



## ANALYSIS OF THE LIPOLYTIC POTENTIAL OF THREE BACTERIAL STRAINS ISOLATED FROM BENTHIC SOIL OF AN AQUACULTURE FARM IN EAST KOLKATA WETLANDS, INDIA.

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**ABSTRACT** Prokaryotic species in the soil microenvironment perform a variety of activities in the circulation of nutrients by breaking down complex detritus molecules into simpler ones by the production of extracellular enzymes. In this study, three bacterial strains were isolated from benthic soil of an aquaculture farm in East Kolkata Wetlands, India, with considerable lipolytic potential. Upon molecular characterization using 16S rDNA analysis the strains were identified as *Enterobacter cloacae* Strain P4cR1, *Klebsiella pneumoniae* Strain P5aR1, and *Serratia marcescens* Strain P5bR1. Enzymatic analyses revealed that *S. marcescens* Strain P5bR1 shows the best enzymatic activity with  $16.82 \pm 0.87$  IU ml<sup>-1</sup>min<sup>-1</sup> followed by *K. pneumoniae* Strain P5aR1 with  $12.53 \pm 1.84$  IU ml<sup>-1</sup>min<sup>-1</sup> and *E. cloacae* Strain P4cR1 showing the lowest activity at  $9.48 \pm 1.05$  IU ml<sup>-1</sup>min<sup>-1</sup>. The enzymes were stable at a pH range of 7 to 8 and a temperature range within 30 to 40°C. This is probably the first report of bacterial strains with lipolytic potential isolated from the benthic soil of an aquaculture farm. These bacteria may find their uses not only in bioremediation purposes but in industrial applications also.

### KEYWORDS

Aquaculture benthos, Bacteria, Lipase, pNpp analysis, 16S rDNA

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### INTRODUCTION:

Bacteria in benthic soil uses complex substances like long-chain fatty acids as the substrate to gain nutrition (Treichel et al., 2010). These molecules are abundant in the soil sediment as detritus materials. Bacteria produce extracellular lipases to degrade the complex lipid molecules to gain nutrition. Lipase producing bacteria has been reported from different geographical conditions and various ecosystems, like lakes (Bergtold & Traunspurger, 2004), coastal region (Bal et al., 2009), riverine ecosystems (Zoppini et al., 2010), continental shelves undersea (Poremba & Hoppe, 1995) and even in the cryosphere of Arctic region (Bienhold et al., 2012; Srinivas et al., 2009). Extracellular lipase producing bacteria finds its application primarily in the field of bioremediation of oil spills (Ogboosingha et al., 2015; Prasad & Manjunath, 2011; Ugochukwu et al., 2008), biodiesel production (Hama et al., 2007; Luo et al., 2006; Winayanuwattikun et al., 2011) and many industrial uses as well (Kanlayakrit & Boonpan, 2007; Treichel et al., 2010). Though several references exist for lipase producing bacteria from several soil sources, the same from the benthic soil of aquaculture farms are very meager.

### MATERIALS AND METHOD

#### SAMPLE COLLECTION:

Benthic soil samples were collected from an aquaculture farm in East Kolkata Wetlands, Kolkata, West Bengal, India (Latitude – 22.5699° Longitude- 88.4394°). Soil samples from the top surface of the benthos were aseptically collected from a depth of 60cm of the water column. The samples were immediately transferred to pre-sterilized polycarbonate containers with a sterile spatula. The samples were marked and taken to the laboratory for further processing.

#### Isolation, Purification, and Molecular Characterization:

Bacterial Strains were isolated from soil using the protocol of Vieira

and Nahas, 2005, with some modifications. 5gm (wet weight) of soil were first homogenized in sterile water and passed through 2mm filter mesh. The filtrate was then serially diluted up to 10<sup>8</sup> times and 100 μl of 10<sup>4</sup> to 10<sup>8</sup> diluted suspensions were spread on Agar plates (tryptone 1%, yeast extract 1%, Agar 2%) supplemented with tributyrin (0.25%) and Tween 20 (0.25%), as an emulsifier. The plates were incubated at 37°C for 24 hours. Extracellular lipase produced by these strains degraded tributyrin, thus resulting in the formation of a halo. The diameter of the halo as well as that of the colony were noted. From the supplementary culture, the bacteria were separated based on colony morphology (shape, size, color, transparency, margin contour, surface topology, etc.) and were transferred to a Tryptone Soya Agar (TSA) plate by streaking. Repeated subcultures were done until the pure culture was obtained. The final isolates were routinely cultured in Nutrient Agar. Molecular characterization was done using 16S rDNA analysis following the protocol as described by De et al., 2018, and standard *In-silico* analyses of the sequenced samples.

