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OPTIMIZATION OF LIPASE PRODUCTION BY *BACILLUS SUBTILIS* 20B

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Abstract

This study aims to screen out the potential lipase-producing bacteria and optimize different culture parameters to produce maximum lipase enzyme. A total of twenty-nine lipase-positive bacteria were isolated from organic waste. Among them, *Bacillus subtilis* 20B was found to be a potential candidate. The optimum pH and temperature of lipase production by the isolate were 7.0 and 37ºC, respectively, and the lipase production was 11.72 ± 0.25 U/ml. When the isolate was cultured in medium supplemented with starch as a carbon source, the isolate produced a better level (28.39 ± 1.19 U/ml) of lipase. On the other hand, lipase production increased up to 21.61 ± 0.75 U/ml when yeast extract was used as a nitrogen source. After optimization of inoculum concentration, enhanced lipase production was 51.17 ± 0.50 U/ml. Through optimization, lipase production increased up to 84.30 ± 0.17 U/ml, which was 7.19-fold higher than before. The present study provides useful information about lipase production by indigenous *B. subtilis* 20B, which may be used as a potential bacterial source of lipase for further study involving lipid-rich wastewater treatment and other purposes.

Introduction

Lipases represent the third most vital class of enzymes, next to carbohydrases and proteases (Kavitha 2016). They are recently given abundant attention with the speedy development of enzyme technology. Lipases are also commercially important enzymes that catalyze the chemical reaction of triglycerides to free fatty acids and glycerol (Habibollahi and Salehzadeh 2018). These enzymes can be produced from plants, animals, and microorganisms. Among them, microorganisms have been shown to produce significant yields of lipases when compared to plants and animals (Mendes *et al*. 2010). Lipases are currently attracting enormous attention due to their being the most versatile and widely used enzymes in biotechnological applications and owing to their unique properties (Kapoor and Gupta 2012). Lipases are now applied in the modern food industry instead of traditional chemical processes and used in the production of a variety of products like fruit juices, baked foods, fermented vegetables, cheese, soups, and sauces (Rasmey *et al*. 2017). Microbial lipases are ubiquitous in nature and are commercially important because they are cheaper to produce, more stable, and more readily available than animal and plant lipases (Sharma *et al*. 2017). Natural or recombinant microbial lipases are widely used in various biotechnological applications, such as in the biological treatment of lipid-rich waste effluents (Sarmah *et al*. 2018). Most of the extracellular lipases are derived from fungi and bacterial species, which are different in enzymological features and specificities (Patel *et al*. 2018, Bharathi and Rajalakshmi 2019).

Lipase production by various bacterial species has been extensively studied and reported. They are found in diverse habitats, including oil processing industries, organic wastes, dairy industries, slaughter-house, decaying foods, oil-contaminated sites, etc. (Mobarak-Qamsari *et al*. 2011). Among different bacteria, *Pseudomonas* sp. and *Bacillus* sp. constitute a major source of lipase enzyme, and members of these bacteria are of interest in biotechnology (Awad *et al*. 2015).

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The potential metabolic activities of bacteria are largely dependent on the culture media compositions. Mostly, bacterial lipases are extracellular, and the production of lipase is influenced by media composition with various nutritional factors (carbon and nitrogen source) and physicochemical factors such as pH, temperature, presence of lipid as inducer, etc. (Ilesanmi *et al*. 2020).

Therefore, different culture media on lipase production have different stimulatory effects (Dhiman and Chapadgaonkar 2013). Generally, bacterial lipases show activity in a large pH range (pH 4-11), and the thermal stability of these lipases ranges from 20 to 60ºC (Gururaj *et al.* 2016). One of the major factors in order to express the lipase enzyme is the carbon source since these are inducible enzymes. Lipases are normally produced in the presence of lipid sources such as oil, fatty acids, tweens, triacylglycerols, glycerols, and hydrolyzable esters in addition to carbon source. On the other hand, the types of nitrogen sources also influence lipase production, especially organic nitrogen (Alhamdani *et al.* 2016). Considering the importance of biotechnologically and industrially significant bacterial lipase enzymes, the study was undertaken to screen potential indigenous lipase producing bacteria and the optimization of media compositions for maximum lipase production.

