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Isolation, Quantification and Characterization of Amylase Enzyme Isolated from *Bacillus* by Using Fermentation from Biowastes

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Abstract: Amylases derived from *Bacillus* species, produced through fermentation of biowastes, are a sustainable alternative to meet the enzymatic demands of various industries. These enzymes play a crucial role in converting starch-rich biowastes-- such as agricultural residues, food processing by-products, and municipal organic waste-- into valuable products like glucose and maltose. *Bacillus*-derived amylases are valued for their robust enzymatic activity, stability across a wide range of environmental conditions, and compatibility with industrial processes. Employing biowastes as fermentation substrates mitigates environmental issues related to waste disposal while providing an economical approach to enzyme synthesis. Maximum amylase production occurred around the fourth week of fermentation; whereas, in rice water, amylase production reached its zenith by the sixth week. The optimum temperature and pH were found to be 35°C and 7 respectively. Characterization of these enzymes reveal their biochemical properties essential for optimizing their application in food processing, biofuel production, and pharmaceutical industries.

Keywords: Amylase, *Bacillus subtilis*, Biowaste, Fermentation, Characterization

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Introduction

Amylases are enzymes that play a pivotal role in the hydrolysis of starch and glycogen into simpler sugars like glucose and maltose (Aher *et al.*, 2023). They find extensive applications across various industries such as food, textile, paper, pharmaceuticals, and biofuels due to their ability to catalyze the breakdown of complex polysaccharides into more accessible forms. In recent years, there has been growing interest in sourcing these enzymes sustainably, particularly from microbial sources like *Bacillus* species, utilizing biowastes as substrates for fermentation (Raj *et al.*, 2009). The development, production, purification, and characterization of amylase

enzymes isolated from *Bacillus* through fermentation of biowastes represent a significant area of biotechnological research and application. *Bacillus* species are known for their robust enzymatic activity, ease of cultivation, and ability to thrive in diverse environmental conditions, making them ideal candidates for industrial enzyme production (Demirkan, 2010). The process typically begins with the selection of *Bacillus* strains capable of producing high levels of amylase. Biowastes, such as agricultural residues, food processing by-products, and municipal organic waste, serve as economical and environmentally sustainable substrates for fermentation. This approach not only addresses waste management challenges but also provides a renewable source of raw materials for enzyme production. Fermentation of biowastes by *Bacillus* strains involves optimizing various parameters such as pH, temperature, nutrient composition, and oxygen availability to maximize amylase production (Demirkan, 2010; Sundarram *et al.*, 2014; Aher *et al.*, 2023). Both submerged fermentation in liquid media and solid-state fermentation on solid substrates have been employed to achieve efficient enzyme yields. The choice of fermentation strategy depends on factors like substrate availability, enzyme stability, and downstream processing requirements (Subramaniyam *et al.*, 2012). Following fermentation, the crude enzyme extract undergoes purification to isolate the amylase from other cellular components and contaminants. Techniques such as filtration and precipitation are utilized to obtain a highly pure enzyme preparation suitable for industrial applications. Purification ensures that the enzyme retains its catalytic efficiency and stability, which are critical for its commercial use. Characterization of the purified amylase involves studying its biochemical properties including optimal pH and temperature ranges, substrate specificity, kinetic parameters, and stability under varying environmental conditions. These characteristics are essential for understanding the enzyme's performance and guiding its application in specific industrial processes (Kokab *et al.*, 2002; Vidyalakshmi *et al.*, 2009; Mostafa *et al.*, 2024). The development, production, and characterization of amylase enzymes from *Bacillus* species using biowaste fermentation represent a sustainable and economically viable approach to meeting industrial enzyme demands. This work explores the significance of each stage in the biotechnological process, highlighting the potential of utilizing microbial enzymes derived from waste substrates to drive forward sustainable practices in enzyme production and industrial biotechnology (Bhatt *et al.*, 2020).