#### BIOCHEMICAL CHARACTERIZATION:

Cumulatively, 14 biochemical tests were performed according to *Bergey's Manual of Systematic Bacteriology* (Garrity & Holt, 2001) including determination of Gram Character, Methyl Red (MR), Voges-Proskauer (VP), Triple Sugar Iron (TSI), etc. to determine the biochemical characters of the strains with lipolytic potential.

#### LIPASE ACTIVITY ASSAY:

For quantitative estimation of the enzymatic activity, the strains selected were first cultured in Nutrient broth overnight. On the following day, 10% of the overnight culture was inoculated in 45ml sterile Minimal Salt medium supplemented with 0.25% tributyrin and were incubated in a shaker incubator at 37°C, 160rpm for 48hours.

Harvesting of the crude enzyme extract was done by centrifugation of the culture at 10000rpm for 20 minutes at 4°C. The supernatant was collected and preserved at 4°C for further studies.

Extracellular lipase activity was measured from the supernatant following the protocol given by Mongkolthanaruk and Dharmstithi, 2002, with some minor modifications. Hydrolysis of p-nitrophenyl palmitate (pNPP) occurred due to the extracellular lipase in the crude extract forming p – nitrophenol (pNP), a chromogenic substance that shows peak absorption at 405nm. The results were interpreted as One Unit of Lipase activity = 1µmol of pNP released per minute per ml of enzyme. The results were expressed in IU ml<sup>-1</sup> min<sup>-1</sup>.

**pH AND TEMPERATURE STABILITY:**

Cell-free supernatants containing the crude enzymes were tested for their optimal pH and thermostability. The same process of enzymatic analysis was done by varying the pH of the enzyme production media and temperature of the enzyme before incubation. The pH was tested within a range of 4 – 10 (7 samples with an interval of 1 pH) and the temperature was tested from 20°C to 70°C (6 samples with 10°C intervals).

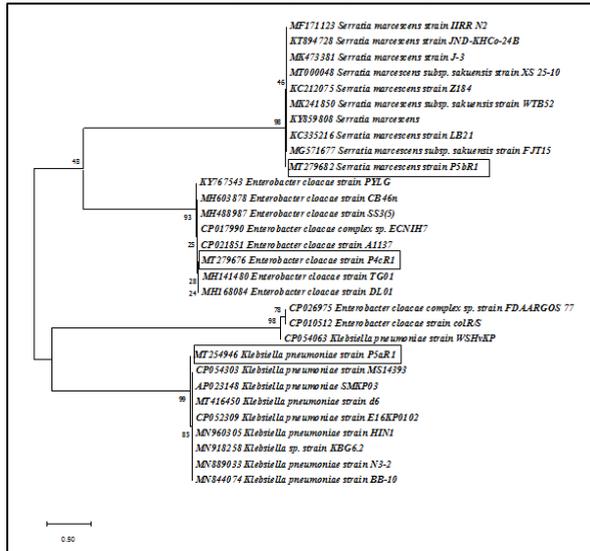
**STATISTICAL ANALYSIS**

Experiments of the enzymatic analysis of lipase were performed in triplicate and values are expressed as mean values ± standard deviation (SD). Statistical analyses were done using GraphPad Prism 7 (GraphPad Software, USA).

**RESULTS:**

**ISOLATION, PURIFICATION, AND MOLECULAR CHARACTERIZATION:**

Three bacterial strains were isolated with lipolytic potential from the benthic soil sample. Upon purification and molecular characterization, the strains were identified as *Enterobacter cloacae* Strain P4cR1, *Klebsiella pneumoniae* Strain P5aR1, and *Serratia marcescens* Strain P5bR1. The sequence data of the three strains were submitted to the GenBank vide Accession Numbers of MT279676, MT254946, and MT279682 respectively. The phylogenetic relationship of the three strains is shown with 10 nearest neighbors obtained from the GenBank in a scaled Neighbor-joining tree in Figure 1.



**Figure 1:** A combined phylogenetic analysis of all the lipase producing strains showing their interrelationship. The tree is drawn to scale using Neighbor-joining method and Maximum Likelihood Composite model.

**BIOCHEMICAL CHARACTERIZATION:**

Detailed biochemical characterization results of the isolates are given in Table 1. The isolates showed a varying degree of fermentation potential in both anaerobic and aerobic conditions. The strains were also able to produce other extracellular enzymes like urease and amylase.

