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ISOLATION, CHARACTERIZATION AND IDENTIFICATION OF POTENT LIPASE PRODUCING BACTERIA FROM OIL SPILLED SOIL OF OIL PROCESSING FACTORY IN BURDWAN, WEST BENGAL

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ABSTRACT
Lipases are glycerol ester hydrolases that catalyze the hydrolysis of triglycerides to free fatty acids and glycerol. Lipases have great importance due to their wide usage in industry. Lipases are produced by microorganisms (bacteria and fungi), plants and animals. However, microbial lipases, especially from bacteria are more useful than their plant and animal derivatives because of several important properties. The primary goal of this study is to isolate and characterize novel lipase producing bacteria from soil sample. We have selected oil spilled soil in Burdwan, West Bengal, India to isolate and characterise novel lipase producing bacteria. The characterisation includes morphological and biochemical characterization of isolates, determination of the enzyme activity and effect of temperature on the growth of the isolates. From biochemical and morphological analysis we found that all isolates were gram positive rods, endospore former, non-citrate utiliser, non-reducer of sulphur compounds etc. From 16s rRNA sequencing four of them was found to be *Bacillus*. To understand the evolution of lipase proteins, we have done a phylogenetic tree with the amino acid sequences from among diverse group of organisms.

KEYWORDS: Lipase producing bacteria, oil factory, *Bacillus.*

INTRODUCTION
Microbial enzymes have a great usage in food, pharmaceuticals, textile, paper, leather and other industries.[16] Their applications have been increasing rapidly. Among industrially important enzymes, hydrolases come in the first place and include enzymes with wide substrate specificity. Carbohydrases, proteases, pectinases and lipases are classified in the group of hydrolases. They catalyze the hydrolysis of natural organic compounds.[5] Lipids play structural roles in membranes and are involved in signalling events. To be able to carry out these functions, lipids require lipolytic enzymes during their metabolism. Lipolytic enzymes are grouped into three main categories, which are esterases, phospholipases and lipases.[2] Lipases (glycerol ester hydrolases triacylglycerol acylhyrolase; EC 3.1.1.3) are defined basically as fat-splitting enzymes that catalyze the hydrolysis of long-chain triacylglycerols to diacylglycerols, monoacylglycerols, fatty acids and glycerols in the presence of excess water. Also, they can catalyze the reverse reaction, synthesis of triacylglycerols, under non-aqueous conditions.[4,6,11] Lipases have many industrial applications, for example, additives in detergents, the elaboration of dietetic foods for use in the food industry and pure optical compounds in chemical synthesis processes as well as modifications of fats and lipids by hydrolysis and esterification reactions.[13] Lipases share a common folding pattern called α/β hydrolase fold.[3] The active site of the α/β hydrolase fold enzymes contains three catalytic residues which are nucleophilic residues (serine, cysteine or aspartate), a catalytic acid residue (aspartate or glutamate) and a histidine residue. In lipases the nucleophile residue has been determined to be a serine residue, but the catalytic acid can be either an aspartate or a glutamate residue. The active site serine residue is located in a highly conserved Gly-X-Ser-X-Ser-pentapeptide.[9,11] In contrast, this well conserved pentapeptide differs in lipases from *Bacillus* strains where the first Gly residue is replaced by an Ala.[7] Another unusual and interesting feature of the structure of most lipases is the presence of a lid-like structure that consists of one or two α-helices. This property results in a conformational change in lipase if there is an interface between oil and water (interfacial activation phenomenon). The lid moves away from the active site, thereby allowing it to become accessible for the substrate.[1] Bacterial lipolytic enzymes are classified into eight families (families i – viii) based on differences in their amino-acid sequences and biological properties. Microbial lipases are mostly extracellular and their production is greatly influenced by medium composition besides physicochemical factors such as temperature, pH, and dissolved oxygen. The major factor for the

expression of lipase activity has always been reported as the carbon source, since lipases are inducible enzymes. These enzymes are generally produced in the presence of a lipid such as oil or any other inducer, such as triacylglycerols, fatty acids, hydrolysable esters, tweens, bile salts, and glycerol.[15]

In this report, we have isolated lipase producing bacteria from oil processing factory at Burdwan, West Bengal, India and from biochemical assay and 16s rRNA sequencing they were found to be *Bacillus* sp.