Materials and Methods

For the isolation of bacteria, soil mixed with organic waste was collected from Mirpur, Dhaka Metropolitan City, Bangladesh. Bacteria were isolated through the serial dilution technique at 37ºC for 48 hrs. Bacterial isolates were randomly selected and purified by repeated streaking on nutrient agar (NA) plates. The cultures were preserved at 4ºC for further use. Pure cultures were grown on two lipid-based media, tributyrin agar (TBA) and tween agar (TA), for 48 hrs at 37ºC for detection of bacterial lipolytic activity. The lipase activities of isolates were observed by clear zone formation in TBA plates and intensity of opaque zone in TA plates around the colonies (Bueno *et al*. 2014).

The isolated bacterium was identified molecularly by 16S rRNA gene sequencing. The 16S rRNA gene from the genomic DNA was amplified by PCR using the universal primers. The heat-thaw method was used for bacterial DNA extraction and preserved at -20°C (Salehi *et al*. 2005). Then PCR amplification was performed in a thermocycler (Aktar *et al*. 2016). PCR products were investigated on 1% 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 identity of the sequence obtained was established by comparing it with the gene sequences in the database using the BLASTn software provided by the National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov) against diverse sequences (Kerbauy *et al*. 2011).

The production of lipase was carried out by submerged fermentation process (Anbu *et al.* 2011). As a liquid culture medium, tryptone soya broth (TSB) was used, and 1% olive oil as a substrate was added with the medium. In this experiment, 50 ml of TSB medium was taken in a 100ml Erlenmeyer flask and inoculated with 1% inoculum. The inoculated flasks were then incubated at 37°C for 48 hrs with constant shaking at 150 rpm in a rotary shaker (Daihan Labtech, England). Samples were then removed aseptically, and cell-free supernatant was recovered by centrifugation at 9,000 rpm for 20 min at 4ºC. The clear supernatant was collected, and it was used as a source of crude enzyme for the determination of lipolytic activity.

Lipase activity 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 with 150 rpm. After

that, the reaction was immediately stopped by submerging the mixture in an ice bath for 10 minutes. About 200 µl of the aliquot was added to the reaction mixture, which contains 1,800 µl of iso-octane and 400 µl of cupric acetate pyridine at pH 6.0. The upper layer of the iso-octane fraction was pipetted, and the quantity of free fatty acid that dissolved in the iso-octane 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 the bacterial lipase enzyme, which was estimated following a well-known oleic acid standard curve. One unit of lipase activity was defined as the amount of lipase enzyme that produced one µmole fatty acid per min. All experiments in this study were carried out in triplicates.

Optimization of different physico-chemical parameters such as pH, temperature, carbon source, nitrogen source, and inoculum concentration were carried out where 1% (v/v) olive oil was used as an inducer for lipase production (Kumar *et al*. 2012, Veerapagu *et al.* 2013). Variations in the medium's pH (5–9) at 37°C were used to test the effects of pH on lipase synthesis. After being added to the lipase production medium, the bacterial isolate was cultured for 48 hrs at 37°C. In order to examine the impact of temperature on lipase production, the bacterial isolate was inoculated in the lipase production medium at pH 7 and incubated for 48 hrs at five different temperatures (20, 30, 37, 40, and 50°C).

Different kinds of carbon sources, such as glucose, sucrose, starch, arabinose, xylose, and mannitol were used as they might induce lipase production. For this reason, these carbon sources were added at a concentration of 1% (w/v) in the lipase production medium and incubated at 37^{o}C for 48 hrs. Different 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 ($NH_4H_2PO_4$) at a concentration of 1% (w/v) were added in the production media as they might affect lipase production. Bacterial culture was inoculated into production media containing each kind of nitrogen source and incubated at 37ºC for 48 hrs. Cell cultures of different concentrations (1 to 7%) were inoculated into production media to evaluate the effect of inoculum concentration on lipase production and incubated at 37ºC for 48 hrs.

