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OPTIMIZATION OF CULTURAL PARAMETERS FOR LIPASE PRODUCTION BY INDIGENOUS *STENOTROPHOMONAS* SP. AND *PSEUDOMONAS AERUGINOSA* ISOLATED FROM LIPID-RICH ENVIRONMENTS

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Abstract

The present study aims to identify prospective lipase-producing bacteria and optimize different culture parameters to produce maximum lipase. A total of 37 lipase-positive bacteria were isolated from lipid-rich environments. Among them, strains S_1N-2 and S_{10}P-1 were found to have potential and were molecularly identified as *Stenotrophomonas* species ICMM10 and *Pseudomonas aeruginosa* BA7823, respectively. For maximum lipase production, the optimum pH was 7 and the temperature was 37ºC. Using these conditions, the isolates were grown on media with additional carbon and nitrogen sources along with different inoculum concentrations. The maximum amount of lipase produced by *Stenotrophomonas* sp. ICMM10 was 136.47 ± 1.63 U/ml, which is 5-fold higher than before. *Pseudomonas aeruginosa* BA7823 produced a maximum lipase of 96.33 ± 0.21 U/ml, which was about 9-fold higher than before. These results would provide useful information about lipase production by indigenous isolates, which may be employed as a potential bacterial lipase source of biotechnological interest.

Introduction

Lipases have recently received much attention with the speedy advancement of enzyme technology. The chemical transformation of triglycerides to free fatty acids and glycerol is catalyzed by lipases, which are also commercially important enzymes for industry (Habibollahi and Salehzadeh 2018). The versatile properties of lipases make it a preferred enzyme for several biotechnological applications, such as food, detergent, cosmetics, leather, textile, and paper industries, and even in the treatment of waste effluents that are lipid-rich (Kapoor and Gupta 2012).

Various types of bacteria, yeasts, and molds produce lipases that differ in their enzymological features and specificities (Sharma *et al.* 2017). However, lipases derived from microbes are most widely used in the industrial world because they are more economical, stable, and easy to cultivate (Indriyani and Herasaric 2021). Bacterial lipases are found to be more stable as well as economical (Sagar *et al.* 2013). Among different microbial origins, bacterial lipases are most frequently used in biotechnological applications because they are simple to extract and cultivate (Ulker and Karaoglu 2012). Many bacterial species produce lipase, which has been extensively investigated and reported. Bacteria that produce lipase can be found in various environments, for instance, organic waste, dairy wastes, slaughter-houses, etc. (Mobarak-Qamsari *et al.* 2011). Bacteria like *Pseudomonas* sp. and *Bacillus* sp. were found to be good sources of lipase (Zouaoui *et al*. 2012).

The potential metabolic activities of bacteria are mainly influenced by substrate compositions. Optimization techniques for enzyme production are often carried out with different variables like nutritional factors and physicochemical factors (Ilesanmi *et al.* 2020). Therefore, on lipase production, different culture media have different stimulatory effects (Dhiman and Chapadgaonkar 2013). Lipases are supposed to be produced when lipid sources like oils, fatty acids, tweens,

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triacylglycerols, etc. are present. Industrial wastes are abundant in a variety of elements that promote the growth of certain bacteria. Considering all the facts, a lipid-rich habitat has been chosen for the isolation of indigenous bacteria that produce lipase, along with their production potential.

Materials and Methods

Bacteria were isolated from the water of the river Turag and the soil of the premises of Kohinoor Chemicals Company Limited in Dhaka, Bangladesh. Bacterial colonies were isolated through the technique of serial dilution at 37ºC for 48 hrs. To purify them, randomly chosen bacterial isolates were repeatedly streaked over Nutrient Agar (NA) plates. For the purpose of detecting bacterial lipolytic activity, pure cultures were grown for 48 h. at 37ºC on Tributyrin Agar (TBA) and Tween Agar (TA), which are two lipid-based media. The formation of clear zones in TBA plates and the intensity of opaque zones in TA plates around the developed colonies were the indicators of the lipase activities of isolates (Bueno *et al.* 2014).

