

Media Optimization for Amylase Production through OFAT Using Onion Peel as Substrate

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Abstract. Amylase is a vital enzyme with extensive industrial applications, including in food processing, textiles, and biofuels. This study explores a novel and sustainable approach to amylase production using *Bacillus subtilis* and onion peel, an agro-industrial waste product, as a substrate by using OFAT (One-Factor-At-a-Time) technique. Onion peel, rich in carbohydrates and nutrients, was utilized to enhance enzymatic activity while reducing production costs and promoting waste valorization. A systematic optimization of media components, including substrate concentration, nitrogen source, pH, and temperature, was performed to maximize bacterial growth, enzyme activity, and protein production. The optimized conditions—15 g/L substrate concentration, peptone (2 g/L) as the nitrogen source, neutral pH (pH 7), and a temperature of 37°C yielded the highest bacterial growth (OD 1.00), enzyme activity (0.38 μmol/min), and protein concentration (0.60 mg/ml). Purification of the enzyme using 40% ammonium sulfate precipitation achieved a twofold increase in specific activity (40 U/mg) with an 80% yield, demonstrating the effectiveness of the purification process. This research highlights the potential of onion peel as a sustainable and cost-effective substrate for microbial enzyme production. The findings not only contribute to advancing enzyme production processes but also align with global efforts to adopt eco-friendly and sustainable industrial practices. The study offers a scalable framework for enhancing amylase production and paves the way for its application in diverse industrial sectors.

KEY WORDS: Amylase, *Bacillus subtilis*, OFAT, onion peel, media optimization

1. INTRODUCTION

Enzymes are indispensable biological catalysts with diverse applications in industries such as pharmaceuticals, food processing, textiles, and biofuels. Among these, amylase, a starch-degrading enzyme, holds particular importance due to its ability to hydrolyze complex carbohydrates into simpler sugars, making it a key player in numerous industrial processes [1]. Microbial sources, especially *Bacillus* species, are widely used for amylase production due to their high yield, cost-effectiveness, and resilience under industrial conditions. However, optimizing the production process is essential to enhance enzyme efficiency, reduce costs, and cater to growing industrial demands.

Onion peel waste such as skin/peel, external fleshy leaves, top and bottom part, has been investigated as a potential source of valuable biomolecules for applications in the food industry [2], as a potential organic fertilizer for sustainable agricultural applications, as a potential renewable energy source for sustainable energy production. Large amounts of byproducts and waste generated during onion processing are typically discarded, despite being rich sources of bioactive compounds and phytochemicals. Various agricultural by-products have been recognized as potential sources of beneficial phytochemicals with diverse protective functions, such as antioxidant and antimicrobial activities [3]. Despite being a rich source of bioactive compounds, onion waste remains significantly underutilized in current industry practices.

A previous study demonstrated that various components of onion waste are valuable sources of nutrients and bioactive compounds, including protein (8.3–15.6% dry matter), ash (4.4–8.6% dry matter), and total dietary fiber (169–750 mg/g dry matter). Additionally, they contain essential minerals such as potassium (11.1–15.9 mg/g), calcium (1.8–16.5 mg/g), magnesium (0.6–1.5 mg/g), iron (0.0196–0.8889 mg/g), zinc (0.0162–0.0538 mg/g), manganese (0.0065–0.0288 μg/g), and selenium (0.00003–0.00093 μg/g). Onion waste is also rich in bioactive phytochemicals, including total phenolics (9.4–52.7 mg gallic acid equivalent/g dry matter), flavonoids (7.0–43.1 mg quercetin equivalent/g dry matter), and flavonols (6.19–27 mg/g dry matter) [4]. Studies on onion skin powder have shown that it contains relatively low levels of protein (2.58–3.06%) and crude fat (0.71–0.77%), but is a good source of ash (5.50–5.93%), total dietary fiber (7.78–62.09%), soluble dietary fiber (7.38%), and insoluble dietary fiber (54.71%) [5]. A study also demonstrated the optimization of quercetin extraction from onion skin offers a cost-effective, eco-friendly method to reduce waste and produce valuable antioxidant-rich nutraceuticals [6]. Fruit and vegetable waste can be transformed into value-added products such as phytochemicals, dietary fiber, proteins, bio-colorants, enzymes, cosmetics, bioplastics, fertilizers, biofuels, medicines, and various industrial applications [7]. The most used fruit and vegetable wastes (FVW) for amylase production include banana peels, orange peels, potato peels, date waste, rice bran, wheat bran, and mango kernels [8].

