Screening and Microbial Characterization of Lipase Producing Organic Solvent Tolerant *Lysinibacillus Fusiformis* C5 (MTCC 11801)

Rajesh.B, Usha Devi.Ch, Raga Aruna Bindu.D, Bhaskar Reddy.I

Abstract -The aim of the present work was to isolate and screen a lipase producing bacterium that can tolerate different organic solvents and its microbial characterization. A total of 12 cultures originating from sun dried spoiled coconut were successfully screened for lipolytic activity by Rhodamine B-Olive oil plate assay. Six cultures shown tolerance to various organic solvents (acetone, benzene, toluene, ethyl benzene, 1-butanol, n-hexane and n-heptane) with hydrolytic zones when flooded on tributyrin agar medium. The maximum zone of hydrolysis is observed in culture C5 on plates flooded with n-hexane with maximum activity of 4.6 U/ml at 37°C after 48 hrs incubation period at 150 rpm. Culture C5 showed growth at temperature range 30°C to 45°C, pH range 5.8 to 9, NaCl concentration 2.5% to 5% and retained 99% of its initial activity upon exposure n-hexane. Later the culture C5 was identified as *Lysinibacillus fusiformis* by morphological, biochemical and molecular characterization and was deposited in Microbial Type Culture Collection (MTCC) under the accession number MTCC 11801.

Keywords- *Lysinibacillus fusiformis*, Lipase, n-hexane, Rhodamine B, Tributyrin, Solvent-tolerant, Spoiled coconut.

1 INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3) are the enzymes that catalyses the hydrolysis of water-insoluble triglyceride at an oil-water interface to release di-acylglyceride, mono-acylglyceride, free fatty acid and glycerol, while in immiscible or anhydrous solvents lipases accelerate other chemical reactions such as esterification, transesterification, aminolysis, acidolysis, and alcoholysis [1]. Lipases are ubiquitous enzymes which are widely distributed in plants, animals and microbes. Lipases especially of microbial origin, have great potential in commercial applications such as detergents (hydrolysis of fat), fine chemicals (synthesis of ester), additives in food (flavour modification), waste water treatment (decomposition and removal of oil substances), cosmetics (removal of lipids), leather (removal of lipids from animal skins), pharmaceuticals (digestion of oil and fats in foods) and analytical (blood triglyceride assay) [2], [3].

The extracellular bacterial lipases are of considerable commercial importance, as their bulk production is much easier [4]. Use of enzyme in organic media has exhibited many advantages: increased activity and stability; regiospecificity and steroselectivity; higher solubility of substrate and ease of products recovery; ability to shift the reaction equilibrium toward synthetic direction [5]. Enzymes are generally not stable in the presence of organic solvents and are apt to denature and inactivated, most organisms lose their functions and cease growing. Therefore, search for organic solvent tolerant (OST) enzymes has been an extensive area of research [6]. Some organic solvent tolerant bacterial strains have been reported in recent years [7].

We hypothesized that extracellular enzymes secreted by organic solvent tolerant microorganisms were stable in the presence of organic solvents. In a similar effort, we have isolated a lipase producing bacterial strain from spoiled coconut which tolerates organic solvents to a certain degree and will be very useful in industry as catalysts for the reactions in the presence of organic solvents. This paper describes the screening, microbial characteristics and phylogenetic tree construction of an isolated OST culture C5.

2 MATERIAL AND METHODS

2.1 Microorganism

An organic solvent tolerant (OST) bacterial strain used in this study for lipase production was isolated from sun dried spoiled coconut and identified as *Lysinibacillus fusiformis.* This identified strain was deposited in Microbial Type Culture Collection (MTCC) as *Lysinibacillus sp.* under the accession number MTCC 11801.

2.2 Isolation and Screening of True Lipase Producing Strains

Samples collected from sun dried spoiled coconut were processed by dilution technique using nutrient agar medium to isolate bacterial strains [8]. Each isolate was tested for its true lipase production by modified sensitive plate assay on Rhodamine B-Olive oil agar medium [9].