The potential bacterial isolates were identified using 16S rRNA sequence based on molecular approach. The universal primers were used to amplify the 16S rRNA sequence. The heat-thaw technique was used for bacterial DNA extraction and preserved at -20°C (Salehi *et al*. 2005). Then PCR amplification was performed (Aktar *et al*. 2016). PCR products were investigated on a 1.0% agarose gel and DNA bands were seen on a UV- transilluminator and captured in photographs using a system of gel documenting (Microdoc DI-HD, MUV21-254/365, Cleaver Scientific). In an automated gene sequencer, DNA sequencing was carried out and sequences were examined. The evolutionary similarities were discovered utilizing a sequence database search using the BLASTn search program analysis of the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/BLAST/) against diverse sequences. After that, in order to receive accession numbers, DNA sequences were submitted to the GenBank database. To confirm the phylogenetic position of the potential isolates, the MEGA 11 program was used to construct the Clustal W phylogenetic evolutionary trees (Tamura *et al*. 2021). The strength of the nodes in the trees was evaluated using a bootstrap technique with 1000 replicates.

The process of lipase production was carried out using a submerged fermentation approach (Anbu *et al*. 2011) using tryptone soya broth (TSB) with 1% olive oil as a substrate. In a 100-ml Erlenmeyer flask, 50 ml of TSB medium was taken and inoculated with 1% inoculum. Inoculated flasks were incubated at 37°C for 48 h. while being constantly shaken at 150 rpm in a rotary shaker (Daihan Labtech, England). The samples were then removed aseptically, and cell-free supernatant was collected by centrifugation at 9,000 rpm for 20 min at 4ºC. To measure the lipolytic activity, the clear supernatant was collected and used as a source of crude enzyme.

The activity of lipase was estimated as described by Marseno *et al.* (1998). About 2 ml of reaction mixture in a screw-cap vial was taken, which contains 60% (v/v) olive oil dissolved in iso-octane. By adding 20 µl of crude enzyme, the reaction was initiated at 30ºC for 30 min at 150 rpm. After that, the reaction was immediately ceased by submerging the mixture in an ice bath for 10 min. About 200 µl of the aliquots were incorporated into the reaction mixture, which was made up of 1800 µl of isooctane and 400 µl of cupric acetate pyridine at pH 6. The upper layer of isooctane fraction was pipetted and the quantity of free fatty acid that dissolved in isooctane layer was estimated spectrophotometrically with the aid of measuring the optical density at 715 nm. Lipase activity was determined by measuring the amount of oleic acid released as free fatty acid from olive oil by bacterial lipase enzyme which was estimated following well known oleic acid standard curve. The volume of lipase enzyme that produces one µmole of fatty acid per min was used to define one unit of lipase activity. All the experiments were performed in triplicates.

In order to optimize several physicochemical parameters, including pH, temperature, carbon and nitrogen sources, and inoculum concentration, 1% (v/v) olive oil was used as a lipase synthesis inducer (Veerapagu *et al.* 2013).

Effects of pH on lipase production were performed at varying pH (5-9) of the medium at 37°C. The bacterial isolates were inoculated into the lipase production medium and incubated at 37°C for 48 h. To test the effect of temperature on lipase production, the bacterial isolates were inoculated in the lipase production medium of pH 7 and incubated at five different temperatures of 20, 30, 37, 40, and 50°C for 48 h.