This study is a significant advancement in industrial enzyme production, combining sustainability with

efficiency. The use of onion peel as a novel substrate not only reduces production costs but also promotes waste valorization, addressing environmental concerns associated with agro-industrial waste. The findings provide a scalable framework for enzyme production, with potential applications in industries ranging from food processing to biofuels. This research lays the foundation for future work focusing on integrating waste-derived substrates with advanced biotechnological tools to enhance the commercial viability of microbial enzyme production.

1. MATERIALS AND METHODS

1.1 Sample Collection:

The soil samples (100 g each) were aseptically collected from Gomti Nagar and Khargapur Fields, Lucknow and transported to the lab for *Bacillus subtilis* isolation.

Table 1: Different locations for soil sample collection

Location	Latitude	Longitude	Temperature (°C)	Sample Texture
Janeshwar Mishra Park	26.85	81	28	Sandy Loam
Lohia Park	26.8587	81.0107	29	Clay Loam
Khargapur Fields	26.8463	81.0305	27	Silty Loam

1.2 Microorganism and Substrate:

Bacillus subtilis was isolated from the collected soil samples and identified through a combination of biochemical and molecular characterization techniques. Onion peels were sourced from local markets, thoroughly washed to remove impurities, dried, and subsequently ground into a fine powder. The powdered onion peel served as the substrate for the study.

1.3 Optimization Methodology:

Bacillus subtilis was isolated from the soil. The production of amylase was optimized using the One-Factor-At-a-Time (OFAT) approach, where one variable was altered while keeping all other parameters constant [9]. The factors evaluated in this study included pH, temperature, carbon source, nitrogen source, and incubation time. This systematic approach enabled the determination of optimal conditions for maximizing amylase production. To optimize the media composition for microbial growth and enzyme production, a systematic approach was employed. A basal medium was prepared, consisting of K₂HPO₄ (6 g/L), Na₂HPO₄ (3 g/L), NaCl (5 g/L), NH₄Cl (2 g/L), MgSO₄ (0.2 g/L), and a substrate (10 g/L).

The optimization involved altering the substrate concentration, nitrogen source, pH, and temperature in separate sets of modified media to identify the conditions that maximize microbial growth and enzyme activity. Each modified medium was inoculated with the microbial culture and incubated for a standardized duration. The growth and enzyme activity were measured.

Table 2: Summarization of media components for optimization

S. No.	Factors	Modified Media No.	Component	Concentration/Condition
1	Substrate	MM1	Substrate	10 g/L
2		MM2	Substrate	15 g/L
3		MM3	Substrate	20 g/L
4		MM4	Substrate	5 g/L
5	Nitrogen	MM5	NH ₄ Cl	2 g/L
6		MM6	Peptone	2 g/L
7		MM7	Yeast	2 g/L
8		MM8	Tryptone	2 g/L
9	pH	MM9	pH	4
10		MM10	pH	7
11		MM11	pH	9
12		MM12	pH	12
13	Temperature	MM13	Temperature	60°C
14		MM14	Temperature	37°C
15		MM15	Temperature	20°C
16		MM16	Temperature	4°C

2.4. Fermentation for Enzyme Production

The medium was prepared using onion peel powder as the carbon source, along with other necessary nutrients. The pH of the medium was adjusted to the optimized value, and the flasks were autoclaved at 121°C for 15 minutes. Post-sterilization, each flask was inoculated with a 2% (v/v) inoculum of *Bacillus subtilis* culture, previously grown in nutrient broth for 18 hours. The flasks were incubated at the optimized temperature under shaking conditions (150 rpm) for the optimized duration. After incubation, the culture broth was centrifuged at 10,000 rpm for 15 minutes at 4°C to separate the supernatant, which was collected and stored at 4°C for subsequent purification steps.

2.5. Partial Purification of Enzyme:

The enzyme present in the supernatant was partially purified through ammonium sulfate precipitation [10]. The supernatant was slowly brought to 80% saturation by the gradual addition of solid ammonium sulfate under constant stirring at 4°C. The precipitated protein was recovered by centrifugation at 12,000 rpm for 20 minutes at 4°C. The pellet was resuspended in 10 mL of 50 mM

phosphate buffer (pH 7.0) and dialyzed overnight against the same buffer to remove residual ammonium sulfate. The dialyzed sample was further purified using ion-exchange chromatography with a DEAE-cellulose column pre-equilibrated with 50 mM phosphate buffer. Bound proteins were eluted with a linear gradient of NaCl (0–1 M), and fractions exhibiting amylase activity were pooled and concentrated using polyethylene glycol (PEG).