The ideal condition was used, taking into account all the criteria for optimizing lipase production. For this, a lipase estimate was done at 12 h intervals while inoculation flasks were incubated for 96 hrs at 37°C. Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) version 20 (SPSS Inc.). Data were analyzed as mean ± standard deviation.

Results and Discussion

Based on morphological differentiation of individual colonies on dilution plates, different types of bacterial isolates were isolated. Then they were screened for lipase production on TBA and TA medium. Among the total isolates, twenty-nine showed lipase positive. After careful screening (based on their clear zone formation in TBA medium and intensity of opaque zone in TA medium), nine isolates (S_3N-3, S_3N-6, S_3P-1, S_3P-2, S_3T-4, S_3T-5, S_3T-7, S_3T-8, and S_3T-9) were selected as better lipase producers. Among them, isolate S_3T-9 was the best possible lipase producer, as it showed the highest clear zone (10.5 mm) in TBA medium (Fig. 1A) and the highest intensity of opaque zone in TA medium (Fig. 1B) after 48 hrs of incubation. Then the isolate was identified molecularly by 16S rRNA gene sequencing as *B. subtilis* 20B. Later on, this strain was used for optimization of lipase production.

The effect of different pH ranging from 5 to 9 on the production of extracellular lipase by *B. subtilis* 20B was observed. The result clearly indicated that the isolate showed the highest activity at pH 7 (Fig. 2). Maximum lipase activity was 11.72±0.25 U/ml at pH 7.0, while minimum activity was 1.78±0.42 U/ml obtained at pH 5. This result inferred that this strain prefers neutral pH for better lipase production. Generally, bacteria prefer neutral pH for their best growth and lipase production (Joyruth and Growthe 2020). Temperature would be one of the important parameters for better lipase production. The effect of temperature was carried out against different temperatures, and the result showed that the isolate gave maximum lipase production (11.72±0.25 U/ml) at a temperature of 37°C (Fig. 3). This isolate showed the lowest activity (1.28±0.35 U/ml) at 20°C. Similarly, *B. subtilis* I-4 isolated from oil-contaminated wastewater had its maximum lipase activity at 37°C (Iqbal and Rehman, 2015). In contrast, 45°C was the optimum temperature for *B. subtilis* PCSIRNL-39 (Mazhar *et al.* 2017).

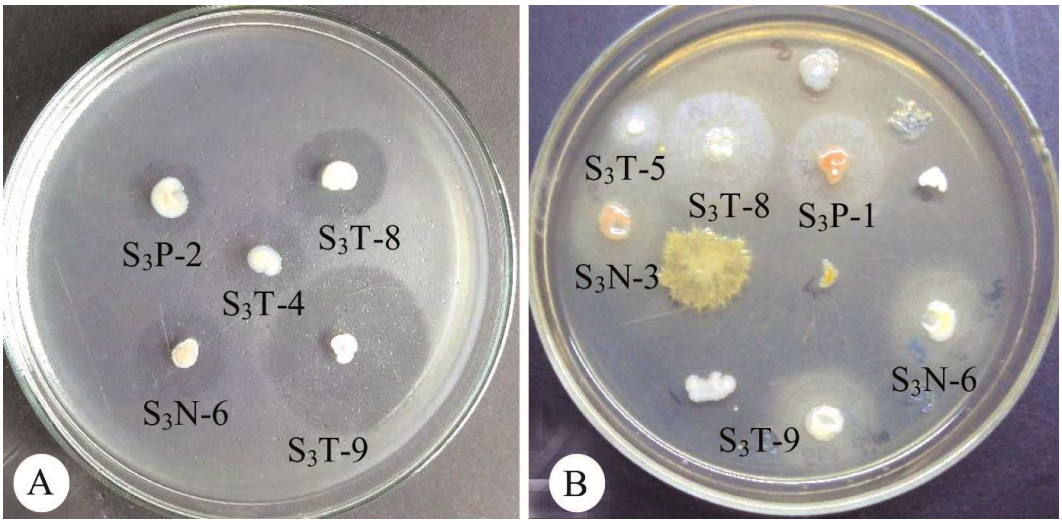

Fig. 1. Clear zone formation in TBA medium (A) and opaque zone formation in TA medium (B) of lipase-positive bacterial isolates around their colonies.