**Table 1: Biochemical test results of the lipase producing strains. [+**

= Positive result, - = Negative result, Y = Sugar Fermenters (Slant – Anaerobic/ Butt – Aerobic) R = Negative result (TSI and MacConkey media)

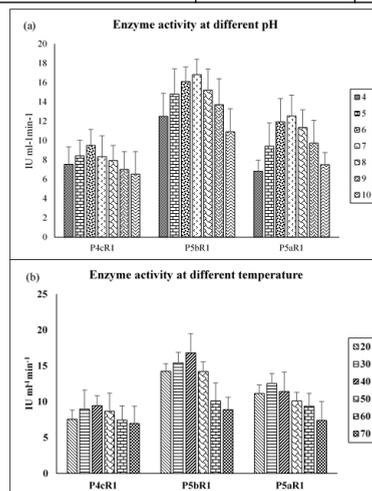
Biochemical Tests		Strain		
		P4cR1	P5aR1	P5bR1
TSI	Slant	Y	Y	Y
	Butt	Y	Y	Y
	H <sub>2</sub> S	-	-	-
	Gas	-	+	-
Oxidase		-	-	-
Catalase		+	+	+
Gram Staining		-	-	-
KOH String Test		-	-	-
MR		+	-	-
VP		+	+	-
Gelatinase		-	-	-
Indole		-	-	-
Motility		+	-	+
DNase		-	-	-
MacConkey		W	W	W
Urease		-	+	-
Citrate		-	+	-
Amylase		+	+	+

**Lipase Activity Assay:**

The screening test result performed in Tributyrin – Tween 20 agar media revealed a glimpse of the comparative account of the lipolytic potential of the strains involved and is shown in Table 2. As revealed by pNpp assay, *S. marcescens* Strain P5bR1 scores the best enzymatic activity with 16.82±0.87 IU ml<sup>-1</sup>min<sup>-1</sup> followed by *K. pneumoniae* Strain P5aR1 with 12.53±1.84 IU ml<sup>-1</sup>min<sup>-1</sup> and *E. cloacae* Strain P4cR1 showing lowest activity at 9.48±1.05 IU ml<sup>-1</sup>min<sup>-1</sup>. Results of temperature and pH stability estimation of the enzyme activity of the three isolates are depicted graphically in Figure 3a and 3b respectively. Both the analyses showed a common pattern for both the enzymes. For pH, the maximum activity of the enzymes obtained from the strains showed the highest activity within a range of 7 to 8 and diminishing at either extremity. In the case of temperature, the highest activity was recorded within 30 to 40°C with the same trend of diminishing activity with either increase or a decrease in the temperature.

**Table 2: The results of screening tests in Tributyrin Agar for extracellular lipase producing isolates. The diameter of the Clearing Zone and that of the colony are shown. Enzymatic activities of extracellular lipase are shown as Mean ± SD.**

Strain	Diameter of the Clearing Zone (Halo) (in mm)	Diameter of the colony (in mm)	Activity (U ml <sup>-1</sup> min <sup>-1</sup> )
P4cR1	8.6	3.4	9.48±1.05
P5aR1	14.7	4.5	12.53±1.84
P5bR1	18.6	4.9	16.82±0.87



**Figure 2:** Graphical representation of the variation of activity of extracellular lipase as revealed by pNPP assay with varying pH ranging from pH 4-10 (a) and temperature ranging from 20°C - 70°C (b).

#### DISCUSSION:

Bacterial strains with lipolytic potential are of biotechnological and ecological importance, hence the efficacy of the strains has been widely studied. From the East Kolkata Wetlands region, several genera of bacteria have already been reported (Ghosh et al., 2007; Sarkar & Saha, 2014). However, this is the first report on the efficacy of extracellular lipase producing strains isolated from benthic soil of aquaculture farms in East Kolkata Wetlands. When compared to previously reported articles the efficacy of the extracellular lipase revealed in this study stands at par —(Gupta et al., 2012; Raina et al., 2019; Srinivas et al., 2009; Zoppini et al., 2010). Lipase-producing bacteria find its industrial application in biodiesel production (Winayanuwattikun et al., 2011), as a biocatalyst in several agro and pharmaceutical industries (Contesini et al., 2010). In the field of bioremediation, lipase producing bacteria can be used for bioaugmentation purposes in neutralizing hydrocarbon pollution that may arise due to oil spills and industrial effluents (Ogboosingha et al., 2015; Prasad & Manjunath, 2011). Since the extracellular lipase produced by the bacterial strains is stable and shows the most efficacy at a range of 30°C to 40°C, these strains can be one of the potential candidates for biotechnological and bioremediation practices in tropical countries.

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