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Isolation and Characterization of Indigenous Lipase Producing Bacteria from Lipid-rich Environment

Lovely Aktar, Farhana Islam Khan[1], Tahmina Islam, Shawon Mitra and Mihir Lal Saha*

Department of Botany, University of Dhaka, Dhaka-1000, Bangladesh

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Abstract

To isolate and characterize lipase producing bacteria from lipid-rich environment and screen the best lipolytic indigenous bacteria a study was made. For the isolation of bacteria, oil based wastewater and soil were collected from ten different sampling sites. Four different media were used for study the aerobic heterotrophic bacterial count. The highest bacterial count (1.56×10^7 cfu/gm) was observed in dairy farm soil and lowest (8.3×10^2 cfu/ml) in the Buriganga river water. The highest percentage (94.51) of lipase producing bacteria was found in edible oil mill soil and lowest (23.44) in the Buriganga river water. Among the total isolates 30 showed better lipase activity. Potential ten lipase producers were taken for molecular identification. Among them, nine genera were matched with their conventional identification but conventionally identified *Acetobacter liquifaciens* was found to be as *Stenotrophomonas maltophilia*. The enzyme produced by the isolated bacteria could be used for the treatment of lipid-rich wastewater.

Introduction

Lipase is one of the most important enzymes in biotechnology which can catalyze ester bonds into fatty acids and glycerol. This versatility makes lipases the enzyme of choice for potential applications in food, detergent, pharmaceutical, leather, textile, cosmetic, paper industries and industrial waste management (Ruggieri et al. 2008). They are currently given much attention with the rapid development of enzyme technology. Source of lipase occurs widely in nature, but only microbial lipases are commercially significant. Compared to plants and animals, microorganisms have been found to produce high yields of lipases within a short period of time (Mendes et al. 2010). Microbial lipases are high in demand due to their specificity of reaction, less

*Author for correspondence: <sahaml@yahoo.com>. [1]Department of Botany, Jagannath University, Dhaka-1100, Bangladesh.

energy consumption, hydrolytic and synthetic activities, high yield and ease of genetic manipulation (Kumar et al. 2012). Many microorganisms such as bacteria, yeast and fungi are known to have lipases. Of all these, bacterial lipases are more economical and stable (Sagar et al. 2013). Lipase producing bacteria have been found in diverse habitats such as industrial wastes, oil processing factories, dairies, slaughterhouse, soil contaminated with oil, decaying foods etc. (Mobarak et al. 2011). Bacterial lipases are mostly extracellular which are greatly influenced by nutritional and physico-chemical factors such as temperature, pH, nitrogen and carbon sources, inorganic salts, agitation and dissolved oxygen concentration (Gupta 2004).

Lipid in domestic wastes that causes severe environmental pollution. Wastewater produced from edible oil refinery, slaughter house, wool scouring and dairy products industry contains a high (> 100 mg/l) concentration of lipids (Dimirel et al. 2005). Wastewater with lipid often cause major problems in biological wastewater treatment process because it can form oil film on water surface, preventing the diffusion of oxygen from air into water and leading to the death of many forms of aquatic life (Cammarota and Freire 2009). It can also block water drainage line and cause high chemical oxygen demand (COD) in wastewater.

Bioremediation, the use of microorganisms or microbial process to detoxify and degrade the oil effluents is among the innovative technologies. Different microbes producing lipases are used for the oil effluent remediation process (Bhumibhamon et al. 2002 and Creencia et al. 2014). Enzymatic treatment technique has gained more attention because of stringent environmental regulations and friendly applications (Prasad and Manjunath 2011).

Lipase currently used in different industrial products and processes and areas of applications are constantly being added. Many attempts have been made to isolate lipase producing bacteria since this enzyme is used in numerous biotechnological processes. The oily environment with organic matter may provide a good environment for isolation of lipase producing bacteria (Mobarak et al. 2011). The present study was aimed at isolating and characterizing indigenous lipase producing bacteria from lipid-rich environment for biotechnological application.