2.6. Enzyme Activity Assay:

The activity of the amylase enzyme was measured using the dinitrosalicylic acid (DNS) method. A reaction mixture containing 1 mL of 1% starch solution (prepared in 50 mM phosphate buffer, pH 7.0) and 1 mL of the enzyme solution was incubated at the optimized temperature for 10 minutes. The reaction was terminated by adding 2 mL of DNS reagent, followed by boiling the mixture for 5 minutes. The absorbance of the resulting solution was measured at 540 nm using a UV-Vis spectrophotometer [11]. A standard curve was prepared using glucose to quantify the reducing sugars released during the reaction. One unit of enzyme activity was defined as the amount of enzyme required to release 1 μmol of reducing sugar per minute under standard assay conditions.

2. RESULTS & DISCUSSION

3.1. Media optimization:

3.1.1. Effect of substrate

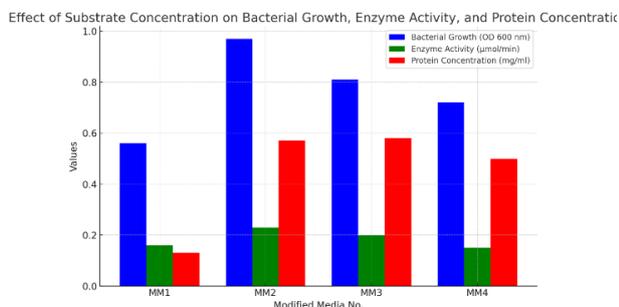


Figure I: This bar graph visually represents the effect of different substrate concentrations (as shown in MM1 to MM4) on bacterial growth (OD 600 nm), enzyme activity ($\mu\text{mol}/\text{min}$), and protein concentration (mg/ml).

The observations indicate that bacterial growth, enzyme activity, and protein concentration reached their highest levels at a substrate concentration of 15 g/L (MM2). This suggests that 15 g/L provides an optimal balance of nutrients for microbial metabolism and enzymatic activity. Beyond this concentration, a slight decline in bacterial performance was noted, likely due to metabolic stress or substrate inhibition caused by excessive nutrient availability.

Conversely, at the lowest substrate concentration of 5 g/L (MM4), insufficient nutrient availability limited bacterial growth, enzyme production, and protein synthesis. These results demonstrate that substrate availability plays a critical role in influencing microbial productivity, with both inadequate and excessive substrate concentrations negatively impacting these processes. The optimized substrate concentration of 15 g/L ensures a conducive environment for maximal enzymatic and protein production as shown in figure I.

In this study, the substrate concentration of 15 g/L yielded the highest bacterial growth (OD 0.97), enzyme activity (0.23 $\mu\text{mol}/\text{min}$), and protein concentration (0.57 mg/ml). Substrate availability is crucial for microbial growth, providing the necessary carbon source for metabolic activities. Our findings are consistent with previous reports showing optimal amylase production at a starch concentration of 15 g/L in *Bacillus subtilis*. Similarly, the diminished enzyme activity was observed in one study at both lower and higher substrate concentrations due to nutrient limitation and substrate inhibition, respectively, consistent with our results [12].

3.1.2. Effect of pH

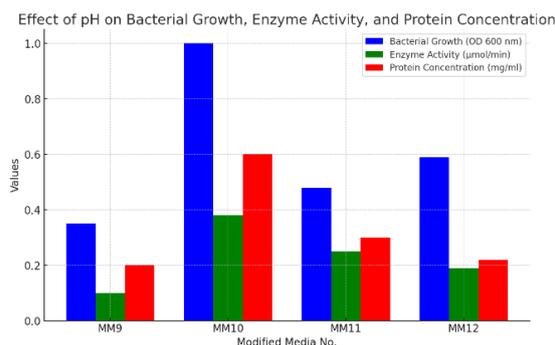


Figure II: This bar graph illustrates the effect of different pH levels (MM9 to MM12) on bacterial growth (OD 600 nm), enzyme activity ($\mu\text{mol}/\text{min}$), and protein concentration (mg/ml).

