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Indigenous Lipase Producing Bacteria for Lipid-rich Wastewater Treatment

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Abstract

This study was undertaken to evaluate the removal of lipid-rich organic matter from wastewater by lipase producing bacteria. Ten potential lipase producing bacteria were isolated from lipid-rich environments in and around Dhaka Metropolitan city. Three of them produced lipase higher than 10 U/ml. These three isolates and their consortium were used for synthetic wastewater treatment in the laboratory. The initial COD value of synthetic wastewater was 1,200 mg/l. COD removal efficiencies in the synthetic wastewater were 74.75, 73.33 and 66.67% by the *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B, respectively. *Stenotrophomonas maltophilia* showed better COD removal performance (74.75%) in case of monoculture. But consortium showed better COD removal (83.33%) than that of monoculture. Therefore, it could be concluded that consortium of three isolates will be more useful for wastewater treatment as seed cultures in the wastewater treatment plant associated with the lipid-rich wastewater.

Introduction

Lipid, characterized by fats, oils and greases (FOG) and long chain fatty acids (LCFA) are major organic components of wastewater (Chipasa and Medrzycka 2006). Lipid in wastewater often cause major problems in natural systems of biological wastewater treatment processes because of oil film on water surface, preventing oxygen diffusion from air into water and leading to the death of many forms of aquatic flora and fauna (Cammarota and Freire 2009). Aggregates formed by the oil droplets and other particles present in wastewater could also block water drainage systems and with high chemical oxygen demand (COD) in wastewater (Mendes et al. 2010).

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Treatment of wastewater is essential to prevent the contamination of surface water as well as drinking water and the entry of contaminants into the food chain. Treating lipid based wastewater sources is very broad, as the oil in the oil industry, oil refining, oil storage, transportation and petrochemical industries in the production process generate lot of oily wastewater(Chen and He 2003, Machin-Ramirez et al. 2008). Therefore, oily wastewater treatment is urgently needed to address the challenges of environmental pollution. These challenges might be ecofriendly solved by the bacteria which are already adapted with the contaminated sites.

The biological process is the main treatment system in wastewater plant. In biological methods, bacteria with high lipase activities can degrade oil. The most common method of treating lipid-rich wastewater is enhancing lipase producers either in monoculture or mixed culture. Different industries generate strong wastewaters characterized with high BOD and COD, and lipid source could increase BOD level. These types of effluents can be treated by cultivating lipase producing bacteria (Nelson and Rawson 2010). Generally *Pseudomonas*, *Bacillus* and *Acinetobacter* are used in the wastewater treatment plant (Mongkolthanaruk and Dharmsthiti 2002).

Considering the importance and applications of biotechnologically and industrially valuable bacterial enzymes, the present study was aimed to screen indigenous lipase producing bacteria isolated from lipid-rich environment for wastewater treatment.

Materials and Methods

The soil and water samples were collected from different lipid-rich environmental areas located in and around Dhaka Metropolitan city, Bangladesh (Aktar et al. 2016). They were subjected to serial dilutions. Actively growing bacterial colonies were randomly selected from dilution plates and grown on two lipid based media-Tributyrin Agar (TBA) and Tween Agar (TA) for 72 hrs at 37°C for detection of lipolytic activity (Kumar et al. 2012). The lipolytic activities of all isolates were compared by measuring the width of the areas of clearing expressed as zone ratio in TBA medium. Zone ratio was determined by calculating zone diameter (mm) divided by colony diameter (mm) (Bueno et al. 2014). Potential ten isolates were identified using 16S rRNA sequence based on molecular technique (Aktar et al. 2016).

For lipase production, the liquid culture medium, Tryptone Soya Broth (TSB) with 1% olive oil substrate was used (Anbu et al. 2011). An Erlenmeyer flask (250 ml) containing 100 ml of TSB medium was inoculated with 1% of the pre culture (0.5 McFarland standards) prepared in Nutrient Broth (NB). The inoculated flasks were incubated for 96 hrs at 37°C at 150 rpm on a shaking incubator (DAIHAN-LABTECH). Samples were removed aseptically at an interval of 24 hrs and cell free supernatant was recovered by centrifugation at 9,000 rpm for 20 min at 4°C (Sagar et al. 2013). The clear supernatant was collected as source of crude enzyme for determination of lipolytic activity.