Various carbon sources at 1% concentration were added to the production medium, and their effects on lipase production of the isolate were evaluated. An effect of the carbon source is shown in Fig. 4. Before adding any carbon source, *B. subtilis* 20B could produce lipase 11.72 ± 0.25 U/ml. In the case of starch, the highest lipase production was 28.39±1.19 U/ml, attained by this isolate, which was 2.42-fold higher than without adding starch. Mannitol showed a minimum response for lipase production (15.45 ± 0.63 U/ml). This result was also supported by Joseph *et al.* (2012) in *B. sphaericus* MTCC 7526 when they used starch for lipase production. In contrast, Bharathi *et al.* (2019) reported that sucrose showed higher lipase production as a carbon source in *Bacillus* sp.

The effect of nitrogen source on lipase production was evaluated by supplementing production medium with different organic and inorganic nitrogen sources. Among different nitrogen sources, the highest activity of lipase by *B. subtilis* 20B was obtained in the case of yeast extract, which attained the enzyme activity up to 21.61±0.75 U/ml (Fig. 5). The lowest amount of lipase was produced with ammonium orthophosphate, which gave 13.28 ± 0.42 U/ml. The result

showed that the supplementation of the nitrogen source enhanced lipase production. Yeast extract gave almost similar results (20 U/ml) in the case of *Bacillus* sp. reported by Bora and Bora (2012). Bhattacharya *et al.* (2016) showed that when organic nitrogen sources such as yeast extract and peptone were used, bacteria, especially various thermophilic *Bacillus* spp., were able to produce greater levels of lipase.

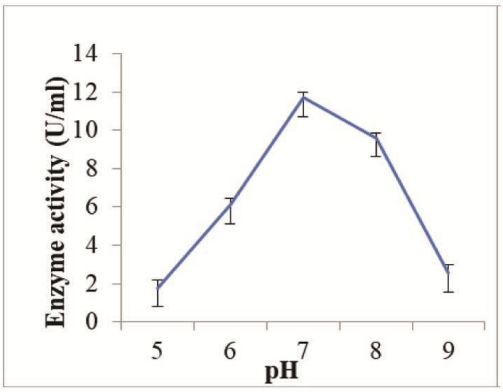

Fig. 2. Effects of pH on lipase production by *B. subtilis* 20B.

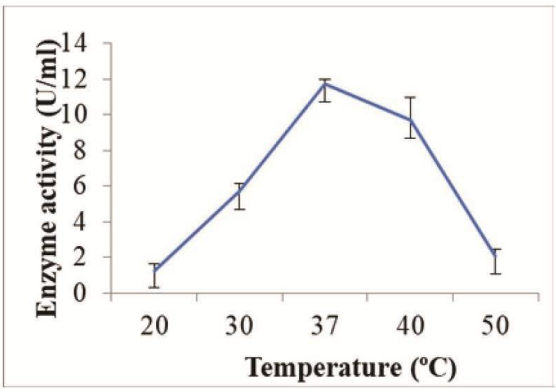

Fig. 3. Effects of temperature on lipase production by *B. subtilis* 20B.

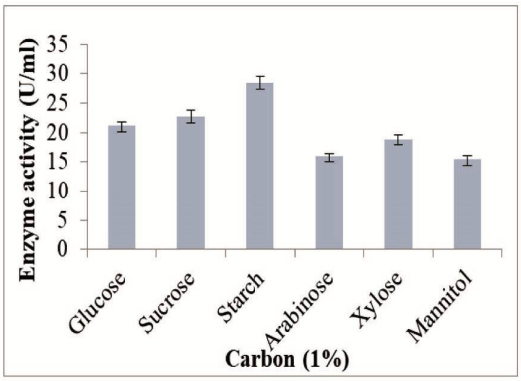

Fig. 4. Effects of carbon source on lipase production by *B. subtilis* 20B.