MATERIALS AND METHODS

Collection of the sample

Sample was collected from oil spilled soil of oil processing factory situated in Alamganj, Burdwan (West Bengal), India. The soil sample was collected in sterile plastic sampling container and labelled properly. The pH of the soil was 7.0.

Isolation of lipase producing organisms

Soil sample was serially diluted and plated in spirit blue agar medium (casein enzymic hydrolysate 10.0 gm, yeast extract 5.0 gm, spirit blue 0.15 gm, agar 2.0 gm) containing 1% (w/v) tributyrin by spread plate method. Plates were incubated at 37°C for two days. Pure cultures of the isolates were maintained on nutrient agar plate (beef extract 3.0 gm (w/v), NaCl 5.0 gm (w/v), peptone 5.0 gm (w/v) and 2% agar, pH 7.0) and were subcultured every 15 days. [12]

Screening of isolates for lipase activity

Lipolytic organisms were screened by qualitative plate assay. Isolates were grown on spirit blue agar with tributyrin and incubated at 37°C for 2 days. The lipase producing isolates took the colour (blue) of the indicator dye (spirit blue). [12]

Culturing and characterization of the isolates

For further analysis, we selected the isolates which took the clear and distinct colour of the indicator dye (spirit blue) and referred as isolates SD-IP_L1 (L_1), SD-IP_ L5 (L_5), SD-IP_L11 (L_{11}), SD-IP_L14 (L_{14}), SD-IP_L23 (L_{23}), and SD-IP_L24 (L_{24}). Morphological and biochemical characteristics of the isolates were studied. Lipase assay of these isolates was done and the effect of temperature on the growth of SD-IP_L_{24} was studied as it showed highest lipase activity than other isolates.

Lipase assay

The selected isolates L_1, L_5, L_{11}, L_{14}, L_{23}, and L_{24} were inoculated separately in tributyrin broth containing 1% (w/v) tributyrin and incubated at 37°C in a shaker at 100 rpm for 48 hours. Then the culture was centrifuged at 10,000 rpm for 20 mins at 4˚C and the cell free supernatant was used as the source of crude extracellular lipase enzyme. Lipase activity was measured by titrimetric method using olive oil as a substrate. Olive oil (10% v/v) was emulsified with gum arabic (5% w/v) in 50mm sodium potassium phosphate buffer pH 7.0. 300 µl of crude extracellular lipase enzyme was added to the emulsion and incubated for 15 minutes at 37^{0}C with continuous agitation. The reaction was stopped and fatty acids were extracted by addition of 2 ml of 95% (v/v) ethanol. The amount of fatty acids liberated was estimated by titrating with 0.05M NaOH until pH 10.5 using a phenophathelin indicator.[8]

Effect of temperature on the growth of the isolate showed maximum lipase activity

The effect of temperature on the growth of isolate showing maximum lipase activity was determined by incubating the culture at different temperatures (10°C, 37°C and 50°C). Inoculum was taken from log phase culture and inoculated in a fresh tributyrin broth so that initial OD becomes 0.2 and incubated at respective temperature keeping the other parameters same. The growth was measured at intervals of 30 minutes by OD at 600 nm.