Different carbon sources, such as glucose, sucrose, starch, arabinose, xylose and mannitol, were added at a concentration of 1% (w/v) in the lipase production medium and incubated at 37°C for 48 h. Thereafter, a better carbon source that supports maximum lipase production was added at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) in the production medium. For optimization, the inoculated medium was incubated at 37ºC for 48 h. Nitrogen sources on bacterial lipase production were studied by adding different organic nitrogen sources such as peptone, tryptone, yeast extract and beef extract, whereas inorganic nitrogen sources, such as ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) and ammonium ortho phosphate ($NH_4H_2PO_4$), were added at a concentration of 1% (w/v) to the medium. The inoculated medium was incubated at 37ºC for 48 h. After that, a better nitrogen source that supports maximum lipase production was added at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) in the production medium and incubated at 37°C for 48 h.

To evaluate the effects of inoculum concentration on lipase production, varied cell concentrations ranging from 1 to 7% were added to the production medium. Then it was incubated at 37 ºC for 48 h., and enzyme activity was estimated.

Considering all parameters for optimization of lipase production, the best condition was employed. For this purpose, inoculated flasks were incubated at 37 °C for 96 h., and lipase estimation was carried out periodically at 12 h. intervals.

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 20 (SPSS Inc.). Data were analyzed as Mean ± Standard deviation. Significant differences between groups were analyzed by Duncan's test. A value of $p = 0.05$ was regarded as statistically significant.

Results and Discussion

Isolated bacteria were screened for lipase production on TBA and TA medium. The lipase-producing isolates showed a clear zone in TBA medium (Fig. 1A) and an opaque zone in TA medium (Fig. 1B). Among the total isolates, 37 showed lipase positivity. Based on their clear zone formation in TBA medium and the intensity of the opaque zone in TA medium, 8 isolates were selected. Among them, two isolates S_1N-2 and S_{10}P-1) were selected as they showed a better clear zone (13mm and 7mm, respectively) in TBA medium and the highest intensity of the opaque zone in TA medium. Isolate S_1N-2 was isolated from the Turag river water and S_{10}P-1 from the soil of Kohinoor Chemicals Company Limited.

The selected two bacterial isolates were identified using 16S rRNA gene sequencing as *Stenotrophomonas* sp. ICMM10 (S_1N-2) and *P. aeruginosa* BA7823 (S_{10}P-1). A phylogenetic tree was constructed using the highest homology of other bacterial species. The relatedness of the strain *Stenotrophomonas* sp. ICMM10 (GenBank accession number OP750511.1) to other *Stenotrophomonas* is illustrated in the constructed phylogenetic tree (Fig. 2). On the other hand, Fig. 3 indicates the relationship of strain *P. aeruginosa* BA7823 (GenBank accession number OP740382.1) with other related *Pseudomonas* species. These two isolates were used for optimization of lipase production.