Rhodamine B- Olive Oil Agar Medium

The screening medium containing 0.8% of nutrient

broth (v/v), 0.4 %(w/v) of sodium chloride, 2 %(w/v) of agar-agar medium, was prepared in distilled water with pH adjusted to 7.0, autoclaved at 121°C for 20 minutes and cooled to 60 °C. The cooled media was added with sterilized 3% olive oil and 1 ml Rhodamine B (10mg/ml) with vigorous stirring and emulsified for one min and was allowed to stand for few minutes to reduce foaming. Aliquots of 20 ml were poured into each petridish to solidify. Each culture was streaked and incubated at 37°C for 48 hrs. The lipase producers were identified by the presence of orange colonies, whereas non-lipase producers were identified by the presence of pink colonies.

2.3 Tolerance of Lipase Producers to Various Organic Solvents

The selected positive cultures obtained were further screened on tributyrin agar medium to check their tolerance to various organic solvents [10].

Tributyrin Agar Medium

The screening medium containing 0.5% (w/v) of peptone, 0.3 %(w/v) of yeast extract, 0.1% (v/v) tributyrin, 2% of agar (w/v), pH 7.5 was prepared in distilled water, autoclaved and cooled to 60°C. Aliquots of 20 ml were poured into each petridish to solidify. The positive colonies were streaked on tributyrin agar plates, upon which each plate was flooded with 7 ml of different organic solvents such as acetone, benzene, toluene, ethyl benzene, 1-butanol, n-hexane and n-heptane. At the same time controls (without flooding of organic solvent) were also prepared for zone comparison. All the plates were now incubated at 37°C for 48 hrs. The ability of the colonies to grow under organic solvent conditions and to produce lipase was determined by D-d values.

D-d value = Diameter of the total clear hydrolytic halo including colony (D) – Diameter of the colony (d)

2.4 Lipase Production by Submerged Fermentation

The lipase production medium used was tributyrin broth composed of 0.5 % (w/v) peptone, 0.3% (w/v) yeast extract, 1% (w/v) glycerol tributyrate, pH 7. The medium was inoculated with 1% inoculum of an overnight grown culture of the selected colony and incubated in orbital shaker (150 rpm) at 37°C for 48 hrs. After incubation time the culture broth was harvested by centrifugation at 10,000 rpm for 15 min at 4°C and the supernatant was used as crude enzyme to assay lipase activity and protein estimation.

Lipase Assay by Titration Method

The alkali titrimetric assay was performed using olive oil as a substrate. The enzyme activity was determined by titration of the free fatty acids liberated from olive oil against standard alkali solution [11].

Lipase assay was done by incubating 5 ml of olive oil emulsion (composed of 1.25ml olive oil and 3.75 ml of 2 % polyvinyl alcohol solution), 4 ml of 0.05 M phosphate buffer and 1 ml of enzyme solution at 37°C for 20 min. After incubation, the reaction was stopped by adding 20 ml of 95% ethanol and the liberated free fatty acid was titrated against 0.05 M NaOH using phenolphthalein as indicator. Blanks were prepared with a heat inactivated enzyme sample (at 100°C for 15 min) and after cooling to ambient temperature, the sample was used as described for the active enzyme sample. Lipase activity was calculated using the following formula

$$\text{Lipase activity (U/ml)} = \frac{(T-C) \times \text{Normality of NaOH} \times 100}{\text{Time of incubation}}$$

One unit of lipase activity was defined as the 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.

Protein Estimation

Protein content was estimated according to the method of Lowry et al., using Bovine serum albumin (BSA) as the standard. OD was measured at 660 nm and expressed in µg/ml [12].

2.5 Microbial Characterization

Phenotypic Characterization

Phenotypic characterization of the selected isolate was studied based on different morphological, physiological and biochemical characteristics. The data was compared with standard description given in Bergey's Manual of Determinative Bacteriology [13].

Molecular Characterization

The genomic DNA was isolated according to the procedure of Weisberg et al [14]. About 50-100 ng of the purified DNA was used for sequencing by PCR using Big Dye® Terminator v3.1 Cycle Sequencing Kits [Applied Bio systems] with two degenerate primers–forward: 5´-AGAGTTTGATCHYGGYTYAG-3´; and reverse: 5´-ACGGCTACCTTGTTACGACTT-3´. Cycling conditions for sequencing PCR were as follows: Denaturation at 96°C for 10s, annealing at 55°C for 10s and extension at 60°C for 4 min. After 35 cycles, the templates were purified by Ethanol/EDTA precipitation method and sequenced on ABI 3730xls Genetic Analyzer (Applied Biosystems). The nucleotide sequence obtained was deposited in DNA Data Bank of Japan (DDJB) with the following accession number AB853119. The deduced sequence was subjected to homology search using basic local alignment search tool (BLAST) programme of the National Centre for Biotechnology Information (NCBI) [15]. Representative sequences of 10 most similar neighbors were retrieved and

aligned using CLUSTAL W for multiple alignments. The multiple alignment file was then used to create neighbor-joining tree using MEGA version 4 software [16].