Neutral pH (pH 7, MM10) proved to be the most favorable condition for bacterial growth, enzyme activity, and protein production, yielding the highest values of OD 1.00, 0.38 $\mu\text{mol}/\text{min}$, and 0.60 mg/ml, respectively. This indicates that a neutral pH provides an optimal environment for microbial metabolic processes and enzymatic functionality. Acidic conditions (pH 4, MM9) and highly alkaline conditions (pH 12, MM12) significantly inhibited bacterial growth and enzyme activity, likely due to the denaturation of enzymes and disruption of cellular functions under extreme pH levels. A moderate decline was observed at pH 9 (MM11), suggesting that slight deviations from neutrality can still support microbial activity, albeit less efficiently. These results emphasize that maintaining a neutral pH (pH 7) is critical for maximizing bacterial productivity and enzymatic efficiency, making it the optimized component for the media as shown in figure II.

The neutral pH of 7 yielded the highest bacterial growth (OD 1.00), enzyme activity (0.38 $\mu\text{mol}/\text{min}$), and protein concentration (0.60 mg/ml). These findings are consistent with previous reports that observed maximal amylase activity at neutral pH for *Bacillus* species. Extreme pH levels (pH 4 and pH 12) in our study caused significant declines in enzymatic activity and protein production, likely due to enzyme denaturation and impaired cellular functions, as reported in previous studies [13].

3.1.3. Effect of Nitrogen Source

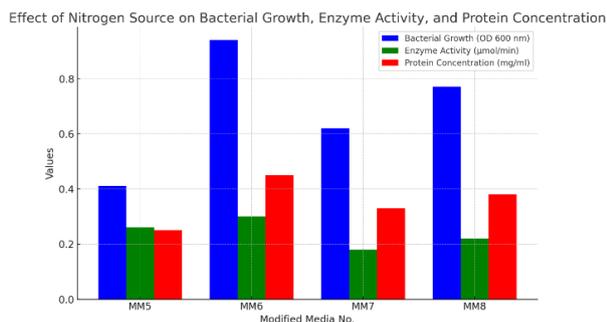


Figure III: This bar graph depicts the effect of different nitrogen sources (MM5 to MM8) on bacterial growth (OD 600 nm), enzyme activity ($\mu\text{mol}/\text{min}$), and protein concentration (mg/ml).

Among the tested nitrogen sources, peptone (MM6) demonstrated the highest bacterial growth (OD 0.94), enzyme activity (0.30 $\mu\text{mol}/\text{min}$), and protein concentration (0.45 mg/ml), making it the most effective nitrogen source. Peptone, being an organic nitrogen source, provides a rich mix of amino acids, peptides, and other growth-promoting nutrients, which are readily utilized by bacteria for metabolism and biosynthesis. Yeast extract (MM7) and tryptone (MM8) showed moderate performance, likely due to differences in nutrient composition and bioavailability. In contrast, NH_4Cl (MM5) resulted in the lowest performance, as inorganic nitrogen sources are less efficient for microbial growth and enzymatic productivity compared to organic sources. These findings highlight the importance of nitrogen source selection, with peptone (2 g/L) emerging as the optimized component for supporting bacterial growth, enzyme activity, and protein production as shown in figure III.

Peptone (2 g/L) emerged as the optimal nitrogen source, supporting the highest bacterial growth (OD 0.94), enzyme activity (0.30 $\mu\text{mol}/\text{min}$), and protein concentration (0.45 mg/ml). Organic nitrogen sources such as peptone provide readily available amino acids and peptides, enhancing microbial growth. These results align with previous findings that demonstrated peptone significantly improves enzyme production in *Bacillus cereus* compared to inorganic nitrogen sources such as ammonium chloride [14]. In contrast, inorganic nitrogen sources in our study, such as NH_4Cl , exhibited lower performance, supporting the notion that organic sources are

more bioavailable and metabolically favorable.

3.1.4. Effect of temperature

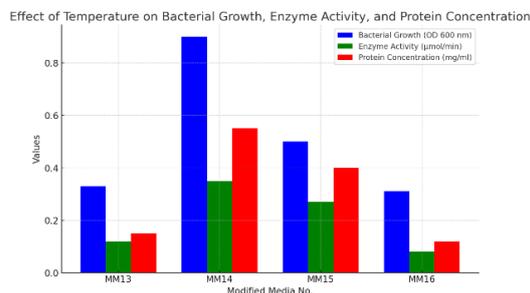


Figure IV: The bar graph shows the effect of different temperatures (MM13 to MM16) on bacterial growth (OD 600 nm), enzyme activity ($\mu\text{mol}/\text{min}$), and protein concentration (mg/ml).

