Volume: 01, Issue: 01, Year: 2025

International Journal of Multidisciplinary Research on Contemporary Issues
(IJMRCI)
journal homepage: https://www.ijmrci.org/index.php

Optimization of cultural conditions of lipase producing bacteria isolated from organic waste

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ARTICLE INFO

***Article history*:**
Received08June 2025
Received in revised form 21 July 2025
Accepted26July 2025
Available online 21 September 2025

***Keywords*:**
Bacillussubtilis,bacteria, enzyme, lipase, optimization

DOI:

ABSTRACT

This study aims to screen out the potential lipase producing bacteria and optimize various culture settings in order to maximize the production of lipase enzyme.From organic waste, a total of 29 lipase positive bacteria were isolated.*Bacillussubtilis* 20B was identified as having potential among them. The isolate produced lipase at an optimal level of 17.28±0.75 U/ml with 0.5% NaCl. The isolate produced a higher amount of lipase (37.39±0.67 U/ml) when it was cultivated in medium supplemented with 1.5% starch as a carbon source. However, when 1.5% yeast extract was utilized as an organic nitrogen source, it climbed to 25.17±0.67 U/ml and when 1.5% ammonium chloride was supplied as an inorganic nitrogen source, lipase production rose to17.22±0.51 U/ml. Following substrate concentration adjustment, 1% olive oil concentration showed the highest activity (11.72±0.25 U/ml). Nutrient Broth (NB) medium was found to be better basal medium for lipase production with maximum activity of 16.28±0.42 U/ml. After 84 h of incubation, the isolate grew at its fastest rate (6.9×10^7cfu/ml), while the number of cells declined during this period. *B. subtilis* produced the most lipase during the exponential growth phase, which lasted 36 h. The current study provides valuable insights into the production of lipase by *B. subtilis* 20B, which could be a viable bacterial source of lipase for future research on treating of lipid-rich wastewater and other applications.

1. Introduction

All synthetic and degradative reactions of living things are catalyzed by enzymes, which are extremely effective biological catalysts. Since its first discovery in the second half of the nineteenth century, enzymes have become much more widely used in a variety of industries (Jamilu et al., 2022).

A class of hydrolytic enzymes known as lipase (triacylglycerol acylhydrolases; EC 3.1.1.3) catalyzes the hydrolysis of triacylglycerol to glycerol and free fatty acids (Gupta et al., 2004). Many lipases are used extensively in the food, detergent, pharmaceutical, leather, cosmetic, textile, dairy, and even biodiesel sectors because of their capacity to carry out both hydrolytic and synthetic processes (Hasan et al., 2006 & Robles-Medina et al., 2009). Although lipases are extensively found in both plants and animals, the majority of lipases that are available commercially are often derived from microbes that generate a diverse range of extracellular lipase (Kexin et al., 2021).

One of the biggest challenges facing the industry was thought to be the cost of the lipase production process. As a result, a lot of work is being done to use wastes as raw materials for the production of lipase. Future biotechnologies will heavily rely on agricultural leftovers for the manufacturing of lipase and other value-added products, primarily because to their environmentally friendly lines and adaptability to both developed and developing nations (Yang et al., 2021).

Every living thing has lipases, which are necessary for their regular functioning. The application of microbial lipases has drawn a lot of interest in recent decades (Rozi et al., 2022). These microbes are potential sources of lipases (Fatima et al., 2021). Many microbial strains have been screened and characterized for their ability to produce enzymes, and microbial biotechnologists have recently turned their focus to the commercial application of lipase of microbial origin. The microorganisms most commonly employed to produce lipase include *Aspergillusniger*,*Penicillium* sp., *Candidarugosa*,

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Rhizopus sp., *Bacillus* sp. and *Pseudomonas* sp. (Lakshmi & Anupudi, 2021; Rodriguez-Salarichs et al., 2021; Helal et. al., 2021). Numerous microorganisms from various bacterial, fungal, and yeast genera have been isolated and screened from various environmentalsettings and these are the potential source of lipases (Yuan et al., 2021 & Pang, 2021).