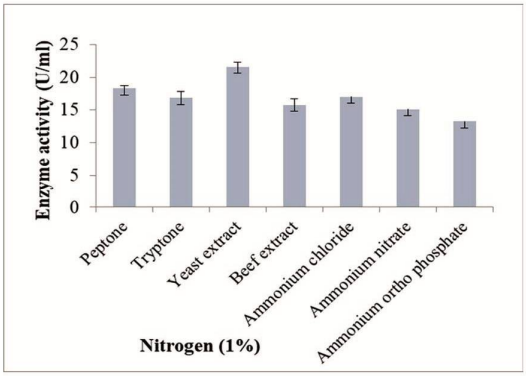

Fig. 5. Effects of nitrogen source on lipase production by *B. subtilis* 20B.

For the increase of lipase production, inoculum concentration was also increased from 1 to 7%, and maximum activity was obtained at 5% inoculum concentration (Fig. 6). In this case, lipase production by the isolate was 51.17 ± 0.50 U/ml, which was found to be 4.37fold higher than before optimization. This study also revealed that increase of inoculum concentration up to 5% in production media there was a considerable increase in the lipase production. Decreased lipase production was noticed when more than 5% inoculum concentration was added into the medium. Mazhar *et al.* (2017) reported the maximum lipase production for the *B. subtilis* PCSIRNL-39 strain occurs at 5% inoculum concentration.

After optimization of all studied parameters, the best conditions were set for the maximum lipase production by the isolated *Bacillus subtilis* 20B. To carry out this work, 5% inoculum was added in the production medium at pH 7.0, which contains 1% starch as a carbon source and 1% yeast extract as a nitrogen source. Inoculated flasks were incubated at 37ºC for 96 hrs, and samples were analyzed in every 12 hrs interval. The result clearly reflected an enhanced production of lipase in the possible optimum condition (Fig. 7). The results showed that the maximum lipase production (84.30 ± 0.17 U/ml) by *B. subtilis* 20B occurred at 36 hrs of incubation, which was 7.19fold higher than before. The isolate showed considerably better performance after optimization. The time of incubation showed a sharp decrease after 36 hrs of incubation, and after this period, the enzyme tends to decrease. Kathiravan *et al.* (2012) have also reported such a trend with *Pseudomonas aeruginosa*. The rate of enzymatic reactions increases with the increase in temperature till optimum temperature, and after that, enzyme inactivation occurs due to denaturation of protein. It causes the slow cell metabolism and ultimately affects the cell growth as well as productivity (Pham *et al.* 2020).

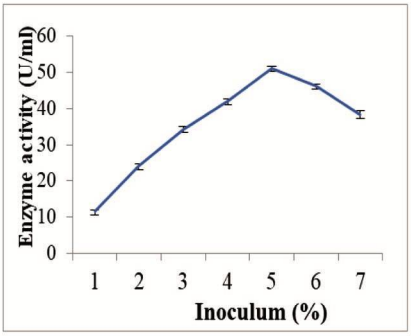

Fig. 6. Effects of inoculum concentration on lipase production by *B. subtilis* 20B.

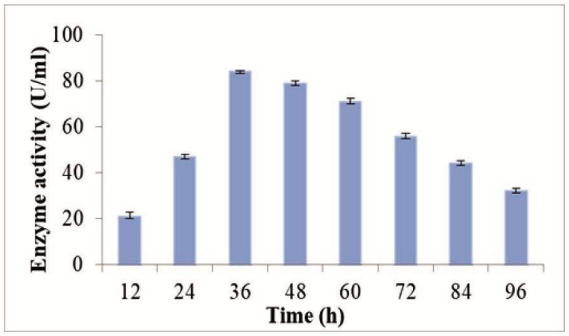

Fig. 7. Lipase production by *B. subtilis* 20B in all studied optimum conditions.

To our best knowledge, this type of work is least explored, in which all optimum conditions were set for maximum production of lipase. Overall, optimum conditions of the culture parameters that were investigated in this study might provide a baseline for further study involving lipid-rich wastewater treatment.

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