3 Results And Discussions

3.1 Isolation and Screening of True Lipase Producers

Among numerous isolated bacterial cultures from sun dried spoiled coconut processed by dilution technique, 12 cultures were recognized as potential true lipase producers using Rhodamine-Olive oil sensitive plate assay. An example of lipolytic activity detected by Rhodamine B-Olive oil plate assay is shown in figure 1. Further all these 12 cultures were checked for tolerance to various organic solvents.

Only one bacteria from spoiled coconut producing lipase was reported till date and it was identified as *B.cereus* [17].

Fig. 1: Orange colonies (positive) and pink colonies (negative)

3.2 Tolerance of Lipase Producers to Various Organic Solvents

Organic solvent tolerance of the selected cultures were investigated by their ability to grow on tributyrin agar plates flooded with different organic solvents (acetone, benzene, toluene, ethyl benzene, 1-butanol, n-hexane and n-heptane) during incubation. Among the 12 cultures, 6 cultures exhibited growth as well as lipolytic zone in presence of all the above solvents. The maximum zone of hydrolysis is observed in culture C5 on plates flooded with n-hexane by comparing with that of controls (absence of n-hexane) which are shown in table 1.

Several authors have reported bacteria tolerant to organic solvents [18], including Benzene tolerant *Rhodococcus sp.* [19] and *Bacillus sp.* [20], butane tolerant *Bacillus sp.* [21]. Whereas, bacteria isolated in this report are found to be capable of tolerating both aromatic and aliphatic organic solvents with maximum tolerance to n-hexane.

Table 1: Represents the D-d values in presence and absence of n-hexane and Lipase activity of OST bacterial cultures.

Culture	D-d values in absence of n-hexane	D-d values in presence of n-hexane	Lipase Activity (U/ml)
C2	18mm	13mm	4.0
C5	29mm	28mm	4.6
C9	21mm	11mm	3.5
C15	16mm	13mm	3.9
C21	22mm	16mm	4.2
C28	20mm	12mm	3.7

3.3 Lipase Production by Submerged Fermentation

The ability of the selected 12 cultures for the production of lipase was measured in liquid medium. Lipase activity of 4.6 U/ml was observed for the culture C5 at 37°C for 48 h, 150 rpm and initial pH 7.0. The results for lipase activity of OST bacterial cultures were shown in table 1.

Tributyrin broth is a basic production medium used in this study and normal fermentation conditions are maintained for lipase production.

3.4 Microbial Characterization

Phenotypic Characterization

The selected colony C5 appeared to be irregular, cloudy, smooth, moist, raised, entire and opaque on nutrient agar medium. The bacterium is a gram positive rod, facultative, spore forming and arranged in 2-3 chains. It was able to produce gas and acid in glucose and sucrose broth. When grown at different temperatures, the culture C5 did not grow at temperature of 5°C and 10°C but showed growth between 30°C and 45°C. Growth was observed in media having initial pH between 5.8 to 9 and in media having NaCl concentrations between 2.5% and 5%. Biochemical characteristics of the culture C5 are shown in

table 2. Based on morphological, physical and biochemical characteristics the above bacteria showed typical characteristic of genus *Bacillus*.

Esculin	Positive
ONPG	Positive

Table 2: Biochemical characteristics of the culture C5

Parameter	Characteristic
Oxidase test	Positive
Catalase test	Positive
Nitrate test	Negative
Hydrogen sulfide formation	Positive
Methyl red test	Negative
Vogue–Proskauer test	Negative
Indole production	Negative
Urease activity	Positive
Citrate utilization	Negative
Hydrolysis of:	
Tributyrin	Positive
Triolein	Positive
Gelatin	Positive
Tween 80	Positive
Starch	Positive
Casein	Negative
Carbon sources for growth:	
Starch	Negative
Raffinose	Negative
Sucrose	Positive
Lactose	Negative
Maltose	Negative
Trehalose	Positive
Cellobiose	Negative
Melibiose	Negative
Ribose	Negative
Arabinose	Negative
Xylose	Negative
Rhamnose	Negative
Dextrose	Positive
Mannose	Positive
Galactose	Negative
Fructose	Positive
Adonitol(ribitol)	Negative
Mannitol	Negative
Sorbitol	Negative
Dulcitol(galacitol)	Negative
Inositol	Negative
Myo-Inositol	Negative
Salicin	Positive
Special Tests:	