The optimization process is typically approached one variable at a time, and the media composition factors that are subject to variation include physicochemical factors (incubation time, temperature, pH, presence of lipid as inducer, cofactors, inhibitors) and various nutritional factors such as carbon and nitrogen sources (Adio et al., 2021). Consequently, the stimulatory effect of various media on lipase synthesis varies (Phukon, et al., 2020). Since most researchers only concentrate on pH and temperature of the enzyme optimization, it was also crucial to evaluate the impact of different circumstances on substrate specificity. Its effectiveness and efficiency may suffer greatly if the impact of other parameters on enzyme production are not examined. Thus, the present work was undertaken to screen indigenous bacteria from organic waste for lipolytic activity and optimize the effects of various cultural factors on lipase synthesis.

2. Methodology

Isolation of lipase producing bacteria

In order to isolate bacteria, soil containing organic waste was collected from Mirpur, Dhaka Metropolitan area, Bangladesh. At 37ºC for 48 h, bacteria were isolated using the serial dilution technique. On Nutrient Agar (NA) plates, bacterial isolates were repeatedly streaked after being chosen at random and purified. The cultures were kept for later use at 4ºC. For 48 h at 37ºC, pure cultures were cultivated on two lipid-based media Tributyrin agar (TBA) and Tween agar (TA), in order to detect bacterial lipolytic activity. The development of a clear zone in TBA plates and the degree of opaque zone surrounding the colonies in TA plates are indicators of the lipase activities of the isolates (Buenoet al., 2014).

Molecular identification

The isolated bacteria was molecularly identified by 16S rRNA gene sequencing. Using the universal primers, the 16S rRNA gene from the genomic DNA was amplified by PCR. Bacterial DNA was extracted using the heat-thaw technique and stored at -20°C (Salehi et al., 2005). Then, using a thermocycler, PCR amplification was carried out (Aktar et al., 2016). The NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNABLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs were used toanalyze the sequence produced by automated sequencing of PCR amplified DNA in order to identify potential similar organisms through alignment of homologous sequences (Kerbaury et al., 2011). A phylogenetic tree was created using the sequence found in the BLAST search. The BLASTN (nucleotide BLAST) sequence was retrieved in FASTA format, and the CLUSTAL OMEGA algorithm software was used to align numerous sequences in order to determine the relationship between each sequence. Neighbor joining (NJ), a distance-based phylogenetic analysis tool was used to create the tree.

Lipase production

To produce lipase, the procedure of submerged fermentation was used (Anbuet al., 2011). Tryptone Soy Broth (TSB) was utilized as the liquid culture medium and 1% olive oil was added as a substrate. In this experiment, 1% inoculum was added to 50 ml of TSB medium in a 100 ml Erlenmeyer flask. A rotary shaker (Daihan Labtech, England) was used to continuously shake the inoculated flasks at 150 rpm for 48 h while they were incubated at 37°C. Following aseptic sample removal, cell-free supernatant was extracted using centrifugation at 9,000 rpm for 20 min at 4ºC. To determine the lipolytic activity, the clear supernatant was collected and utilized as a source of crude enzyme.

Estimation of lipase activity

Lipase activity was estimatedas stated by Marseno et al. (1998). About 2 ml of reaction mixture, which contains 60% (v/v) olive oil dissolved in iso-octane, were taken and placed in a screw-cap vial. The reaction was started by adding 20 µl of crude enzyme and running at 150 rpm for 30 min at 30ºC. The combination was then immersed in an ice bath for ten minutes to instantly cease the reaction. The reaction mixture, which contains 1800 µl of iso-octane and 400 µl of cupric acetate pyridine at pH 6.0, was mixed with around 200 µl of the aliquots. The top layer of the iso-octane fraction was pipetted and the amount of free fatty acid that dissolved in the iso-octane layer was measured spectrophotometrically by measuring the optical density at 715 nm. The amount of oleic acid that liberated as free fatty acid from olive oil by the bacterial lipase enzyme was measured in order to quantify lipase activity. Thiswas estimated using a well-known oleic acid standard curve. The quantity of lipase enzyme that generated oneµmole fatty acid per minute was considered to beoneunit of lipase activity. In thisinvestigation, every experiment was conducted in triplicate.

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Optimization of different cultural parameters on lipase production

Optimization of different cultural parameters such as NaCl, concentration of carbon source, organic and inorganic nitrogen source, substrate and medium were carried out (Kumar et al., 2012 & Veerapagu et al., 2013). In this case, 1% (v/v) olive oil was used as an inducer for lipase production.

Effects of NaCl on lipase production

Different NaCl concentrations (0.5, 1.0, 1.5, 2.0, and 2.5%) were added to the lipase production medium while maintaining the same levels of all other parameters in order to examine the effects of NaCl on lipase production. After being added to the medium, the bacterial isolate was cultured for 48 h at 37°C.