Characterisation of isolates showing highest lipase activity by 16s rRNA sequencing

The highest lipase activity was shown by the following isolates: L_1, L_{14}, L_{23} and L_{24}. For further characterization by 16S rRNA sequencing, genomic DNA of these isolates were prepared using CTAB method and PCR was done on genomic DNA using primers 9F-5′-AGAGTTTGATCMTGGCTCAG-3′and 1492R-5′-TACGGYTACTTGTTACGACTT-3′. The PCR product was purified using GeNet Bio Prime Prep Gel Purification Kit and 16S rRNA sequencing was done using primer 704F 5′TGTGTAGCGGTGAAATGCGTAGA-3′and 907R 5′CCGTCAATTCMTTTGAGTTT-3′ (M=A or C).

Bioinformatic analysis of bacterial, viral and human lipases

Sequence analysis of lipases

Amino acid sequences of lipase from bacteria, virus and human origins were taken from NCBI protein database. The alignment of the amino acid sequences were done in JustBio hosted tools (www.justbio.com) tools with ClustalW2.0.12 multiple sequence alignment.

Phylogenetic analysis of the isolates and lipases

For phylogenetic analysis of isolates, phylogenetic tree was constructed based on 16S rRNA gene sequences of isolate with different species of *Bacillus.* Phylogenetic tree was constructed using p-distance matrix of neighbour-joining (NJ) algorithms through mega (v4.0). To provide confidence level for the tree topology and statistical reliability of individual nodes, bootstrap analysis were performed with 1000 replications.

For phylogenetic analysis of amino acid sequences of lipase from few selected organisms including bacteria, viruses and humans were aligned in mega (v4.0) software package using CLUSTALW and automatically edited in the software (option-w/o gaps was selected). Phylogenetic tree was constructed using p-distance

matrix of neighbour-joining (NJ) algorithms through mega (v4.0). To provide confidence level for the tree topology and statistical reliability of individual nodes, bootstrap analysis were performed with 1000 replications.

RESULTS AND DISCUSSION

Isolation and characterization of lipase positive bacteria from soil sample

Lipase producing bacteria were isolated from oil spilled soil and screened by tributyrin-spirit blue agar spread plate method. Tributyrin was used as lipase substrate and spirit blue is the indicator dye of lipase activity. The formation of blue colour is as a result of pH changes occurred due to the release of free fatty acids produced from triacylglycerols during lipolysis resulting in changing the colour of the lipase positive colonies into blue one whereas non lipase producing colonies remain as white as it can't take the color of the indicator dye (spirit blue). Six isolates were selected in screening as the most effective producer of lipase.

The morphological, biochemical and microbiological characteristics of the selected isolates were done and summarized (Table 1). All isolates are Gram positive rods and except L_1 all isolates are motile. All the isolates utilises glucose, sucrose as carbon source but L_{24} also utilised lactose. $L_{11,}$ L_{14} and L_{23} are VP positive whereas $L_{1,}$ L_5 and L_{24} are MR positive. $L_{1,}$ L_5 and L_{24} were urease positive. All the isolates were nitrate reductase positive. All the isolates do not degrade sulphur containing amino acids as they do not produce hydrogen sulphide.

Table 1: Morphological, physiological and biochemical characteristics of the selected strains. + sign indicates positive and – sign indicates negative.

Characteristics		Result					
		L_1	L_5	L_{11}	L_{14}	L_{23}	L_{24}
Gram staining		+	+	+	+	+	+
Morphology		Small rods	Very small rods	Large rods	Rods	Small rods	Large rods
Motility		Non-motile	Non-motile	Motile	Motile	Motile	Motile
Endospore		+	+	+	+	+	+
Citrate utilisation		-	+	-	-	-	-
Catalase		+	+	+	+	+	+
H_2S production		-	-	-	-	-	-
Gelatin liquefaction		+	+	Weak +	Weak +	Weak +	+
Starch hydrolysis		+	+	+	+	+	+
Nitrate reduction		+	+	+	+	+	+
Oxidase		+	+	+	+	+	+
Fermentation of sugars							
Glucose	Acid	+	+	+	+	+	+
	Gas	-	-	-	-	-	-
Sucrose	Acid	+	+	+	+	+	+
	Gas	-	-	-	-	-	+
Lactose	Acid	-	-	-	-	-	+
	Gas	-	-	-	-	-	-
Indole		-	-	-	-	-	-
Methyl red		-	-	+	+	+	-
Voges-Proskauer		+	+	-	-	-	+
Urease		+	+	-	-	-	+
Decarboxylation of lysine		+	+	-	-	-	+
Phenylalanine deamination		-	-	-	-	-	-