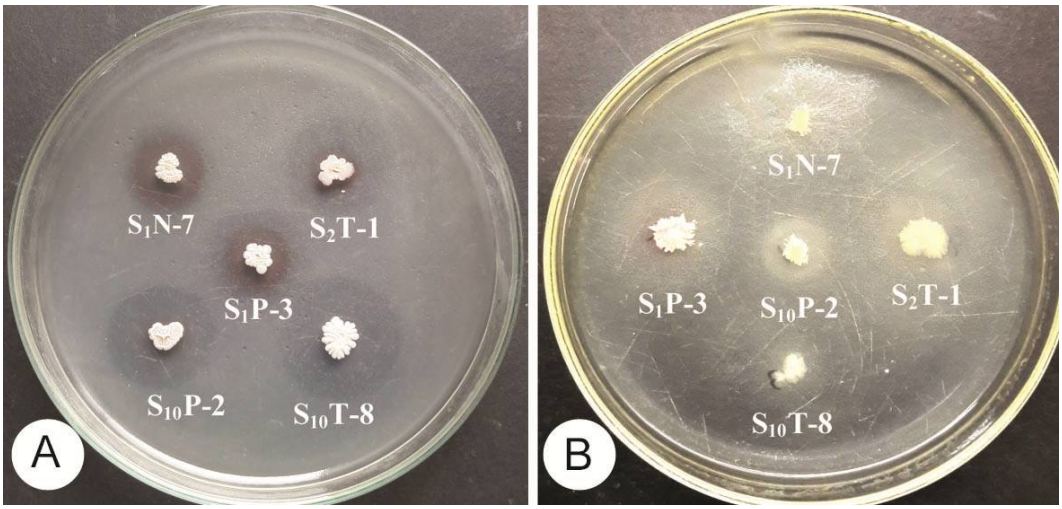

Fig. 1. Clear zone formation in TBA medium (A) and opaque zone formation in TA medium (B) of lipase positive bacteria around their colonies.

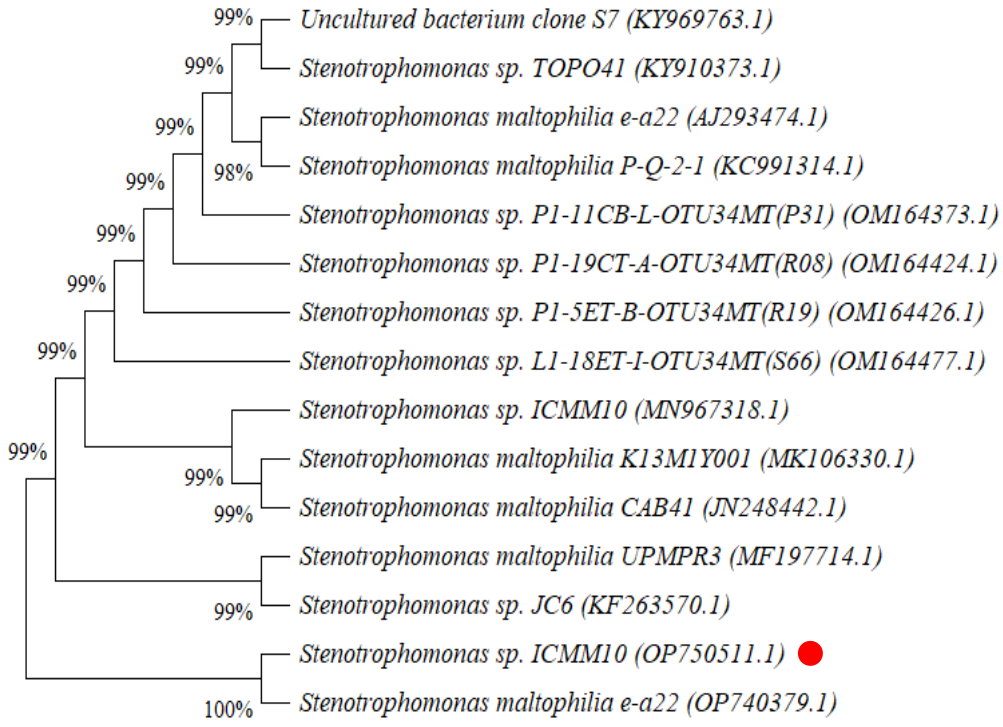

Fig. 2. Phylogenetic tree of *Stenotrophomonas* sp. strain ICMM10 with other 14 bacteria according to the program MEGA 11. The procedure performed 1000 bootstraps under the neighbor-joining method.