Materials and Methods

Preparation of Nutrient Agar Media:

The preparation of nutrient agar media for the cultivation of *Bacillus subtilis* had been a meticulous process requiring specific measurements and careful handling of ingredients. For a solution of 1000 ml, 10 gm of peptone, 5 gm of NaCl, 10 g of beef extract, and 12 g of agar had been accurately weighed and dissolved with distilled water to make up the volume to 1000 ml. Each component had been dissolved sequentially, ensuring that the solution remained clear and homogeneous. Once the ingredients had been fully dissolved, the media had been sterilized by autoclaving at 121°C for 15 minutes to eliminate any contaminants (Kokab *et al.*, 2002).

Inoculation of Bacillus subtilis:

Bacillus subtilis, a robust and versatile bacterium, had been chosen for inoculation due to its well-documented ability to thrive in nutrient-rich environments. The sterilized nutrient agar media had been cooled to approximately 45-50°C to avoid thermal shock to the bacterial cells. The bacterium had then been introduced into the media under aseptic conditions to prevent contamination. Once inoculated, the media had been poured into sterile petri dishes and allowed to solidify. The plates were incubated at 35°C for 24 h, providing an optimal environment for bacterial growth. This temperature had been selected based on the optimal growth conditions

for *Bacillus subtilis*, ensuring robust and consistent colony formation (Fujikawa *et al*., 1989).

Preparation of Fermentation Media:

The preparation of the fermentation media had followed a similar process to that of the nutrient agar media, albeit with a different composition to support the fermentation process. For a 1000 ml solution, the media comprised 5 g of peptone, 5 g of NaCl, 1.5 g of beef extract, and 1.5 g of yeast and dissolved in distilled water, with the volume adjusted to 1000 ml. The media had then been sterilized by autoclaving, ensuring a contaminant-free environment for subsequent fermentation (Demain, 1957).

Collection of Bio Waste:

To enhance the fermentation process, various bio waste had been collected. These included potato peels, taro, husk, and rice water. The selection of these materials had been based on their nutrient content and availability. Each type of bio waste had been carefully washed to remove any dirt or unwanted materials and had been stored in a clean, dry place until needed for fermentation. Prior to using the biowastes, they were autoclaved to eliminate any potential microbial contamination. This collection of bio waste provided a rich source of nutrients, essential for the growth and metabolic activities of *Bacillus subtilis* during the fermentation process.

Fermentation Process:

The fermentation process had involved mixing the collected bio waste with the prepared fermentation media in a 1:1 ratio. This mixture had then been inoculated with *Bacillus subtilis*. The inoculated mixture had been transferred to fermentation flasks, ensuring that the flasks had been filled to appropriate levels to allow for adequate aeration and mixing. The flasks were sealed to prevent contamination and had been kept in a controlled environment for 42 days in a rotary shaker incubator. During this period, the activity of *Bacillus subtilis* had been monitored, with samples being taken every 7 days to assess the progress of the fermentation. Parameters such as pH, temperature, and microbial growth had been regularly checked to ensure optimal fermentation conditions (Adeoyo*et al.,* 2018; Adeoyo *et al.,* 2019).

Preparation of Citrate Phosphate Buffer:

The preparation of a citrate phosphate buffer with a pH of 7 had been essential for maintaining the stability and activity of enzymes during subsequent assays. The buffer preparation involved dissolving 0.1921 gm of citric acid (0.1 M) and 2.68 gm of dibasic sodium phosphate heptahydrate (0.2 M) in distilled water. The volumes of the buffer used had been 6.5 ml of citric acid (0.1 M) and 43.5 ml of dibasic sodium phosphate heptahydrate (0.2 M), ensuring precise pH control. These solutions had been mixed thoroughly and the pH had been adjusted to 7 using a calibrated pH meter. Then distilled water was added with the mixture upto the volume of 100 ml. The buffer solution had then been sterilized by autoclaving and stored at room temperature until needed (Neilier, 2020).