Materials and Methods

Samples were collected from ten different lipid-rich environments. Water and soil samples were collected in sterile bottles and polythene bags. The samples were labeled properly and brought into the laboratory as soon as possible and analyzed within 1 hr of collection.

Serial dilution technique (Clesceri et al. 1998) was used for the isolation of aerobic heterotrophic bacteria. Plating in duplicated plates was made for each diluted sample. The plates were then incubated at 37°C in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach) for 48 hrs. After incubation colonies were counted by a colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan). Nutrient agar (NA), peptone yeast extract glucose agar (PYG), tributyrin agar (TBA) and Luria-Bertani (LB) media were used for the enumeration and isolation of aerobic heterotrophic bacteria. In addition, lipase producing bacterial load and their percentage was also determined using TBA medium. Actively growing bacterial colonies were randomly selected from dilution plates and grown on two lipid based media TBA and Tween agar for detection of lipase activity. The single colony capable of lipase production was screened by the appearance of clear zone in TBA medium and opaque zone in Tween agar medium. Then strains with widely zone were selected. Following standard manuals Gram stain and essential biochemical tests were performed (Eklund and Lankford 1967, SAB 1957, Collins and Lyne 1984). Characterization and identification of the isolates were made through standard microbiological methods (Sneath et al. 1986, Kreig and Holt 1984, WHO 1987).

Potential ten isolates were identified using 16S rRNA sequence based on molecular technique. 16S rRNA sequence was amplified by using the universal primers CC(R): 5′- CCAGACTCCTACGGGAGGCAGC-3′ and CD(F): 5′- CTTG-TGCGGGCCCCCGTCAATTC -3′. The genomic DNA was isolated from bacteria and PCR was carried out. The amplified products were separated electro-phoretically on 1% agarose gel. DNA bands were observed on UV-transilluminator and photographed by a gel documentation system (Microdoc DI-HD, MUV21-254/365, Cleaver Scientific). DNA sequencing was performed in an automated gene sequencer and sequences were analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioin-formatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs.

Results and Discussion

Ten samples were collected from different lipid-rich industrial areas in and around Dhaka metropolitan city. The bacterial load of different samples is shown in Table 1. The load of the collected samples ranged in between 4.6×10^3 and 1.37×10^7, 1.65×10^3 and 1.25×10^7, 1.28×10^3 and 1.56×10^7, 8.3×10^2 and 2.64×10^5 cfu/gm or cfu/ml in NA, PYG, TBA and LB media, respectively. The maximum bacterial count (1.56×10^7 cfu/gm) was observed in TBA medium from dairy farm soil. On the other hand lowest bacterial load (8.3×10^2 cfu/ml) was observed in LB medium from the Buriganga river water. Lipase positive bacterial load and

Table 1. Bacterial load of collected samples of different lipid rich environment.

Sample No.	Date	Sampling sites	Samples	Bacterial load (cfu/ml of water and cfu/gm of soil)			
				NA	PYG	TBA	LB
1	08.02.15	The Turag river, Gabtoli, Dhaka	Water	3.25×10^4	4.51×10^4	1.07×10^7	ND
2	16.02.15	Dairy farm, Savar, Dhaka	Soil	1.37×10^7	1.25×10^7	**1.56×10^7**	ND
3	22.02.15	Kitchen waste, Mirpur, Dhaka	Soil	6.80×10^5	6.90×10^6	3.10×10^6	ND
4	09.03.15	Tannery, Hazaribagh, Dhaka	Water	8.30×10^5	6.80×10^6	1.36×10^6	ND
5	31.03.15	Bus depot, Kalyanpur, Dhaka	Water	1.39×10^5	1.15×10^5	7.30×10^4	4.40×10^4
6	12.04.15	The Buriganga river, Dhaka	Water	4.60×10^3	1.65×10^3	1.28×10^3	**8.30×10^2**
7	30.04.15	Navana car workshop, Tejgaon, Dhaka	Water	1.80×10^6	2.10×10^6	2.91×10^5	2.64×10^5
8	11.05.15	Navy dockyard, Narayanganj	Water	1.21×10^4	2.41×10^4	7.60×10^3	6.80×10^3
9	25.05.15	Edible oil mill, Elephant road, Dhaka	Soil	1.18×10^4	1.11×10^4	9.10×10^3	1.18×10^4
10	17.06.15	Kohinoor chemicals company, Tejgaon, Dhaka	Soil	3.93×10^5	3.70×10^5	2.40×10^5	3.87×10^4