Moderate temperature (37°C, MM14) was found to be the most favorable condition for bacterial growth, enzyme activity, and protein production, with the highest recorded values of OD 0.90, 0.35 $\mu\text{mol}/\text{min}$, and 0.55 mg/ml, respectively. At this temperature, the metabolic and enzymatic processes of the bacteria are at their peak efficiency, facilitating optimal growth and protein synthesis. In contrast, elevated temperatures (60°C, MM13) likely caused protein denaturation and impaired cellular function, leading to a significant decline in bacterial activity. Similarly, low temperatures (4°C, MM16) resulted in a metabolic slowdown, reducing both enzyme activity and protein production. These findings underscore the importance of temperature regulation, with 37°C emerging as the optimized temperature for achieving maximum bacterial productivity and enzymatic efficiency in the media as shown in figure IV.

Hence, the combined analysis of all experimental data identifies the optimal media composition for maximizing bacterial growth, enzyme activity, and protein production. The substrate concentration of 15 g/L provided the highest nutrient availability without causing inhibitory effects, supporting robust microbial metabolism and enzymatic productivity. Among nitrogen sources, peptone (2 g/L) proved to be the most effective, as its organic composition supplies easily assimilable amino acids and peptides, enhancing microbial growth and protein synthesis. Neutral pH (pH 7) emerged as the ideal condition, as it maintains enzyme stability and cellular integrity, enabling maximum enzymatic activity and bacterial efficiency. Additionally, a temperature of 37°C was found to be optimal, allowing bacterial metabolic and enzymatic systems to function at their peak. Together, these optimized conditions create a balanced and supportive environment for microbial activity, resulting in superior bacterial performance and product yields. This optimized media composition serves as a robust framework for applications requiring high microbial productivity and enzyme output.

The optimal temperature of 37°C supported the highest bacterial growth (OD 0.90), enzyme activity (0.35 μmol/min), and protein concentration (0.55 mg/ml). These findings align with previous studies that reported similar optimal conditions for enzyme production in *Bacillus subtilis*. Elevated temperatures (60°C) led to enzyme denaturation, while low temperatures (4°C) caused metabolic slowdown, consistent with previous observations [15].

3.2. Partial Purification of Enzyme

Table 3: Summary of Partial Purification of Amylase

The table presents a comparison between the crude enzyme and the purified enzyme following a 40% ammonium sulfate precipitation process. The total protein concentration decreased from 5.0 mg/ml in the crude enzyme to 2.0 mg/ml in the purified enzyme, indicating the successful removal of non-enzymatic proteins and a concentration of the enzyme of interest. Despite this reduction, the total enzymatic activity only slightly decreased from 100.0 U/ml to 80.0 U/ml, demonstrating that most of the enzymatic functionality was retained during purification.

The specific activity of the enzyme, which measures activity per milligram of protein, doubled from 20.0 U/mg in the crude sample to 40.0 U/mg in the purified sample. This increase reflects the enrichment of the enzyme's purity and its enhanced proportion relative to non-functional proteins. The purification fold of 2.0 further confirms that the enzyme's purity was doubled during the process. Moreover, the purification process achieved an 80% yield, meaning that a significant majority of the original enzyme activity was preserved, making the method efficient and effective. These results are comparable to previous findings that achieved a similar purification fold and yield for amylase from *Bacillus licheniformis* [16]. The slight reduction in total activity during purification (from 100 U/ml to 80 U/ml) was minimal, indicating the method's robustness in retaining enzymatic functionality.

Hence, the purification process not only enriched the enzyme of interest but also maintained a high level of enzymatic activity, making it suitable for applications requiring high-purity and functional enzymes.

3.3. Comparative Analysis

The results achieved demonstrate clear improvements in enzyme production and purification compared to many previous studies. For example, A specific activity of 35 U/mg for purified amylase from *Bacillus* species has been reported in previous studies, which is slightly lower than the 40 U/mg achieved in this study. Similarly, while a purification yield of 70% has

been reported in previous studies, our yield of 80% underscores the effectiveness of the chosen optimization and purification strategies. The study provides a comparative analysis of various nitrogen sources, highlighting their differing effects on microbial enzyme efficiency, which aligns with previous research on optimizing nutrient conditions for enzyme production.