Molecular Characterization

The identity of the selected culture C5 was confirmed by sequencing of 16S rRNA gene. The sequence of the 16S rRNA gene was compared with GenBank entries, using BLAST programme and the sequence showed a similarity of 99% with the *Lysinibacillus fusiformis* and *Lysinibacillus sphaericus*. From the multiple sequence alignment data, length of sequence was 1266 nucleotides with 1147 conserved and 115 variable nucleotides. It is inferred that the culture *Lysinibacillus sp*. C5 is showing affliation towards *Lysinibacillus fusiformis* with 74% boot strap confidence values (fig. 2) and hence the culture C5 under study is conclusively confirmed as *Lysinibacillus fusiformis*.

Organic solvent tolerant *Lysinibacillus sphaericus* producing lipase was reported by Chin John Hunin *et al.*, [22]. Here we reported organic solvent tolerant *Lysinibacillus fusiformis* producing lipase from spoiled coconut.

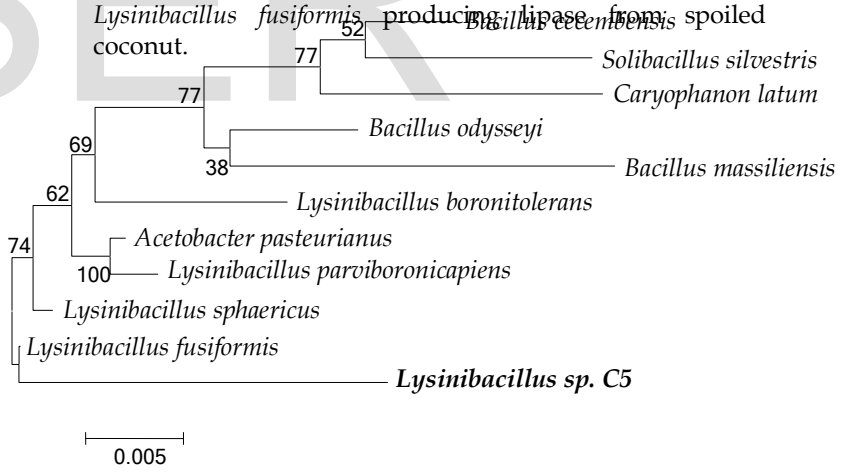

Fig. 2: Neighbor-joining tree of isolate C5 and their closest GenBank entries

4 Conclusion

In the present study a lipase producing bacteria was isolated from sun dried spoiled coconut by Rhodamine B-Olive oil plate assay. It showed tolerance to various organic solvents flooded on tributyrin agar medium with maximum tolerance for n-hexane. The culture was identified as *Lysinibacillus fusiformis* C5 by morphological, biochemical and molecular characterization. This was the

first report to isolate organic solvent tolerant *Lysinibacillus fusiformis* producing lipase from sun dried spoiled coconut. The result of the present study indicated scope for utilizing lipase producing solvent tolerant bacteria for various industrial applications.

ACKNOWLEDGMENT

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] B.Joseph, P.W.Ramteke and G.Thomas,"Cold active microbial lipases: Some hot issues and recent developments,"*Biotechnology Advances*, vol. 26, pp.457–47, 2008.

[2] N.R Kamini, Fujii, T.Kurosu and H.Lefuji,"Production, purification and characterization of an extracellular lipase from the yeast, Cryptococcus sp. S-2,"*Process Biochem*., vol. 36, pp. 317-324, 2000.

[3] T.F.Burkert, F.Maugeri and M.I.Rodrigues," Optimization of extracellular lipase production by Geotrichum sp. Using factorial design,"*Bioresource Technology*, vol. 910, pp. 77-84, 2004.

[4] R.Gupta, N.Gupta and P.Rathi,"Bacterial lipases: an overview of production, purification and biochemical properties," *Appl Microbiol Biotechnol*, vol. 64, pp. 763–781, 2004.

[5] A.L.Serdakowsli and J.S.Dordick,"Enzyme activation for organic solvents made easy,"*Trends in Biotechnology,* vol. 26, pp. 48–54, 2008.