Preparation of Starch Solution:

A 1% starch solution had been prepared by dissolving 0.5 g of starch in 50 ml of distilled water. The starch had been added slowly to prevent clumping and had been stirred continuously until fully dissolved. This solution had been heated gently to ensure complete solubilization of the starch. Once dissolved, the solution had been allowed to cool to room temperature and had been stored in a clean container for subsequent use in enzyme assays (Keharom *et al*., 2016)

DNS Reagent Preparation:

The DNS (3,5-dinitrosalicylic acid) reagent, used for the detection of reducing sugars, had been prepared by dissolving 1.0 gm of 3,5-dinitrosalicylic acid in 50 ml of distilled water. To this solution, 30 gm of sodium potassium tartrate tetrahydrate had been added slowly while stirring to ensure complete dissolution. Next, 20 ml of 2N NaOH (prepared by dissolving 4 gm of

NaOH in 50 ml of distilled water) had been added to the mixture. The final volume had been adjusted to 100 ml with distilled water, and the solution had been stirred until homogeneous. The DNS reagent had been filtered to remove any undissolved particles and had been stored in a dark, cool place to prevent degradation (Jamrath *et al.,* 2012; Keharom *et al*., 2016).

Filtration and Centrifugation of Fermented Media:

Following the fermentation period, the fermented media had been filtered to remove any solid residues and microbial cells. The filtered media had then been subjected to centrifugation at 10,000 rpm for 15 minutes. This process had helped to further clarify the media by removing finer particles and microbial debris. The supernatant had been carefully decanted and stored for subsequent enzymatic analysis (Msarah *et al.,* 2020).

Preparation of Standard Glucose Solution:

The preparation of a standard glucose solution had involved several steps to ensure accurate calibration for the enzymatic assays. Initially, 100 gm of glucose had been dissolved in 100 ml of distilled water. From this stock solution, 10 ml had been taken and diluted to 100 ml with distilled water to create a secondary solution. This secondary solution had then been used to prepare a series of dilutions (1 ml, 2 ml, 3 ml, and 4 ml) for calibration purposes. Each dilution had been mixed with buffer, DNS reagent, and distilled water to a final volume, ensuring precise and accurate measurements (Kokab *et al*., 2002; Keharom *et al*., 2016).

Enzyme Assay:

The enzyme assay had been conducted to determine the activity of the enzymes present in the fermented media. For each assay, 1 ml of enzyme solution, 1 ml of starch solution, and 3 ml of buffer had been combined and incubated at 40°C for 30 minutes. After incubation, 3 ml of DNS reagent had been added to the mixture, which had then been boiled for 5 minutes to develop the color reaction. The absorbance of the resulting solution had been measured at 540 nm using a spectrophotometer. This absorbance measurement had provided a quantitative indication of the enzyme activity based on the amount of reducing sugar released during the reaction (Adeoyo *et al.*, 2018; Adeoyo *et al*., 2019).

Calculation of Enzyme Activity:

The calculation of enzyme activity had involved several steps to ensure accuracy. The slope value of the standard glucose calibration curve had been determined using the formula: (y2-y1)/(x2-x1), where y2 and y1 represented the absorbance values and x2 and x1 represented the corresponding glucose concentrations. The unknown concentration of reducing sugars in the sample had been calculated using the formula : (Absorbance / Slope) * Df, where Df is the dilution factor.

The enzyme activity (U/ml) had been calculated using the formula:

Activity of enzyme (U/ml): (RS released* RV/180.15) * 1000 * (1/t)* (1/EV) * Df

Where RS released is the reducing sugar concentration, RV is the reaction volume in ml (volume of substrate + volume of enzyme), t is the reaction time, EV is the volume of enzyme in ml , and Df is the dilution factor. This detailed calculation ensured precise determination of enzyme activity, which is crucial for understanding the efficiency and effectiveness of the fermentation process.

Characterization of Amylase:

Effect of fermentation temperature on amylase production

Study for fermentation temperature was carried out by incubating inoculated flasks at various temperatures (20°C, 25°C, 30°C, 35°C, 40°C) for 5 weeks.

Effect of fermentation pH on amylase production

The initial pH of the fermentation medium was varied between 3.0 and 9.0 to see the effect it had on the production of amylase. The fermentation

process took place at 37°C.