Optimization of concentration of better carbon and nitrogen sources

Better carbon and nitrogen sources which support maximum lipase production were added at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) in the production medium. The inoculated lipase production medium was incubated for 48 h at 37ºC in order to optimize it.

Effects of substrate concentration on lipase production

Using various olive oil concentrations (0.5, 1.0, 1.5, 2.0, 2.5, and 3.0% v/v) in the medium, the effects of olive oil concentration on lipase activity were ascertained. For 48 h, the inoculated medium was incubated at 37ºC.

Effects of media on lipase production

Five distinct basal media *viz.* Nutrient Broth (NB), Luria Bartani (LB), Tributyrin Broth (TBB), Tween Broth (TB), and Tryptone Soya Broth (TSB) were investigated for lipase production., The inoculated media were incubated at 37ºC for 48 h.

Determination of bacterial growth

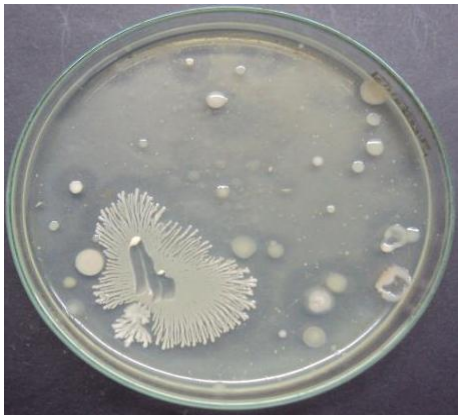
By counting bacterial colonies, the growth of bacteria was ascertained. Using the dilution plate technique, the number of bacteria was measured as colony forming unit (cfu/ml). Serially diluted 1 ml culture mixed with molten nutrient agar. For 24 h, the inoculated plates were incubated at 37°C. A colony counter was used to count the number of bacterial colonies that had grown in the inoculated plates following incubation.

Statistical analysis

The Statistical Package for the Social Sciences (SPSS) v.20.0 for Windows (SPSS, SAS Institute Inc. Cary, USA) was used to conduct the statistical analysis. Descriptive statistics like mean and standard deviation were calculated by analyzing the data. The one-way ANOVA test with a 95% confidence interval using Duncan's Multiple Range Test was used to evaluate group differences. Value of p <0.05 was regarded as statistically significant.

3. Results

Different bacterial strains were isolated based on the morphological distinction of individual colonies on dilution plates. They were then tested on TA and TBA media for lipaseproduction. Of all the isolates, 29 were found to be lipase positive. Nine isolates demonstrated improved lipase production following rigorous screening (based on the severity of the opaque zone in TA medium and the creation of clear zone in TBA medium). The clear zone formation in TBA medium by the isolate is shown in Fig. 1. The strain that produced the most lipase among them was S_3T-9. Photomicrograph of this isolate is shown in Fig. 2. The bacteria was then molecularly identified as *Bacillussubtilis* 20B using 16S rRNA gene sequencing. Phylogenetic tree was constructed by neighbor joining method and shown in Fig. 3. This strain was later employed to optimize the production of lipase.



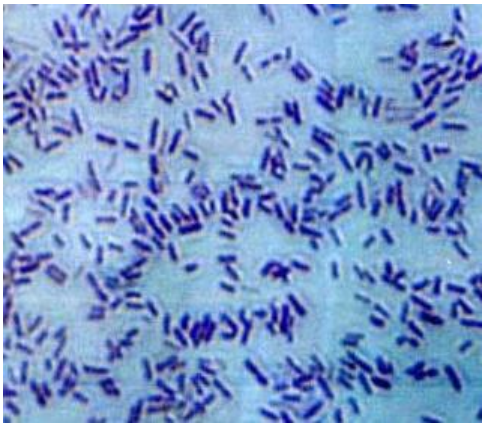


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Fig. 1: Photograph showing lipase producing bacterial colonies developed on TBA plate.

Fig. 2: Photomicrograph of strain S_3T-9.