Standard method was followed for the estimation of lipase activity of the bacterial isolates (Marseno et al. 1998). Lipase activity was estimated in a screw cap vial containing 2 ml of reaction mixture containing 60% (v/v) olive oil in iso-octane. The 60% (v/v) olive oil in iso-octane was prepared by mixing 60 ml of olive oil with 40 ml of iso-octane. The reaction was started by adding of 20 µl of crude enzyme solution at 30 ^{o}C at 150 rpm for 30 min. Then the reaction was stopped by placing the reaction mixture in an ice bath for 10 min. About 200 µl of the aliquots was added to the mixture containing 1800 µl of iso-octane and 400 µl of cupric acetate pyridine at pH 6.0. The upper layer (iso-octane fraction) was pipetted and the amount of free fatty acid that dissolved in iso-octane layer was determined spectrophotometrically by measuring the optical density at 715 nm. Lipase activity was estimated by quantification of oleic acid released as free fatty acid from olive oil by bacterial lipase which is determined following oleic acid standard curve. Released oleic acid was estimated up to 96 h and results were recorded every 24 hrs intervals. One unit of lipase activity was defined as the amount of enzyme that produced one µmole fatty acid per min.

A synthetic wastewater containing the following compositions was prepared as described by Chen et al. (2009): (g/l) glucose (7.5), NH_4Cl(1.5), K_2HPO_4(0.2), KH_2PO_4 (0.2),$MgSO_4.7H_2O$(0.25), $CaCl_2.2H_2O$(0.3). Trace element solution (0.5 ml) was added for this medium with the following compositions (mg/L): H_3BO_3(50), $ZnCl_2$(50), $CuCl_2$(30), $MnSO_4.H_2O$(500), $CoCl_2.H_2O$(50), $(NH_4)_6MoO.24H_2O$(50). The solution was diluted to 100 times (1 ml stock + 99 ml distilled water). One percent olive oil was added in the diluted medium. The pH was adjusted to 7.0 and the media was autoclaved at 121°C for 15 minutes. In the laboratory synthetic wastewater treatment was carried out by two different ways. Firstly, the wastewater treatment was done by monoculture. Secondly, by consortium or mix culture of bacterial isolates. Seed culture was prepared in NB medium. Inoculated Erlenmeyer flasks (250 ml) containing 100 ml synthetic wastewater with 5% (v/v) seed culture at OD value with 600 nm =1 were then incubated in a shaking incubator at 37°C at 150 rpm for 96 hrs. COD analysis was done periodically at 24 hrs intervals.

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v.20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Mean and Standard deviation (SD). Evaluation on differences between groups was based on the oneway ANOVA test at 95% confidence interval with Duncan's Multiple Range Test. Value of p <0.05 was regarded as statistically significant.

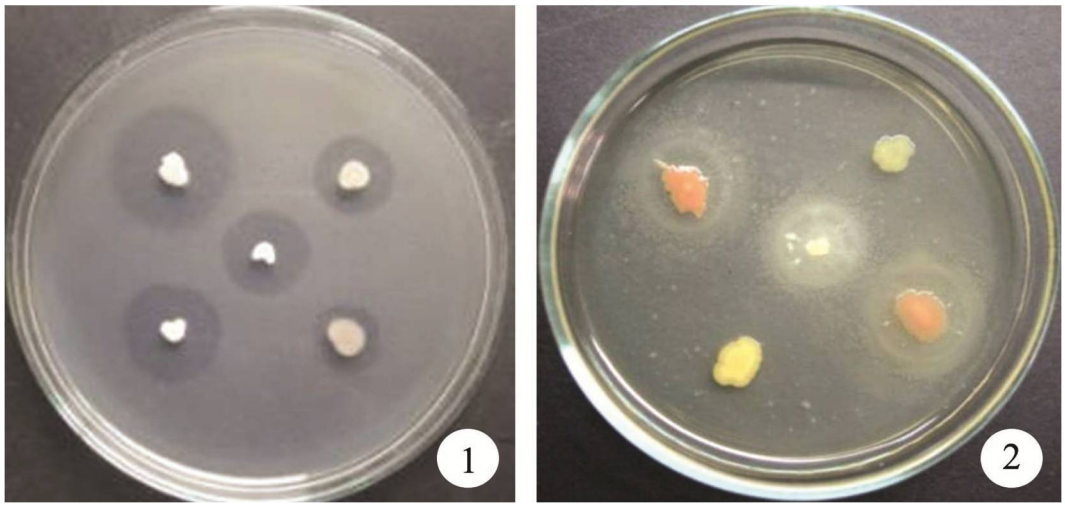
Results and Discussion

For analysis of the degree of lipolysis, zone ratio of 10 selected isolates was calculated from TBA plates and is shown in Table 1. Among the isolates, the highest zone ratio showed by the isolate S_4P-4 and the measured zone ratio was 4.08 ± 0.47.The formation of

clear zone due to hydrolysis of tributyrin on TBA medium is shown in Fig. 1. The formation of opaque zone around the inoculated bacterial colony on TA medium after 72 h of incubation is shown in Fig. 2.