Antibiotic sensitivity test of the isolates were done and summarized (Table 2). L_{14} and L_{24} were sensitive to all antibiotics but other isolates were resistant to maximum antibiotics listed.

Table 2: Antibiotic sensitivity test of the selected isolates. R indicates resistance and S indicates sensitive.

Antibiotic susceptibility	L_1	L_5	L_{11}	L_{14}	L_{23}	L_{24}
Penicillin G	R	R	S	S	S	S
Clindamycin	S	S	R	S	R	S
Co- Trimoxazole	R	R	S	S	S	S
Erythromycin	R	R	R	S	R	R
Vancomycin	S	S	S	S	S	S
Ampicillin / subactam	R	R	S	S	S	S

Enzymatic activity of the selected isolates was determined using titrimetric lipase assay method. One unit of lipase activity is defined as the amount of enzyme that catalyses the release of 1 μmol of fatty acid per minute per ml under the specified assay conditions. L_{24} isolate showed the highest enzyme activity (U/ml) among all the isolates.

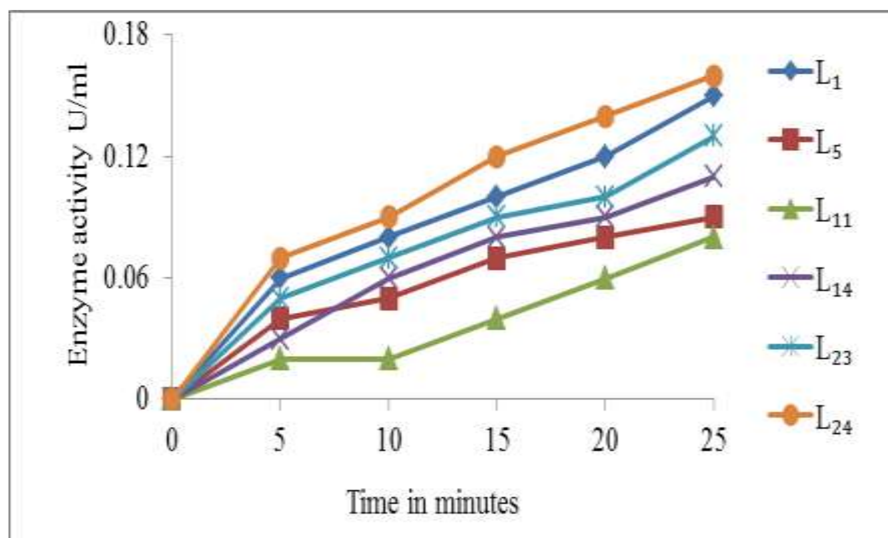

Fig.1. Comparison of lipase activity of isolates L_1 (blue), L_5 (brown), L_{11} (green), L_{14} (purple), L_{23} (sky), and L_{24} (orange). Y axis denotes the enzyme activity (U/ml) while X axis denotes the time.

Growth pattern of the isolate L_{24} was studied at different temperatures. Though the isolate is able to grow at a wide range of temperatures from 10-50^{0}C, the optimal temperature for growth was 37°C. So it can be concluded that the experimental isolate is a mesophilic one (Fig. 2).