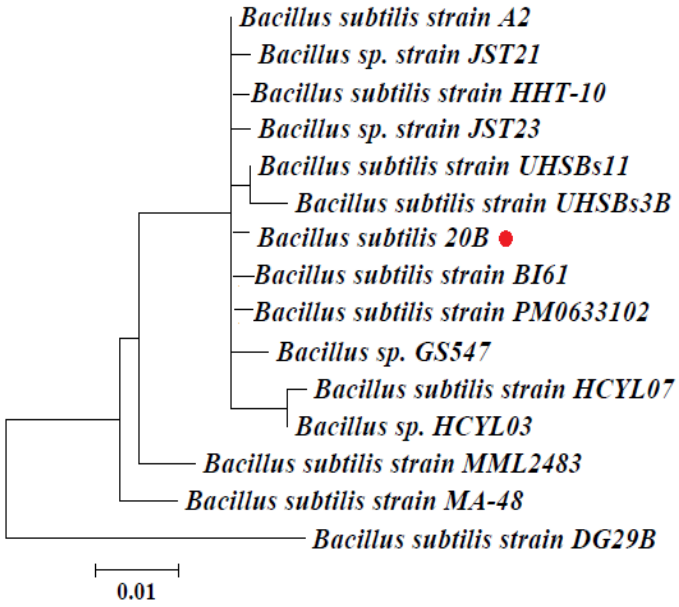

Fig. 3: A neighbor-joining phylogenetic tree of *B.subtilis* 20B.

Effects of NaCl concentration on lipase production

The effect of NaCl concentration on lipase production is shown in Fig. 4. The result indicated that lipase synthesis was impacted by increased salt content. A decrease in lipase synthesis was noted in response to increased NaCl concentration. At 0.5% NaCl, the maximum enzyme production (17.28±0.75 U/ml) was observed.

Optimization of better carbon source concentration on lipase production

Since starch was shown to be appropriate for lipase production, different quantities of starch (0.5 – 3.0%, w/v) were added to the medium to examine the impact on lipase production by the *B. subtilis* isolate. At 1.5% starch, the maximum enzyme activity (37.39±0.67 U/ml) was observed (Fig 5).

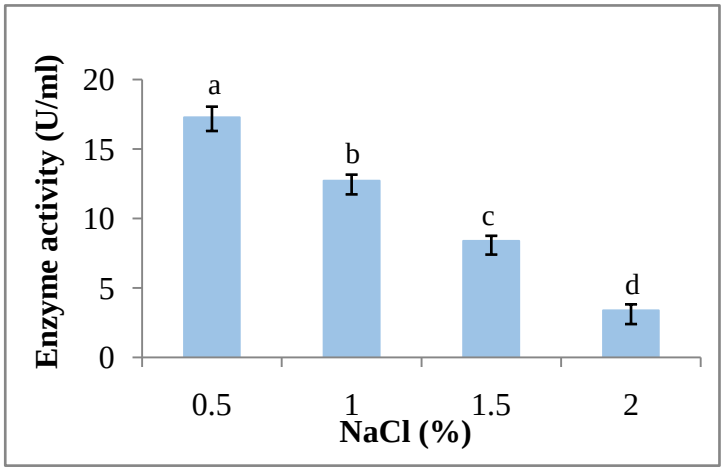

Fig. 4: Effects of NaCl on lipase productionof *B.subtilis* 20B.

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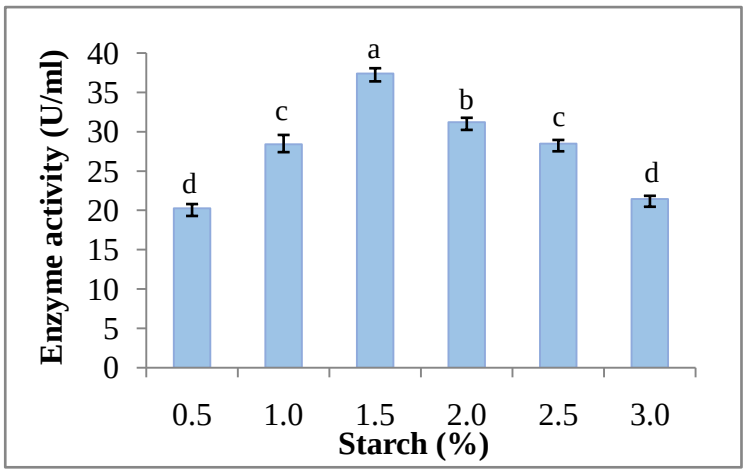

Fig. 5: Effects of starch concentration on lipase production of *B.subtilis* 20B.