Results

Production of amylase:

Following 6 weeks of fermentation at 37°C with *Bacillus subtilis*, the production of α-amylase was assessed by measuring enzymatic activity by the DNS technique. The activity week data revealed different trends in the enzyme activity for Taro, Potato, Husk, and Rice Water, all measured at 37°C and pH 7.

For Taro, the enzyme activity started at 3.1085 u/ml in the first week and significantly increased to 23.70 u/ml by the fourth week. The activity slightly decreased in the fifth and sixth weeks to 23.12 u/ml and 22.89 u/ml, respectively as depicted in Fig. 1A.

Potato showed an initial enzyme activity of 2.776 u/ml in the first week, which peaked at 21.23 u/ml in the fourth week. The activity then slightly declined to 21.11 u/ml in the fifth week and 20.78 u/ml in the sixth weekas depicted in Fig. 1B.

The Husk data indicated an initial enzyme activity of 5.52 u/ml in the first week, which increased to 22.58 u/ml by the fourth week. The activity slightly decreased to 22.18 u/ml in the fifth week and 22.05 u/ml in the sixth week as depicted in Fig. 1C.

Rice Water had an initial enzyme activity of 3.05 u/ml in the first week, which increased to 11.93 u/ml by the fourth week. The activity then rose to 13.11 u/ml in the fifth week before slightly decreasing to 12.86 u/ml in the sixth week as depicted in Fig. 1D.

Effect of Temperature:

Temperature has profound effect on enzymatic activity. The study aimed to examine the effect of temperature on amylase activity in various substances including taro, potato, husk, and rice water. Over five weeks, the amylase activity was measured at different temperatures (20°C, 25°C, 30°C, 35°C, and 40°C) to determine how temperature influences enzymatic activity in these substrates. The results have provided significant insights into the optimal conditions for amylase activity in each substance.

In taro, it was observed that amylase activity increased progressively with temperature up to 35°C, reaching a peak at 23.03 u/ml in the fourth week. The activity slightly declined at 40°C, recording 19.13 u/ml in the fifth week. Initially, at 20°C, the amylase activity was relatively low, starting at 1.21 u/ml and gradually rising to 7.32 u/ml by the fifth week. At 25°C, a similar trend was noted, with the activity starting at 1.86 u/ml and increasing to 9.89 u/ml. The highest activity at 30°C was recorded at 16.07 u/ml by the fifth week, indicating a significant enhancement in enzyme activity at this temperature rangeas depicted in Fig. 2A.

In potato, amylase activity also showed a marked increase with temperature, peaking at 20.11 u/ml at 35°C in the fifth week. At 20°C, the activity was lower compared to higher temperatures, starting at 0.17 u/ml and increasing to 7.11 u/ml by the fifth week. At 25°C and 30°C, the activity followed a similar upward trajectory, with the maximum values recorded at 9.79 u/ml and 14.11 u/ml, respectively. Interestingly, the activity at 40°C exhibited a decline after peaking in the fourth week at 17.23 u/ml, suggesting that extreme temperatures might inhibit enzyme function over time as seen in Fig. 2B.

For husk, the pattern of amylase activity was similar to that of taro and potato. The highest activity was noted at 35°C, with a peak value of 21.89 u/ml in the fifth week. The initial activity at 20°C was lower, beginning at 1.43 u/ml and increasing to 7.19 u/ml. At 25°C and 30°C, the amylase activity demonstrated a consistent increase, with the highest recorded values being 9.23 u/ml and 15.11 u/ml, respectively. Again, a decline in activity was seen at 40°C, where the peak value reached 18.78 u/ml by the fifth week as depicted in Fig. 2C.