[6] G. A.Sellek and J.B.Chaudhuri,"Biocatalysis in organic media using enzymes from extremophiles,"*Enzyme and Microbial Technology,* vol. 25, pp. 471–482, 1999.

[7] S.Isken and J.A.M.De Bont,"Bacteria tolerant to organic solvents,"*Extremophiles*, vol. 2, pp. 229–238, 1998.

[8] A.J.Salle,"Text book of bacteriology (7th ed.). New Delhi,"*Tata McGraw-Hill*, pp. 274, 1974.

[9] G. Kouker and K.E.Jaeger," Specific and sensitive plate assay for bacterial lipases.,"*Appl. Environ. Microb*, vol. 53, no. 1, pp. 211-213, 1987.

[10] E.H.Ahmed, T. Raghavendra and D.Madamwar,"An alkaline lipase from organic solvent tolerant Acinetobacter sp. EH28: application for ethyl caprylate synthesis,"*Bioresource Technology*, vol. 101, no. 10, pp. 3628–3634, 2010.

[11] J.G.YANG, et al,"Degumming of Vegetable Oil," *Food Technol. Biotechnol,* vol. 44, no. 1, pp. 101–104, 2006.

[12] O.H.Lowry, N.J.Rosenbrough, A.L.Farr and R.I. Randal,"Protein measurement with Folin phenol reagent," *Journal of Biological Chemistry,* pp. 193-265, 1951.

[13] J.G.Holt, N.R.Krieg, H.A.S.Peter and D.H.Bergey,"Bergey's manual of determinative bacteriology (9th ed.)," M.D.Baltimore: Williams & Wilkins, 1994.

[14] W.G.Weisburg, S.M.Barns, D.A.Pelletier and D.J. Lane," 16S ribosomal DNA amplification for phylogenetic study," *Journal of Bacteriology*, vol. 173 pp. 697-703, 1991.

[15] S.F.Altschul, W.Gish, W.Miller, E.W Myers and D.J. Lipman,"Basic Local Alignment Search Tool,"*Journal of Molecular Biology,* vol. 215 pp. 403-410, 1980.

[16] K.Tamura, J.Dudley, M.Nei and S.Kumar,"MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0,"*Molecular Biology and Evolution* vol. 24, pp. 1596-1599, 2007

[17] Dutta Sanghamitra and Ray Lalitagauri,"Production and Characterization of an Alkaline Thermostable Crude Lipase from an Isolated Strain of Bacillus cereus C7," *Appl Biochem Biotechnol, vol*. 159, pp. 142–154, 2009.

[18] S. Torres, A.Pandey and G.R.Castro,"Organic solvent adaptation of Gram positive Bacteria, Application and Biotechnological potential,"*Biotechnol. Adv,* vol. 29, no. 4, pp. 442-52, 2011.

[19] M.L.Paje, B.A.Neilan and I.Couperwhite,"A Rhodococcus species that thrives on medium saturated with liquid benzene," *Microbiol*, vol. 143 pp. 2975–2981, 1997.

[20] K.Moriya and K.Horikoshi,"A benzene-tolerant bacterium utilizing sulfur compounds isolated from deep sea," *J Ferment. Bioeng*, vol. 76 pp. 397–399, 1993.

[21] X.Z.Li and K.Poole, "Organic solvent - tolerant mutants of Pseudomonas aeruginosa display multiple antibiotic resistance," *Can. J. Microbiol*, vol. 45 pp. 18-22, 1999.

[22] J.H. Chin, N.Z.A.R.Raja, B.S.Abu and B.Mahiran, "A newly isolated organic solvent tolerant Bacillus sphaericus 205y producing organic solvent-stable lipase," *Biochemical Engineering Journal,* vol. 15, pp. 147–151, 2003.

- Rajesh.B, Dept. of Biochemistry, College of Science, GITAM, Visakhapatnam-530045, AP, India
- Usha Devi.Ch, Dept. of Biochemistry, College of Science, GITAM, Visakhapatnam-530045, AP, India.
- Raga Aruna Bindu.D Dept. of Biochemistry, College of Science, GITAM, Visakhapatnam-530045, AP India.
- Bhaskar Reddy.I, Professor and Head, Dept. of Biochemistry, College of Science, GITAM, Visakhapatnam-530045,AP, India, Email: ibrgitam@gitam.edu