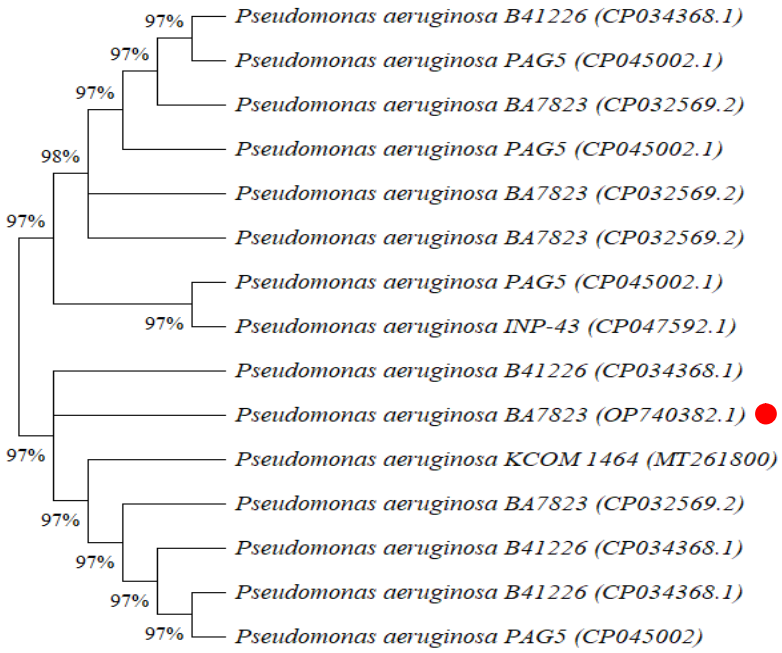

Fig. 3. Phylogenetic tree of *Pseudomonas aeruginosa* strain BA7823 with other 14 bacteria according to the program MEGA 11. The procedure performed 1000 bootstraps under the neighbor-joining method.

The initial pH of the production medium is one of the most critical parameters affecting both bacterial growth and lipase production. Result of the lipase production at different pH values clearly indicated that both isolates showed the highest activity at pH 7 (Fig. 4). Maximum lipase activity was 23.11 ± 1.42 and 5.50 ± 0.72 U/ml by *Stenotrophomonas* sp. and *P. aeruginosa*, respectively, at pH 7. Generally, bacteria prefer neutral pH for their best growth and lipase production (Carvalho *et al.* 2013). The effect of temperature was carried out against different temperatures, and the result showed that the both isolates gave maximum production of lipase at a temperature of 37°C (Fig. 5). The incubation temperature revealed that increasing the incubation temperature up to 37°C resulted in increasing enzyme activity up to 23.11 ± 1.42 and 5.50 ± 0.72 U/ml by *Stenotrophomonas* sp. and *P. aeruginosa*, respectively. Similarly, *P. aeruginosa* was reported to produce maximum lipase at 37°C by Padhiar and Kommu (2016). In a study, Yuan *et al.* (2016) reported that a slight increase in incubation temperature up to 38°C enhanced lipase production.

The maximum enzyme activities of 47.33 ± 0.50 and 32.00 ± 0.50 U/ml were observed by *Stenotrophomonas* sp. and *P. aeruginosa*, respectively, with glucose (Fig. 6A). Using a better carbon source, various concentrations of glucose (0.5 - 3.0% w/v) were tested for better lipase production (Fig. 6B). Results showed that there was an increase in lipase production in these two isolates with an increase in the concentration of glucose up to 2.0% (w/v) and thereafter, a decrease in the enzyme concentration. The highest lipase activities were observed at this concentration by *Stenotrophomonas* sp. (57.33 ± 0.44 U/ml) and *P. aeruginosa* (43.28 ± 0.51 U/ml), which were 2.05 and 5.82-fold higher than without adding glucose. Kathiravan *et al.* (2012) reported that glucose showed the optimum enzymatic activity of *P. aeruginosa* at 1.0%

(w/v) carbon source. Maximum enzyme production with glucose suggests that glucose could be the best inducer for lipase than other carbon sources used. According to Novototskaya-Vlasova *et al*. (2013), olive oil and glucose together boost lipase activity. The present investigation significantly increased the lipase activity of both the isolates by adding olive oil and carbon sources to the growing medium.