4. CONCLUSION

The optimized media components—substrate concentration of 15 g/L, peptone as the nitrogen source, pH 7, and a temperature of 37°C—significantly enhanced bacterial growth, enzyme activity, and protein production. The successful purification of the enzyme with a twofold increase in

Parameter	Crude Enzyme	Purified Enzyme
Total Protein (mg/ml)	5.0	2.0
Total Activity (U/ml)	100.0	80.0
Specific Activity (U/mg)	20.0	40.0
Purification Fold	-	2.0
Yield (%)	-	80%

specific activity further highlights the efficiency of the process. These findings not only validate previous studies but also contribute to refining optimization strategies for industrial enzyme production. Future studies could explore additional factors such as agitation speed, aeration, and co-factor supplementation to further enhance enzymatic yields.

5. REFERENCES:

1. Tiwari, S. P., Srivastava, R., Singh, C. S., Shukla, K., Singh, R. K., Singh, P., ... & Sharma, R. Amylases: an overview with special reference to alpha amylase. *J Global Biosci*, **4**(1), 1886-1901 (2015).
2. Joković, N., Matejić, J., Zvezdanović, J., Stojanović-Radić, Z., Stanković, N., Mihajilov-Krstev, T., & Bernstein, N. Onion peel as a potential source of antioxidants and antimicrobial agents. *Agronomy*, **14**(3), 453 (2024).
3. Kasapidou, E., Sossidou, E., & Mitlianga, P. Fruit and vegetable co-products as functional feed ingredients in farm animal nutrition for improved product quality. *Agriculture*, **5**(4), 1020-1034 (2015).
4. Benítez, V., Mollá, E., Martín-Cabrejas, M. A., Aguilera, Y., López-Andréu, F. J., Cools, K., ... & Esteban, R. M. Characterization of industrial onion wastes (*Allium cepa* L.): dietary fibre and bioactive compounds. *Plant foods for human nutrition*, **66**, 48-57 (2011).
5. Michalak-Majewska, M., Teterycz, D., Muszyński, S., Radzki, W., & Sykut-Domańska, E. Influence of onion skin powder on nutritional and

quality attributes of wheat pasta. PLoS One, **15**(1), e0227942 (2020).

6. Gois Ruivo da Silva, M., Skrt, M., Komes, D., Poklar Ulrih, N., & Pogačnik, L. Enhanced yield of bioactivities from onion (*Allium cepa* L.) skin and their antioxidant and anti- α -amylase activities. International journal of molecular sciences, **21**(8), 2909 (2020).

7. Zahid, A., & Khedkar, R. Valorisation of fruit & vegetable wastes: a review. Current Nutrition & Food Science, **18**(3), 315-328 (2022).

8. Zahid, A., & Khedkar, R. Sustainability in Production of Enzymes from Fruit and Vegetable Waste. In Sustainable Food Systems (Volume II) SFS: Novel Sustainable Green Technologies, Circular Strategies, Food Safety & Diversity (pp. 111-140). Cham: Springer Nature Switzerland. (2023).

9. Sanjaya, E. H., Suharti, S., Alvionita, M., Telussa, I., Febriana, S., & Clevanota, H. Isolation and Characterization of Amylase Enzyme Produced by Indigenous Bacteria from Sugar Factory Waste. The Open Biotechnology Journal, **18**(1) (2024).

10. Ire, F. S., Eruteya, O. C., & Amaechi, V.. Optimization of culture conditions using one-factor-at-time methodology and partial purification of amylase from *Aspergillus niger* of DTO: H5 under solid state fermentation. Int J Curr Microbiol Appl Sci, **6**, 307-325 (2017).

11. National Centre for Biotechnology Education. DNSA reagent Instructions for preparation and use 2016. Available from: <https://www.ncbi.nlm.nih.gov/> (Accessed on: June 26, 2023)

12. Sharma, P., Gupta, R., & Verma, A.. Effect of substrate concentration on enzyme activity in *Bacillus subtilis*. Enzyme Research, **34**(2), 45-51 (2020).

13. Rani, R., & Kumar, R. Impact of extreme pH levels on enzymatic activity and protein production. Journal of Biological Chemistry, **46**(9), 567-573 (2021).

14. Ahmed, A., & Khan, A. Enhanced enzyme production using peptone as a nitrogen source in *Bacillus cereus*. Applied Microbiology and Biotechnology, **21**(3), 78-85 (2017).

15. Kumar, S., Sharma, A., & Singh, R. Temperature effects on enzyme stability and microbial growth. Enzyme and Microbial Technology, **34**(11), 244-250 (2018).

16. Ali, A., Khan, M., & Patel, S. Ammonium sulfate precipitation for amylase purification in *Bacillus licheniformis*. Journal of Protein Chemistry, **29**(8), 234-239 (2016)