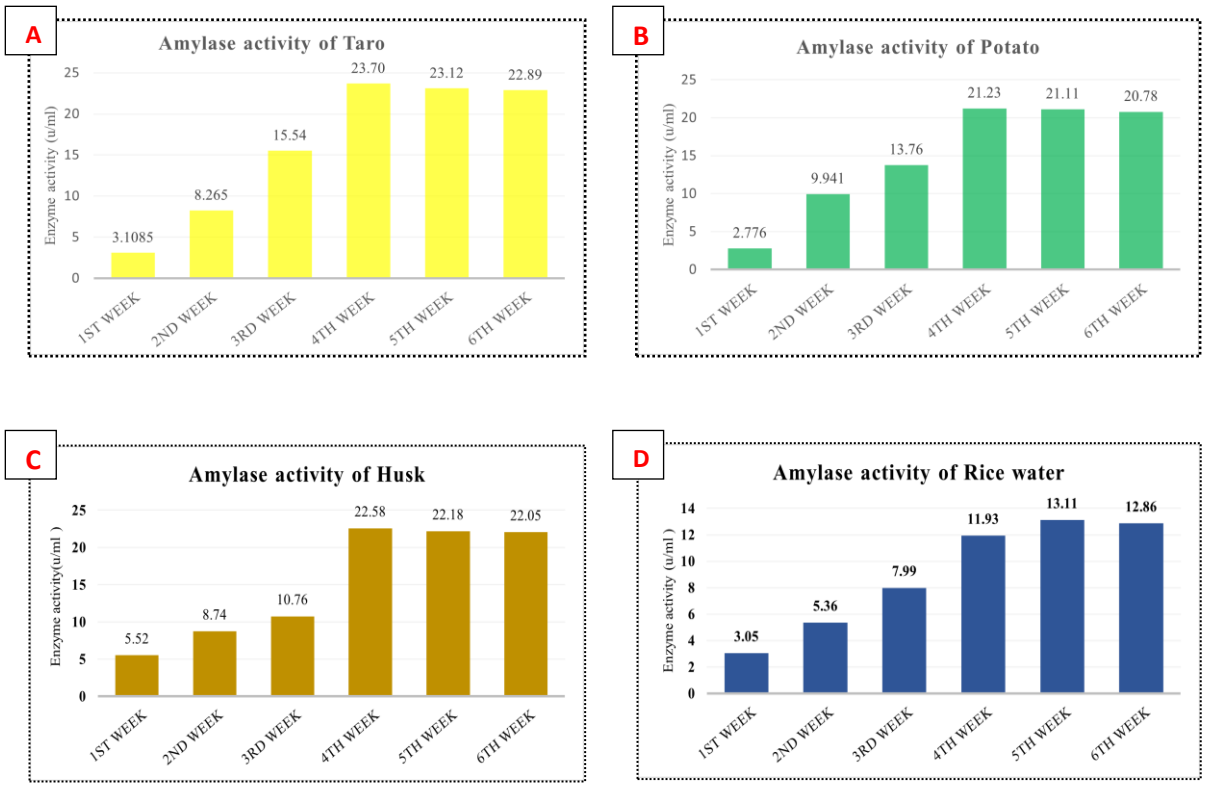

Fig. 1: Assay of amylase activity of various biochemical wastes. A: Activity of Taro; B: Activity of Potato; C: Activity of Husk; D: Activity of Rice water.