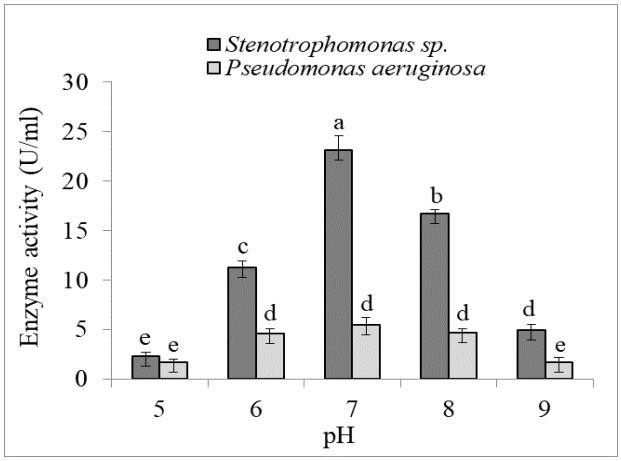

Fig. 4. Effects of pH on lipase production of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* in TSB medium with 1% (v/v) olive oil.

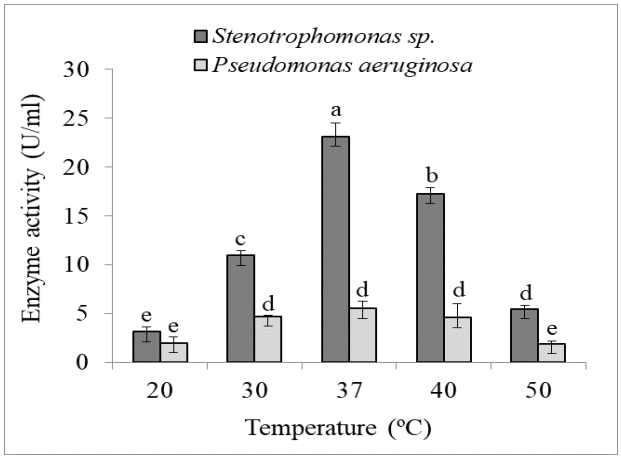

Fig. 5. Effects of temperatures on lipase production of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* isolates in TSB medium with 1% (v/v) olive oil.

Like carbohydrate, the effect of nitrogen sources was evaluated with different organic and inorganic nitrogen sources. Among them, the highest activity of lipase by *Stenotrophomonas* sp. and *P. aeruginosa* was obtained in the case of peptone, which attained enzyme activity up to 42.17 ± 0.84 and 21.45 ± 0.75 U/ml, respectively (Fig. 7A). For maximum lipase production, the best nitrogen source was used in different concentrations for optimum production. Results showed that there was an increase in lipase production with an increase in the concentration of peptone up to

1.5% (w/v) for both the isolates (Fig. 7B). The enzyme activities showed at 1.5% peptone concentration were 48.11 ± 0.84 and 27.33 ± 0.34 U/ml by *Stenotrophomonas* sp. and *P. aeruginosa*, respectively. Bhattacharya *et al.* (2016) showed that when organic nitrogen sources such as yeast extract and peptone were used, the bacteria, especially various thermophilic *Bacillus* spp. and *Pseudomonas* spp., were able to produce greater levels of lipase. Noormohamadi *et al.* (2013) reported that the use of olive oil in combination with other nitrogen sources enhanced bacterial lipase production.