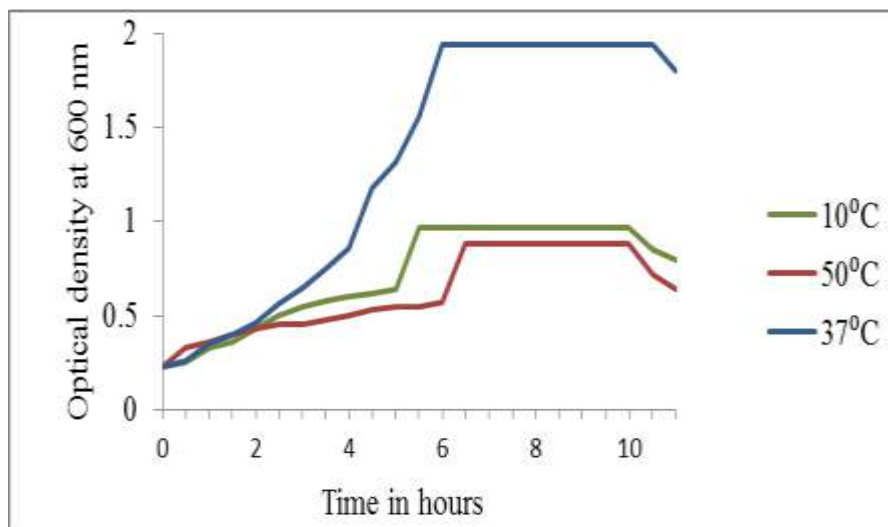

Fig.2.Growth curve: Effect of temperature on the growth of lipase producing strain (L_{24}). Y axis denotes the Optical density at 600 nm while X axis denotes the time. Three different temperatures were taken, 10^{0}C (green), 37^{0}C (blue) and 50^{0}C (brown).

Isolates showing different phenotypes in microscopic and biochemical studies were further characterised. From the sequencing analysis of 16s rRNA sequencing data, L_1 was identified as *Bacillus licheniformis,* L_{14} isolate was identified as *Bacillus cereus,* L_{23} and L_{24} isolates were *Bacillus* sp.

Phylogenetic analysis of 16s rRNA

Phylogenetic tree was constructed based on 16S rRNA gene sequences of four isolates (Fig. 3). Sequences are submitted in Gene bank. Fig. 3 shows the phylogenetic relationship of the isolates among the different species of *Bacillus.*

Fig.3. Phylogenetic tree based on 16S rRNA gene sequences of isolates L_1, L_{14}, L_{23} and L_{24} with different species of *Bacillus*. Bootstrap analysis was performed with 1000 replications.

Bioinformatic analysis of amino acid sequences of lipases from diverse groups of organisms

Lipases belong to many different protein families but they have the same architecture, the α/β hydrolase fold. Their activities rely mainly on a catalytic triad usually formed by ser, his and asp residues.[3] Also, lipases share a consensus sequence of Gly-X-Ser-X-Gly (Fan et al., 2008, Joseph et al., 2008) where x may be any amino acid residue. Sequence alignment of amino acid sequences of various lipases from diverse group of organisms including bacteria, virus and human origin were done to determine the similarity and diversity among them. We found that despite a highly conserved tertiary fold, the protein sequences are less similar and lipolytic enzymes display a wide diversity. Phylogenetic tree was constructed to show the diverse nature of the lipase enzyme. From the unrooted phylogenetic tree of lipases of diverse group including bacteria, humans and viruses, it was seen that three groups were formed. Group1 was mixtures of bacterial, viral and human lipases but mainly consists of viral lipases and Group2 was the mixtures of bacterial, viral and human lipases while Group3 consists of only bacterial and humans lipases (figure 4, tables 3). Presence of bacterial lipases in all the groups is probably due to the highly conserved domain of lipases.

Fig.4. Radial representation of phylogenetic tree of lipases (amino acid sequences) from bacteria, viruses and human origin. Bootstrap analysis was performed with 1000 replications.Group1- orange Group 2- blue and Group 3- red.