Table 1. Lipolytic activity as measured by zone ratio (mean ± SD) on TBA plate (n=3).

Strain	Isolates	Zone diameter (mm)	Colony diameter (mm)	Zone ratio
S_3T-5	*Bacillus* sp. BTM ASC2	22.83 ± 1.53	9.67 ± 0.58	2.36 ± 0.10
S_3T-9	*B. subtilis* 20B	25.00 ± 4.09	9.50 ± 2.18	2.63 ± 0.46
S_4P-4	*B. subtilis* HRBS-10TDI13	24.50 ± 0.87	6.00 ± 0.50	4.08 ± 0.47
S_{10}P-2	*B. subtilis* CI1	29.83 ± 2.75	15.33 ± 1.15	1.95 ± 0.15
S_3P-1	*B. pumilus* Ja02	19.50 ± 4.77	7.00 ± 0.50	2.79 ± 0.62
S_7N-1	*Staphylococcus epidermidis* 6E02	16.83 ± 4.48	5.67 ± 0.76	2.97 ± 0.90
S_1N-2	*Stenotrophomonas maltophilia* e-a22	17.17 ± 1.89	7.83 ± 0.29	2.19 ± 0.28
S_1N-7	*Serratia rubidaea* 9B	23.17 ± 1.89	6.33 ± 0.58	3.66 ± 0.29
S_{10}P-1	*Pseudomonas aeruginosa* 12	23.50 ± 3.00	14.69 ± 1.76	1.60 ± 0.13
S_{10}T-8	*Acinetobacter johnsonii* 372	21.50 ± 1.76	8.33 ± 0.76	2.58 ± 0.24



Figs 1-2: 1. Photograph showing growth and clear zone on TBA medium. 2. Photograph showing growth and opaque zone on TA medium.

For lipase production, six Gram positive and four Gram negative bacterial isolates were selected. Lipase production of Gram positive bacterial isolates is shown in Fig. 3. The lipase producing capabilities of Gram positive isolates ranged from 0.89±0.63 to 13.50

± 0.14 U/ml. Lipase production of Gram negative bacterial isolates is shown in Fig. 4. In case of Gram negative isolates lipase production was in between 1.28 ± 0.25 and 26.89 ± 2.68 U/ml. Among the bacterial isolates, the Gram negative *Stenotrophomonas maltophilia* e-a22 was found to be the most potent isolate for lipase production which could produce lipase up to 26.89 ± 2.68 U/ml at 72 hrs of incubation. *Pseudomonas aeruginosa* 12 could produce lipase up to 10.89 ± 0.30 U/ml at 24 h of incubation. Padhiar and Kommu (2016) reported almost similar type of observation by *Pseudomonas aeruginosa* with lipase production 8.32 U/ml. Another isolate *Bacillus subtilis* 20B could produce lipase up to 13.50 ± 0.14 U/ml at 24 hrs of incubation. Musa and Tayo (2012) found lipase production of *Bacillus* sp. as 3.8 U/ml. In another research, *Bacillus subtilis* isolated from oil-contaminated wastewater could produce lipase 9.33 U/ml (Iqbal and Rehman 2015). These three isolates were selected for wastewater treatment.

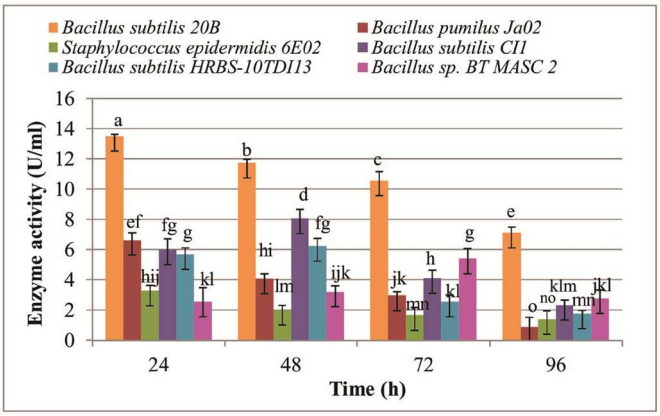


Fig. 3. Lipase production of 6 Gram positive bacterial isolates. Error bar representing the experimental error of Sd. Means with different letters designations within the column are significantly different at $p < 0.05$ by DMRT (n=3).