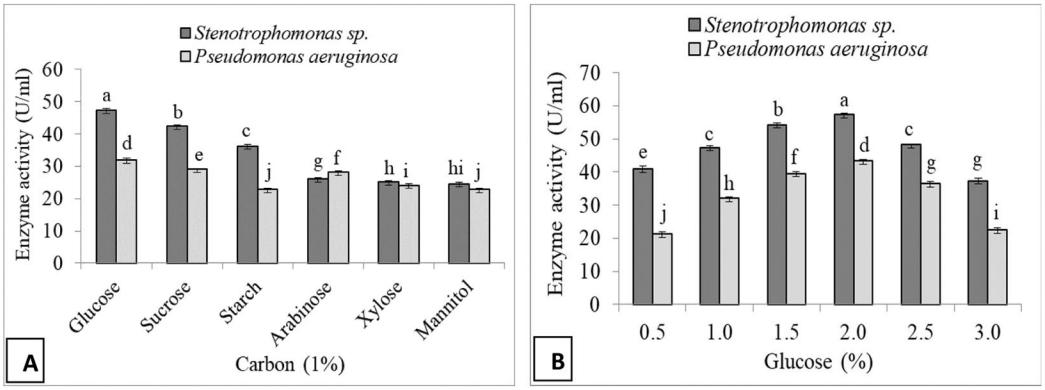

Fig. 6. Effects of carbon sources (A) and glucose concentrations (B) on lipase production of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* isolates in TSB medium with 1% (v/v) olive oil.

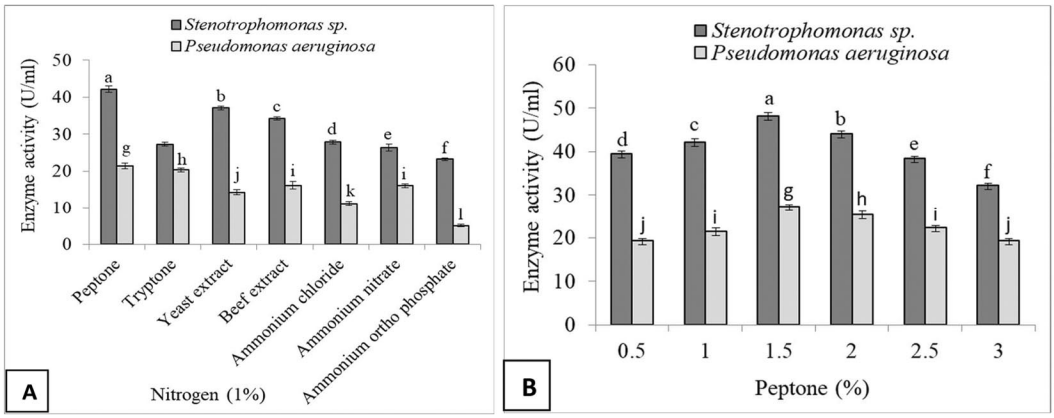

Fig. 7. Effects of different nitrogen sources (A) and peptone concentrations (B) on lipase production of two isolates in TSB medium with 1% (v/v) olive oil.

The effect of inoculum concentration were tested for lipase production by the isolates. For this purpose, 1-7% fresh inoculum was used, and maximum activity was obtained at 5% inoculum concentration (Fig. 8). In this case, lipase production in *Stenotrophomonas* sp. and *P. aeruginosa* was recorded at 73.50 ± 0.44 and 37.05 ± 0.75 U/ml, respectively, which were found to be 3.18 and 6.74-fold higher than before. Similarly, the maximum output of lipase was reported by Mazhar *et al*. (2017) for the *Bacillus subtilis* PCSIRNL-39 strain at 5% inoculum concentration.

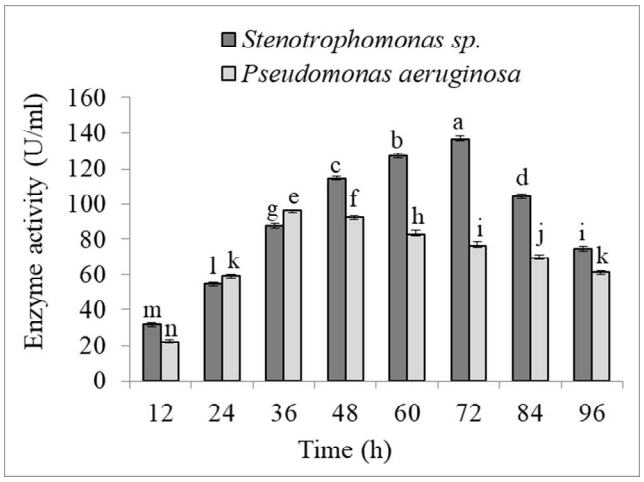

Fig. 8. Effects of inoculum concentrations on lipase production of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* isolates in TSB medium with 1% (v/v) olive oil.