ND = Not done.

their percentage were observed in TBA medium (Table 2). Lipase positive bacterial load in TBA medium ranged in 3×10^2 to 1.17×10^7 cfu/gm or cfu/ml. The highest percentage (94.51) was found in edible oil mill soil and the lowest (23.44) was in the Buriganga river water. The results revealed that lipase producing bacteria are widely distributed in oily environment.

Table 2. Lipase positive bacterial load and their percentage.

Sampling sites	Samples	Bacterial load (cfu/ml of water and cfu/gm of soil)		% of lipase positive bacteria
		TBA medium	Lipase positive	
The Turag river, Gabtoli, Dhaka	Water	1.07×10^7	6.80×10^6	63.55
Dairy farm, Savar, Dhaka	Soil	1.56×10^7	1.17×10^7	75.00
Kitchen waste, Mirpur, Dhaka	"	3.10×10^6	2.92×10^6	94.19
Tennary, Hazaribagh, Dhaka	Water	1.36×10^6	5.35×10^5	39.34
Bus depot, Kalyanpur, Dhaka	"	7.30×10^4	3.50×10^4	47.95
The Buriganga river, Dhaka	"	1.28×10^3	3.00×10^2	**23.44**
Navana car workshop, Tejgaon, Dhaka	"	2.91×10^5	1.16×10^5	39.86
Navy dockyard, Narayangang	"	7.60×10^3	6.80×10^3	89.47
Edible oil mill, Elephant road, Dhaka	Soil	9.10×10^3	8.60×10^3	**94.51**
Kohinoor chemicals company, Tejgaon, Dhaka	"	2.40×10^5	1.90×10^5	79.17

The bacterial colonies in dilution plates were subjected to qualitative screening for lipase producing strains. Primarily, 273 bacteria were isolated based on their different colonial morphology. They were point inoculated in two lipid based media (TBA and Tween agar) to separate the lipase producing bacteria by formation of clear zone on TBA medium due to hydrolysis of tributyrin and opaque zone on Tween agar medium due to hydrolysis of Tween 80. Among them, 186 isolates showed lipase positive and 30 showed better lipase activity and selected for detailed study. Out of 30 isolates, 13 were Gram positive rods, 3 were Gram positive cocci and 14 were Gram negative rods. Important biochemical tests for provisional identification are shown in Table 3 and 4. The isolated Gram positive rods were provisionally identified as *Bacillus* sp. and cocci were *Staphylococcus intermedius*, *Planococcus citreus and Micrococcus lylae*. On the

other hand, Gram negative members were identified as the genera of *Alcaligenes*, *Acetobacter, Acinetobacter, Pseudomonas* and *Serratia*.

Bacterial abundance of the isolates is shown in Table 5. Among the isolates *Bacillus* sp. was the highest abundance (43.34%). The lowest abundance represented by *Micrococcus lylae, Staphylococcus intermedius, Planococcus citreus* and *Serratia rubidaea* (3.33%). Similar bacterial lipases have been studied for lipid degradation and the bacterial strains were *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., *Streptococcus* sp., *Bifidobacterium* sp., *Yersinia* sp., *Acinetobacter* sp., *Acetobacter* sp. and *Lactobacillus* sp. (Musa and Tayo 2012).

Table 3. Major biochemical tests and provisional identification of Gram positive bacteria.