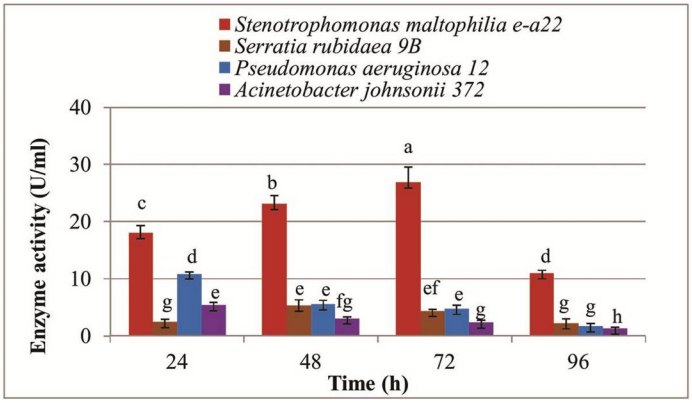


Fig. 4. Lipase production of 4 Gram negative bacterial isolates. Error bar representing the experimental error of Sd. Means with different letters designations within the column are significantly different at $p < 0.05$ by DMRT (n = 3).

Wastewater treatment in terms of COD was carried out in the laboratory. The initial COD concentration of synthetic wastewater was 1,200 mg/l. COD measurement was carried out at 96 h of treatment under aerobic condition. The COD value of wastewater reduced from 1,200 mg/L to 303, 320 and 400 mg/L after 96 h of treatment by the individual isolates of *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B, respectively. The strain *Stenotrophomonas maltophilia* e-a22 was found to be the best with COD removal rate of 74.75% followed by *Pseudomonas aeruginosa*12 (73.33%) and *Bacillus subtilis* 20B (66.67%).

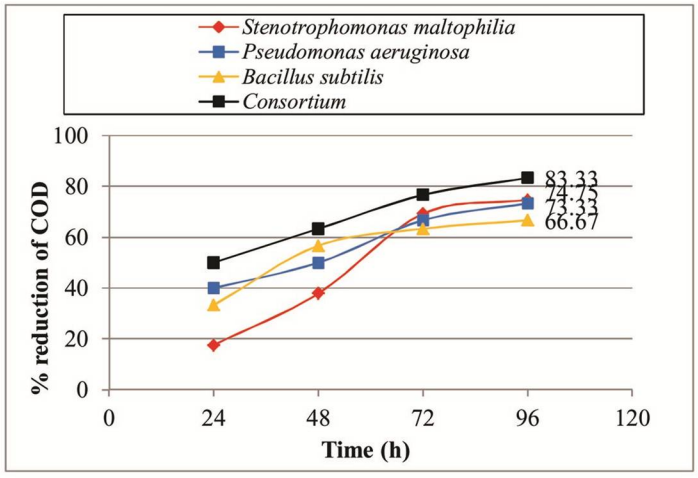


Fig. 5. Wastewater treatment using monoculture and consortium.

Consortium of *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B was found to be better than that of monoculture. There was a considerable decrease in COD (200 mg/l) on the 4th day of the treatment. The consortium attained maximum COD removal up to 83.33% (Fig. 5). Since consortium gave better result due to their synergistic effect and could reduce COD efficiently from synthetic wastewater. Like present study similar work had been carried out with other lipase-producing strain *Pseudomonas aeruginosa* SL-72 (Verma et al. 2012). The crude lipase of *Pseudomonas aeruginosa* SL-72 was added to wastewater contaminated with crude oil, resulting 86.39% reduction of COD after 7 days of treatment. Orapin and and Kriangkrai (2010) reported that single culture of *Pseudomonas* sp. was highly efficient and showed COD removal of 95.81% within 8 days of treatment. Serikovna et al. (2013) reported FOG removal by *Pseudomonas aeruginosa* G23 with the degradation rate of 62 - 66%. Hu et al. (2018) had found that *Pseudomonas* sp. was very effective in removing organic matter from industrial wastewater. Three bacterial isolates, *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B could be used as seeding inocula to treat oily wastewater.

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