Optimization of better organic nitrogen source concentration on lipase production

Effects of the concentration of better organic nitrogen source (yeast extract) on lipase production is shown in Fig 6. Since yeast extract was shown to be more effective for *B. subtilis*, different amounts of yeast extract (0.5–3.0%, w/v) were added to the medium to examine the impact on lipase production. At 1.5% yeast extract concentration, the maximum level of lipase production (25.17±0.67 U/ml) was observed.

Optimization of better inorganic nitrogen concentration on lipase production

Among different inorganic nitrogen sources, ammonium chloride was the most effective for lipase production of *B. subtilis*. Consequently, the medium was supplemented with varying amounts of ammonium chloride. It was shown that up to 1.5% ammonium chloride concentration boosted lipase synthesis, after which it decreased (Fig. 7). Ammonium chloride achieved lipase activity of 17.22±0.51 U/ml at this concentration.

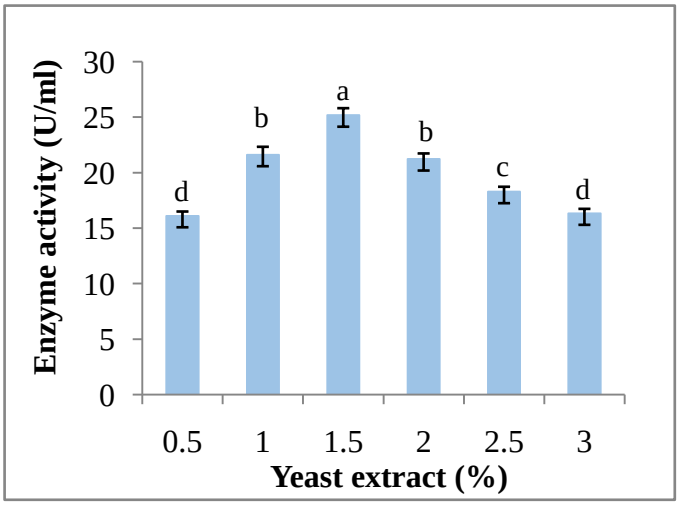

Fig. 6: Effects of yeast extract concentration on lipase production of *B.subtilis* 20B.

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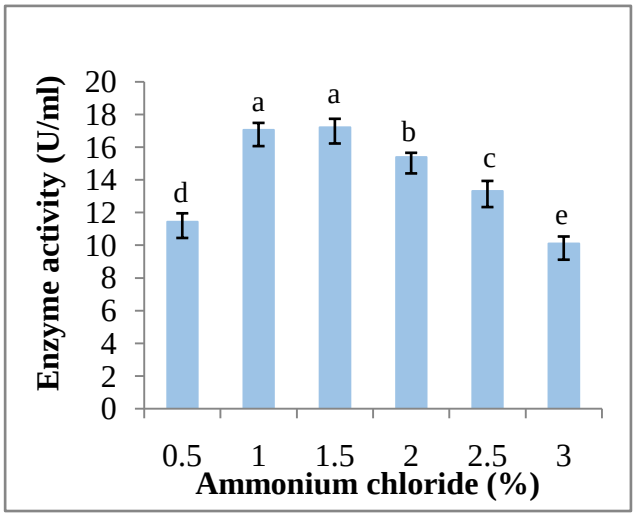

Fig. 7: Effects of ammonium chloride concentration on lipase production of *B.subtilis* 20B.

Effects of substrate concentration on lipase production

By incorporating different concentrations of olive oil (0.5–3%, v/v) into the medium, the impact of lipid as substrate on lipase synthesis was investigated. Lipase production was not significantly impacted by the content of olive oil, and the maximum activity (11.72±0.25 U/ml) was seen at 1% olive oil concentration (Fig. 8).

Effects of basal media on lipase production

Five standard basal media were examined for improved lipase production. Nutrient Broth (NB) medium shown to be more effective basal medium for lipase synthesis in this experiment. In NB medium with olive oil as the lipid substrate, *B. subtilis* demonstrated the highest lipase activity of 16.28±0.42 U/ml (Fig. 9).