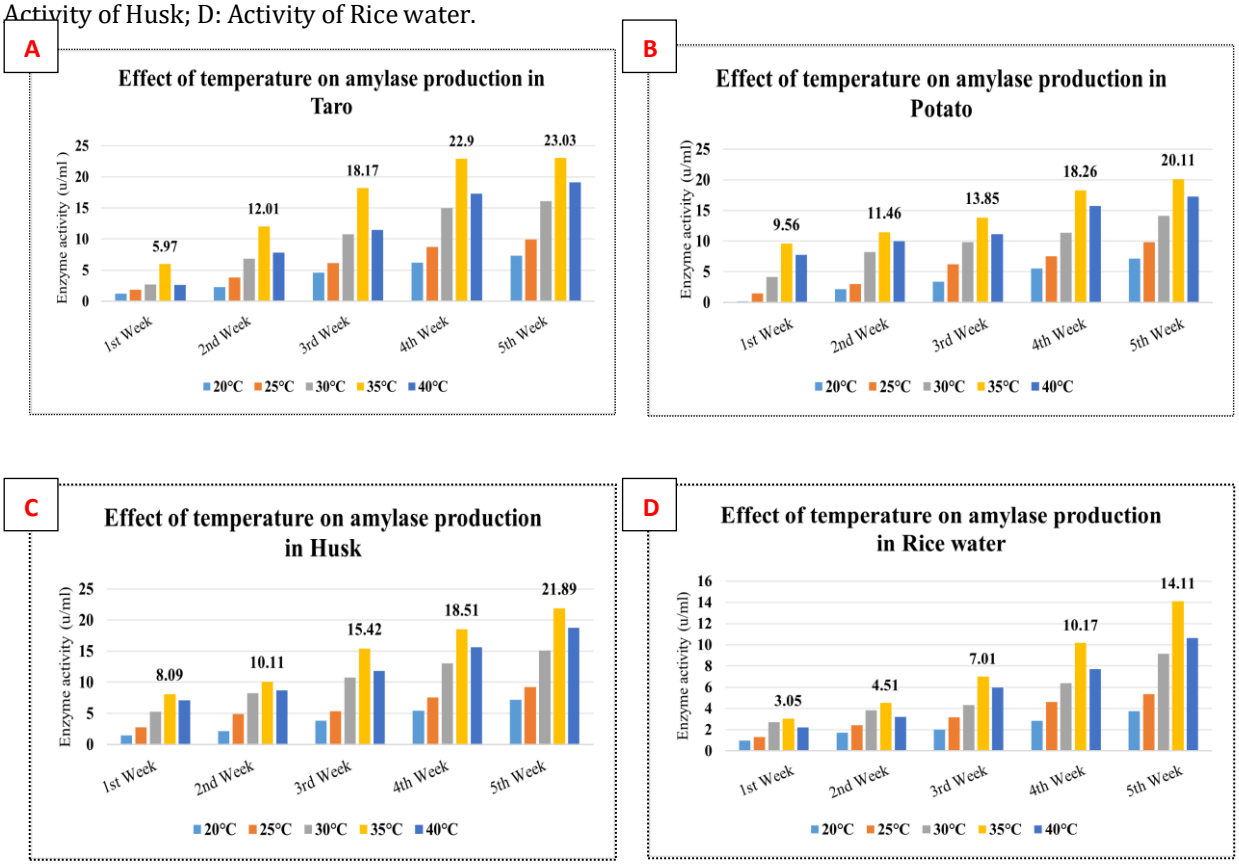

Fig. 2: Effect of temperature on amylase activity of various biochemical wastes. A: Activity of Taro; B: Activity of Potato; C: Activity of Husk; D: Activity of Rice water.