Isolates	Biochemical tests							Provisional names
	Catalase	VP	Starch	Case in	Citrate	Pro-pionate	Nitrate	
S_3T-8	+	+	+	+	-	-	+	*Bacillus subtilis*
S_3T-9	+	+	+	+	-	-	+	*B. subtilis*
S_3T-7	+	+	+	+	-	-	+	"
S_8L-1	+	+	+	+	-	-	+	"
S_9N-1	+	+	-	+	-	-	-	"
S_3N-3	+	+	-	+	-	-	-	*B. pumilus*
S_3N-6	+	+	-	+	-	-	-	"
S_3P-1	+	+	-	+	-	-	-	"
S_3P-2	+	+	-	+	-	-	-	"
S_3T-4	+	+	+	+	+	-	+	*B. polymyxa*
S_3T-5	+	+	+	+	+	-	+	"
S_4P-4	+	+	+	+	+	-	+	"
S_{10}P-2	+	+	+	+	-	-	-	*B. lentus*
S_6P-1	+	+	-	+	-	-	+	*Micrococcus lylae*
S_9T-1	+	-	-	+	-	+	+	*Planococcus citreus*
S_7N-1	+	-	-	+	-	-	+	*Staphylococcus intermedius*

+ = Positive result. - = Negative result.

All the isolated strains with positive lipase test were characterized by conventional biochemical techniques. Potential ten isolates were further characterized by PCR amplification. From Fig. 1 it is seen that, lane M is 1.0 kb ladder and lanes 1 to 10 are representing 10 bacterial isolates. The size of the amplified DNA band was approximately 600 bp. Among ten isolates, nine genera were matched with their conventional identification except *Acetobacter liquifaciens* where conventionally identified *Acetobacter liquifaciens* was found to be *Stenotrophomonas maltophilia* in case of molecular identification (Table 6).

Table 4. Major biochemical tests and provisional identification of Gram negative bacteria.

Isolates	Biochemical tests							Provisional names
	Catalase	Oxidase	Arginine	Indole	H_2S	Tyrosine	Levan	
S_1P-4	+	-	-	-	-	+	-	*Acinetobacter calcoacetius*
S_1T-1	+	-	-	-	-	-	-	*A. baumanni*
S_2T-1	+	-	-	-	-	+	-	*A. calcoacetius*
S_4T-5	+	+	+	-	-	-	-	*A. lwoffi*
S_6L-1	+	+	-	-	-	+	-	*A. aceti*
S_1N-2	+	-	+	-	-	+	-	*Acetobacter liquifaciens*
S_1P-3	+	-	+	-	-	-	-	*A. pasteurianus*
S_{10}T-8	+	-	-	-	-	+	-	*A. johnsonii*
S_5L-1	+	+	-	-	-	+	-	*Pseudomonas pseudoalcaligenes*
S_5L-2	+	+	-	-	-	+	-	*P. pseudoalcaligenes*
S_{10}P-1	+	+	+	-	+	+	-	*P. aeruginosa*
S_4T-2	+	+	-	-	-	-	-	*Alcaligenes paradoxus*
S_6T-1	+	+	-	-	-	-	-	*A. faecalis*
S_1N-7	+	-	+	-	-	+	+	*Serratia rubidaea*

+ = Positive result. - = Negative result.

The results demonstrated that lipase producing bacteria are widely distributed in environment. In the present study, *Bacillus* sp. occupied the highest number (13) of the total isolates. Cipinyte et al. (2009) reported more or less same result where *Bacillus* was the dominating genus among 724 strains isolated from soil rich in organic matter like lipid. Other genera of bacteria such as *Pseudomonas, Staphylococcus, Serratia, Lactobacillus, Acinetobacter, Escherichia* are known to have the ability to degrade lipid as like as *Bacillus*.

Table 5. Number of bacterial isolates and their abundance percentage.