Estimation of bacterial growth

The serial dilution plate technique was used to conduct this experiment at 12 h intervals for a total of 96 h. The bacterial growth pattern during lipase synthesis under all evaluated ideal conditions is displayed in Fig. 10. Initial cell density of the isolate was 1.56×10^3 cfu/ml. Increasing the incubation time showed that lipase production had been initiated slowly after 12 h and at 48 h the bacteria had obtained logarithmic growth phase. The viable count result showed that there was an increase in the number of cells from time 84 h in *B.subtilis* and then decreased as the time progressed. The isolate showed highest growth (6.9×10^7cfu/ml) at 84 h of incubation and cell number decreased over this time. Lipase production was the highest in exponential growth phase at 36 h of incubation by the isolate.

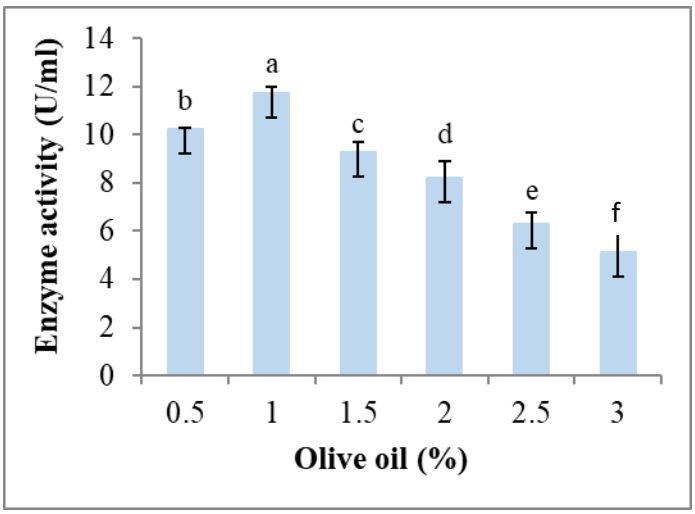

Fig. 8: Effects of substrate (olive oil) concentration on lipase production of *B.subtilis* 20B.

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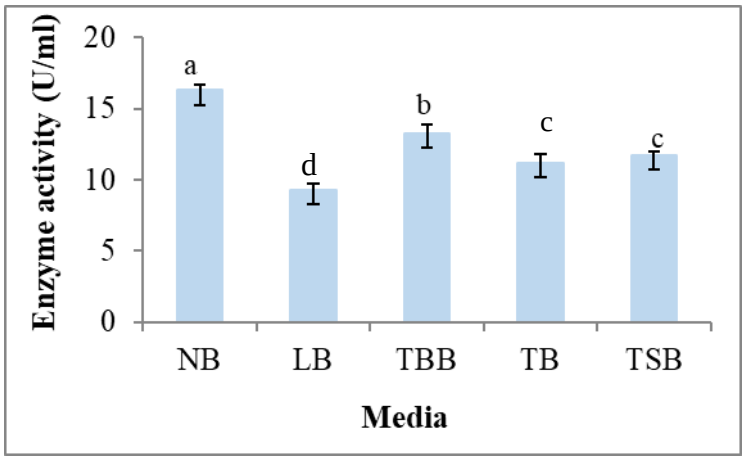

Fig. 9: Effects of basal media on lipase production of *B.subtilis* 20B.

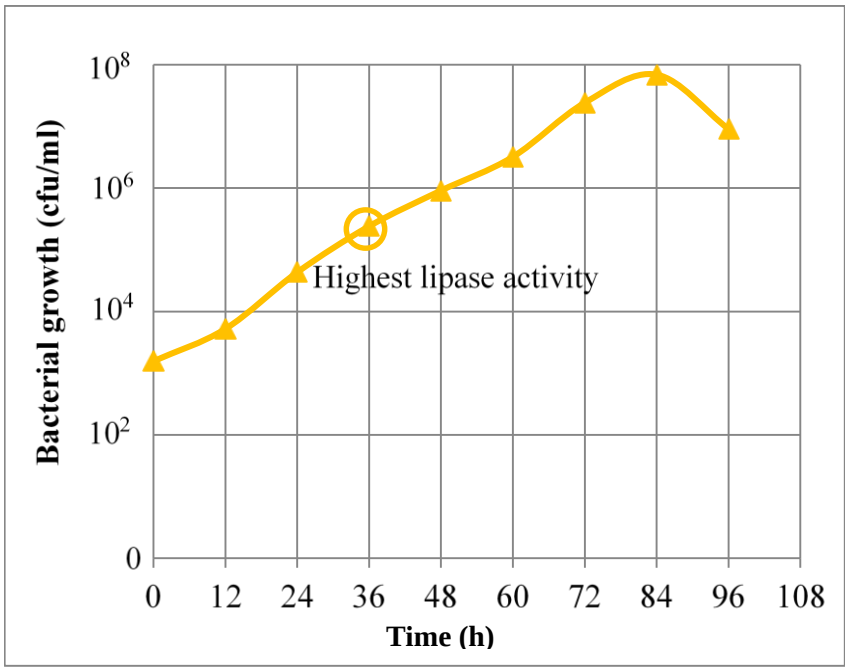

Fig. 10: Growth pattern and lipase activity of *B.subtilis* 20B.