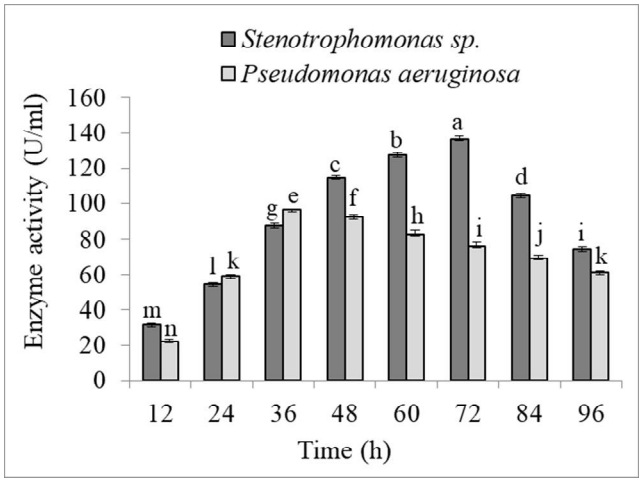

Fig. 9. Lipase production of *Stenotrophomonas* sp. and *Pseudomonas aeruginosa* isolates at different time intervals optimum conditions (pH 7, temperature 37°C, glucose 2.0% and 1.5% peptone as nitrogen source, 5% inoculum in TSB medium with 1% (v/v) olive oil).

For maximum lipase production, pH 7, glucose 2.0% and peptone 1.5% were used as production medium with 5% fresh inoculum. Inoculated flasks were incubated at 37ºC for 96 h., and samples were analyzed at 12 h. intervals. The results clearly reflected an enhanced production of lipase in the possible optimum condition (Fig. 9). The maximum lipase production by *Stenotrophomonas* sp. occurred at 72 h. of incubation, with a production of 136.47 ± 1.63 U/ml. This was 5-fold higher than before optimization. On the other hand, *P. aeruginosa* showed the highest activity at 36 h. of incubation, and lipase activity was 96.33 ± 0.21 U/ml, which is about 9-fold higher than before optimization. The time of incubation showed a sharp decrease in lipase activity after 72 h. of incubation in *Stenotrophomonas* sp. and 36 h. in *P. aeruginosa.* After these periods, the enzyme tends to decrease, indicating that the enzyme may have been either broken

down or lost its ability to function due to a reduction in the amount of lipidic substrate. In the present investigation, lipase activity was 96.33 ± 0.21 U/ml by the isolated *Pseudomonas aeruginosa* BA7823, which is higher than 66.26 U/ml by *Pseudomonas* sp. (Tembhurkar *et al*. 2012) and 81.83 U/ml by *Pseudomonas* sp. BWS-5 (Sooch and Kauldhar 2013). Isolated *Stenotrophomonas* sp. ICMM10 showed lipase activity of 136.47 ± 1.63 U/ml, which was higher than the 114 U/ml produced by *Pseudomonas gessardii* reported by Veerapagu *et al*. (2013). After adjusting various culture settings, a significant amount of lipase production was attained by the two indigenous isolates, especially *Stenotrophomonas* sp. ICMM10. According to the best knowledge of the authors, *Stenotrophomonas* sp. ICMM10 is a novel strain as a potential lipase-producing bacterium in Bangladesh. Results of the present analysis can therefore, undoubtedly have an impact on future biotechnological applications.

Two indigenous bacterial isolates, *Stenotrophomonas* sp. ICMM10 and *Pseudomonas aeruginosa* BA7823, were found to be potential lipase producers. The best culture parameters for the indigenous isolates to produce maximum lipase were examined in this work, and they may serve as a baseline for future research involving the treatment of lipid-rich wastewater.

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