurement of microbial lipase activity: A review. *Critical Reviews in Food Science and Nutrition*. 39(2), 165–187.