4. Discussion

Different bacterial strains were separated based on the morphological distinction of individual colonies on dilution plates. They were then tested on TA and TBA media for lipase production. *Bacillussubtilis* 20B, a molecularly identified lipase producer, was the most promising. This isolate was then chosen in order to optimize the synthesis of lipase.

Different NaCl concentrations were added to the production medium in order to optimize the NaCl concentration in lipase production. *B. subtilis* had the maximum activity (17.28±0.75 U/ml) in this instance at *B. pumilus*. 0.5% NaCl. Sangeetha et al. (2014) similarly achieved this result, obtaining a high lipase yield in *Bacilluspumilus* SG2 with 14.98 U/ml at 0.5% salt concentration.

The carbon source, which also serves as an inducer for lipase formation, makes up the majority of the media used to produce lipase (Lotti et al., 1998). At 1.5% starch, the isolate reached its maximal lipase (37.39±0.67 U/ml). When Joseph et al. (2012) employed starch to produce lipase in *Bacillussphaericus* MTCC 7526, they likewise obtained this result (23.67 U/ml). Sahasrabudhe et al. (2021) showed the similar result in

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In this study, *B. subtilis* attained maximum lipase (25.17±0.67 U/ml) at 1.5% yeast extract. Similar outcome (20 U/ml) was obtained with yeast extract for *Bacillus* sp., as reported by Bora & Bora (2012). Sharma et al. (2002) and Bhattacharya et al. (2016) demonstrated that the bacteria, particularly different thermophilic *Bacillus* spp. and *Pseudomonas* spp., were able to produce larger amounts of lipase when organic nitrogen sources such peptone and yeast extract were utilized. According to Noormohamadi et al. (2013), the production of lipase was increased when olive oil was combined with other nitrogen sources. It was discovered that organic nitrogen sources outperformed inorganic ones. This result was consistent with studies on *Pseudomonas* sp. lipase production (Gupta et al. 2004). It has also been shown that certain bacteria respond well to inorganic nitrogen sources like ammonium chloride.

The synthesis of lipase was significantly influenced by the substrate, particularly the lipid concentration of the fermentation medium. According to Novototskaya-Vlasova et al. (2013),lipase activity is increased when olive oil and carbon sources are combined. Olive oil and carbon source in the growing medium significantly increased the isolate's lipase activity in the current investigation. Several thermophilic *Bacillus* species have been shown to produce large amounts of lipase when olive oil is added to the growth medium as a carbon source (Eltaweel et al., 2005). According to Muralidhar et al. (2001), olive oil was a more effective carbon source for lipase synthesis than glucose. In their investigation, Habibollahi and Salehzadeh (2018) noted that the medium's composition had a significant impact on lipase production.

During the estimation of bacterial growth, the time of incubation showed sharp decrease in lipase activity after 36 h in *Bacillus subtilis*. Following these times, the enzyme tends to decline, indicating that it may have either been broken down or rendered inoperable as a result of a decrease in lipidic substrate or an increase in acidity following medium ingestion. Kathiravan et al. (2012) have similarly documented this tendency with *Pseudomonasaeruginosa*.

5. Conclusion

About 29 bacterial isolates were obtained from organic waste which were evaluated for their ability to produce lipase. Among them, strain S_3T-9 exhibited significant lipolytic activity which was molecularly identified as *B. subtilis* 20B. This study looked into improving the culture conditions to help the isolate produce more lipase. It was discovered that the factors of NaCl, carbon source, nitrogen source, substrate concentration and basal media had a substantial impact on lipase synthesis. The best culture parameters for the indigenous isolate 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.

6. Acknowledgements

The authors would like to express thanks to the department of Botany, University of Dhaka, for providing research facility to carry out the work. The authors are sincerely grateful to the Ministry of National Science and Technology (NST) for financial support.

7. Conflict of interest

The authors declared there is no conflict of interest.

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