Table 3: Phylogenetic analysis

Source organisms	Sequence abbreviation	NCBI Accession number
Photobacterium leiognathi	*Photobacterium*	WP_008987564.1
Aeromonas hydrophila	*Aeromonas*	BAK14340.1
Glaciecola sp. 4h-3-7+ye-5	*Glaciecola*	WP_013753730.1
Rheinheimera sp. A13l	*Rheinheimera*	WP_008898153.1
Shewanella frigidimarina	*Shewanella*	WP_011638021.1
Vibrio parahaemolyticus rimd 2210633	*Vibrio*	NP_800369.1
Staphylococcus aureus subsp. Aureus cn1	*Staphylococcus*	AGU60450.1
Escherichia coli 0.1288	*Escherichia*	EKJ53908.1
Mycoplasma penetrans hf-2	*Mycoplasma*	NP_757985.1
Pseudomonas mandelii	*Pseudomonas*	AFK24472.1
Serratia marcescen	*Serratia*	ABI13521.1
Photorhabdus temperata j3	*Photorhabdus*	ERT12637.1
Xanthomonas oryzae pv. Oryzae maff 311018	*Xanthomonas*	YP_452204.1
Proteus mirabilis hi4320	*Proteus*	YP_002151060.1
Erwinia billingiae	*Erwinia*	WP_013204543.1
Bacillus cereus vd021	*Bacillus*	EOO74037.1
Desulfotalea psychrophil	*Desulfotalea*	WP_011190235.1
Trichodesmium erythraeum	*Trichodesmium*	WP_011613246.1
Pantoea ananatis	*Pantoea*	WP_014604579.1
Salmonella enterica subsp. Enterica serovar typhimurium var. Copenhagen str. 0084	*Salmonella*	ETC72336.1
Monkeypox virus zaire-96-i-16	*Monkeypox*	NP_536458.1
Ectromelia virus	*Ectromelia*	NP_671542.1
Cowpox virus	*Cowpox*	ADZ30654.1
Yaba monkey tumor virus	*Yaba*	NP_938271.1
Gallid herpesvirus 2	*Gallid*	AFM74937.1
Meleagrid herpesvirus 1	*Meleagrid*	NP_073291.1
Acanthamoeba polyphaga mimivirus	*Acanthamoeba*	AAV51163.1
Spodoptera frugiperda ascovirus 1a	*Spodoptera*	CAL44613.1
Vaccinia virus	*Vaccinia virus*	ABD52507.1
Human metapneumovirus	*Metapneumovirus*	AAS48482.1
Bile salt-activated lipase precursor [Homo sapiens]	Human 1	NP_001798.2
Lipase maturation factor 1 [Homo sapiens]	Human 2	NP_073610.2
Hormone-sensitive lipase [Homo sapiens]	Human 3	AAA69810.1
Carboxyl ester lipase [Homo sapiens]	Human 4	AAA51973.1
Lipase [Homo sapiens]	Human 5	AAA60129.1
Diacylglycerol lipase, beta [Homo sapiens]	Human 6	AAH27603.1
Hepatic lipase precursor [Homo sapiens]	Human 7	AAA59520.1
Endothelial lipase precursor [Homo sapiens]	Human 8	NP_006024.1
Lipase maturation factor 2 [Homo sapiens]	Human 9	NP_149977.2
Lipoprotein lipase precursor [Homo sapiens]	Human 10	NP_000228.1

CONCLUSION

The highest lipase activity shown by the four lipase producing isolates SD-IP_L1, SD-IP_L14 , SD-IP_L23 and SD-IP_L24 isolated from oil spilled soil in Burdwan, West Bengal, India were identified as Bacillus by biochemical assay and 16S rRNA sequencing.

From bioinformatic analysis of lipase sequence of diverse group, we found that lipases share a consensus sequence of Gly-X-Ser-X-Gly, highly conserved tertiary fold, but the protein sequences are less similar and lipolytic enzymes display a wide diversity.

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