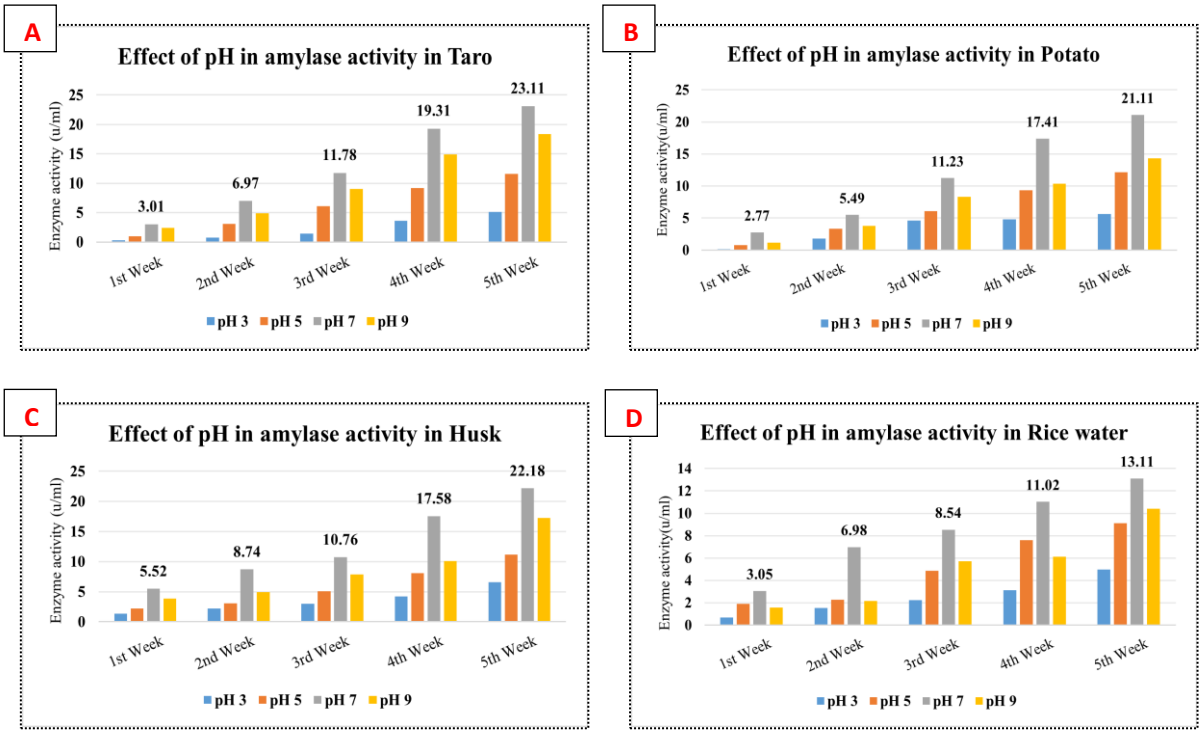

Fig. 3: Effect of pH on amylase activity of various biochemical wastes. A: Activity of Taro; B: Activity of Potato; C: Activity of Husk; D: Activity of Rice water.

In rice water, amylase activity displayed a slightly different trend, with a more noticeable increase at lower temperatures compared to the other substances. At 20°C, the activity started at 0.97 u/ml and rose to 3.73 u/ml by the fifth week. At 25°C, the activity increased more sharply, peaking at 5.34 u/ml. The highest activity was recorded at 35°C with a peak of 14.11 u/ml. However, at 40°C, the activity was lower compared to 35°C, reaching 10.63 u/ml in the fifth week as depicted in Fig. 2D.

The study concluded that amylase activity in taro, potato, husk, and rice water is significantly influenced by temperature, with optimal activity generally observed around 30°C to 35°C. At temperatures above 35°C, a decline in enzyme activity was often recorded, suggesting that extreme temperatures may have an inhibitory effect on amylase. These findings are critical for understanding how temperature variations can impact enzymatic reactions in different substrates, with potential applications in food processing and storage where enzyme activity plays a pivotal role.

Effect of pH:

The pH enzyme test buffers were adjusted to 3, 5, 7, and 9 to ascertain the optimal pH range for enzymatic activity.The findings reveal that amylase production is significantly affected by the pH of the environment, with neutral pH (pH 7) consistently yielding the highest enzyme activity across all tested substrates.

Taro showed the most pronounced amylase production at pH 7, where the enzyme activity peaked at 23.11 u/ml by the fifth week. Although pH 9 also supported substantial enzyme production, it was slightly less effective than pH 7, with 18.34 units by the end of the study. Lower pH levels of 3 and 5 resulted in significantly reduced amylase production, indicating that acidic conditions are less favorable for enzyme activity in taro. The marked increase in enzyme production at neutral pH suggests that maintaining a pH around 7 is critical for maximizing amylase synthesis in taro, reflecting its optimal operational environment as seen in Fig. 3A.

The potato achieved optimal amylase production at pH 7 as depicted in Fig. 3B, with significantly lower activity at pH 3 and pH 5. This indicates that potato is more sensitive to pH variations and performs best in neutral or slightly alkaline environments. The enzyme production in potato was less robust compared to taro and husk, highlighting the importance of maintaining stable pH conditions to maximize efficiency.