Gram positive	Name of organisms	No. of occurrence	Percentage
	Bacillus sp.	13	43.34
	Planococcus citreus	1	3.33
	Micrococcus lylae	1	3.33
	Staphylococcus intermedius	1	3.33
Gram negative	*Acinetobacter* sp.	5	16.67
	Acetobacter sp.	3	10
	Pseudomonas sp.	3	10
	Alcaligenes sp.	2	6.67
	Serratia rubidaea	1	3.33

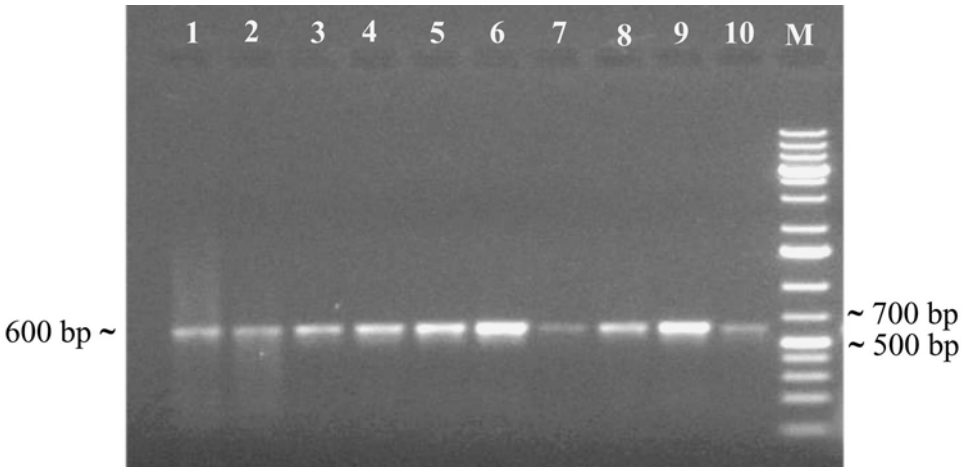

Fig. 1. PCR amplification of part of the 16S rRNA gene. Here, lane 1 = *Stenotrophomonas maltophilia* e-a22, 2 = *Serratia rubidaea* 9B, 3 = *Bacillus pumilus* Ja02, 4 = *Bacillus* sp. BT MASC2, 5 = *Bacillus subtilis* 20B, 6 = *Bacillus subtilis* HRBS-10TDI13, 7 = *Staphylococcus epidermidis* 6E02, 8 = *Pseudomonas aeruginosa* 12, 9 = *Bacillus subtilis* CI1, 10 = *Acinetobacter johnsonii* 372 and M = 1.0 kb ladder.

Table 6. Molecular identification of ten isolates.

No.	Isolates	Conventional identification	Molecular identification			
			Scientific name	Strain	Max. score	Identity match (%)
1	S_1N-2	*Acetobacter liquifaciens*	*Stenotrophomonas maltophilia*	e-a22	1013	98
2	S_1N-7	*Serratia rubidaea*	*Serratia rubidaea*	9B	672	99
3	S_3P-1	*Bacillus pumilus*	*Bacillus pumilus*	Ja02	996	98
4	S_3T-5	*Bacillus polymyxa*	*Bacillus* sp.	BT MASC 2	1024	99
5	S_3T-9	*Bacillus subtilis*	*Bacillus subtilis*	20B	619	89
6	S_4P-4	*Bacillus polymyxa*	*Bacillus subtilis*	HRBS-10TDI13	784	95
7	S_7N-1	*Staphylococcus intermedius*	*Staphylococcus epidermidis*	6E02	869	95
8	S_{10}P-1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*	12	982	98
9	S_{10}P-2	*Bacillus lentus*	*Bacillus subtilis*	CI1	1016	99
10	S_{10}T-8	*Acinetobacter johnsonii*	*Acinetobacter johnsonii*	372	1024	99

As the world oil demand is increasing, generation of oil is also increasing. There is a need of appropriate waste minimization or recycling technology which should be easy to operate and cost effective using the microbial resources. The use of lipases in industries is enormous and increasing. The new areas of applications are constantly being added (Veerapagu et al. 2013). Screening of new lipase producing bacteria will open new, simple routes to solve environmental pollutions with lipid or fat or oil. Therefore, it may be inferred that bacteria with lipase activity can be used as a potent candidate for bioremediation of lipid pollution.

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