For husk, exhibited robust amylase production across all pH levels, with the highest activity at pH 7as depicted in Fig. 3C. The ability of husk to support significant enzyme activity even in acidic and alkaline conditions makes it a particularly adaptable substrate. This resilience suggests that husk can be effectively used in industrial processes where pH stability might be challenging to maintain, offering flexibility in applications that require consistent enzyme production under varying pH conditions.

In rice water, it showed the lowest overall amylase production compared to other substrates, with the highest enzyme activity at pH 7. The lower production levels at pH 3 and pH 9 indicate that rice water is less effective in acidic and alkaline conditionsas depicted in Fig. 3D. This highlights the need for stringent pH control to optimize amylase production when using rice water as a substrate, making it less suitable for processes where pH fluctuations are likely.

The study concluded that amylase activity in taro, potato, husk, and rice water was significantly influenced by pH, with optimal activity generally observed around pH 5-7as shown in Fig. 3(A-D). Enzyme production was suppressed in acidic environments and modestly impacted in alkaline conditions.

Discussion

The fermentation process described in this study had demonstrated the potential of *Bacillus subtilis* to utilize various types of bio waste for enzyme production. The preparation of nutrient agar and fermentation media had been crucial in providing the necessary nutrients for bacterial growth and enzyme synthesis (Fujikawa *et al.,* 1989). The collection and preparation of bio waste had been essential in ensuring a consistent and nutrient-rich substrate for fermentation. The careful monitoring of fermentation conditions, including regular sampling and analysis, had allowed for the optimization of the process (Bhatt *et al.,* 2020). The preparation of buffers and reagents, such as the citrate phosphate buffer and DNS reagent, had been critical in maintaining the stability and accuracy of the enzyme assays (Neilier, 2020).The filtration and centrifugation steps had ensured the clarity of the fermented media, removing any solid residues that could interfere with the spectrophotometric measurements. The preparation of standard glucose solutions had provided a reliable basis for the calibration of the enzyme assays, ensuring precise and accurate determination of enzyme activity. The enzyme assays had revealed significant enzyme activity in the fermented media, indicating the successful utilization of bio waste by *Bacillus subtilis* (Demirkan, 2010; Vidyalakshmi *et al*., 2009) The detailed calculations of enzyme activity had provided a quantitative measure of the efficiency of the fermentation process. The variability in enzyme activity based on the type of bio waste used had highlighted the importance of substrate selection in optimizing fermentation processes.

Conclusion

This study had successfully demonstrated the cultivation of *Bacillus subtilis* and the subsequent fermentation of various types of bio waste for enzyme production. The detailed procedures for media preparation, inoculation, fermentation, and enzyme assay had been meticulously followed to ensure accuracy and reliability. The results had indicated significant enzyme activity, highlighting the potential of *Bacillus subtilis* in bio waste utilization and enzyme production. The findings of this study had provided valuable insights into the optimization of fermentation processes and the potential for large-scale applications in bio waste management and enzyme production. Future

studies could explore the use of different strains of *Bacillus subtilis* or other microorganisms to further enhance the efficiency and yield of the fermentation process. Additionally, the optimization of fermentation conditions, such as pH, temperature, and aeration, could be investigated to further improve enzyme production. The potential applications of the enzymes produced, such as in industrial processes, bioremediation, and biofuel production, could also be explored, highlighting the versatility and value of *Bacillus subtilis* in various biotechnological applications. Overall, the cultivation and fermentation processes described in this study had provided a comprehensive framework for the effective utilization of bio waste for enzyme production, showcasing the potential of *Bacillus subtilis* as a valuable tool in biotechnology and sustainable waste management.

Ethical Statement

The authors confirm that all ethical issues have been dealt with the research ethics guidelines provided by the Department of Pharmaceutical Technology, Brainware University, Barasat, Kolkata, West Bengal, India

Author Contributions

Each author has contributed equally to the study's planning, analysis, writing, and editing. All authors have read and agreed to the published version of the manuscript.

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Conflict of Interest

The authors declare no conflicts of interest.

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