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Amylase Production from Eco-Enzyme Filtrate of *Citrus maxima:* It's Purification, Characterization, and Activity

[1]Rima Paul, [2]Nayan Talukdar, [3]Jyotchna Gogoi*

[1]PhD Scholar, [2]Associate Professor, [3]Associate Professor

[1]Affiliation Address: **Faculty of Science,** Assam down town University, Sankar Madhab Path, Gandhi Nagar, Panikhaiti, Guwahati, Assam, PIN – 781026, India
[2]Affiliation Address: **Faculty of Science,** Assam down town University, Sankar Madhab Path, Gandhi Nagar, Panikhaiti, Guwahati, Assam, PIN – 781026, India
[3]Affiliation Address: **Faculty of Allied and Healthcare Sciences,** Assam down town University, Sankar Madhab Path, Gandhi Nagar, Panikhaiti, Guwahati, Assam, PIN – 781026, India
[1]Email: rimapaul4568@gmail.com, [2]Email: nayan.talukdar@adtu.in,
[3]Email: gogoijyotchna@gmail.com
***Corresponding Mail:** gogoijyotchna@gmail.com*

Abstract

Background: Making enzymes from agro-waste has been promoted as a sustainable and cost-effective solution to the growing demand in the industry. One promising use for the plentiful agro-residue known as *Citrus maxima* peel is in the manufacturing of eco-enzymes and amylases.

Material and Method: In an anaerobic fermentation process, an eco-enzyme was produced using a 3:1:10 ratio of *Citrus maxima* peel, jaggery, and water. The enzyme amylase was isolated by employing a combination of solvent precipitation, ammonium sulphate precipitation, DEAE-Sephadex ion-exchange chromatography, and Sephadex G-100 gel filtration chromatography. Methods such as SDS-PAGE, zymogram, and western blot analysis were employed to characterise the enzymes. It was determined how enzyme activity changed in response to changes in substrate concentration, temperature, thermal stability, and pH.

Result: Amylase that had been purified with a recovery of 10.14 %, fold purification 8.34, and a specific activity of 16.39 U/mg was shown. An approximation of 50 kDa was validated and western blot analysis confirms the presence of α-amylase. Phosphorus 7.4, temperature 40°C, and a starch concentration of 1% were the conditions that produced the highest levels of enzyme activity. Once heated to 40°C, the enzyme showed modest thermal stability.

Conclusion: The study found that *Citrus maxima* peel waste may be used to make amylase in a sustainable and environmentally beneficial way. Within the context of the circular bio-economy, the results show that it could be a cheap substrate for enzyme production and the valorization of agro-waste.

Keywords: Amylase; *Citrus maxima*; Eco-enzyme; Enzyme purification; Bioprocess optimization

1. Introduction

α-Amylases (EC 3.2.1.1) are endo-acting enzymes that cleave internal α-1,4-D-glycosidic bonds within amylose chains at random positions and produce small dextrins and oligosaccharides having an α-anomer configuration at the C1-OH group. They are used in widely varied industrial fields, including textiles, detergents and food processing (Far et al. 2020; Ariaeenejad et al. 2021). The wide application of α-amylase is attributable to its being an efficient and environmentally friendly alternative to traditional chemical catalysts (Mehta & Satyanarayana, 2016). Despite their widespread application, the industrial use of α-amylases is often limited by their functional stability and catalytic efficiency under harsh industrial conditions (Jaiswal & Jaiswal 2024). Therefore, extensive research activity focuses on searching for novel species of α-amylases with thermophilic and alkaliphilic properties as well as optimising sustainable production methods and stabilisation techniques (Abd-Elhalim et al. 2023; This, therefore, typically includes mining the microbial world for new and improved enzymes and improving fermentation processes using mainly low-cost substrates (Şahutoğlu & Kızıloluk 2025).

Eco-enzyme (Bio-enzyme) is a complex organic solution produced from waste peel of vegetables and fruit through fermentation, containing fruit or vegetable waste in brown sugar and water (3:1:10) to produce this enzymatic solution (Varshini and Gayathri, 2023). The fermentation process is usually three months and results in a multipurpose liquid that can be used in household cleaning as well as in agricultural upgrades (Wuni & Husaini, 2021). These systems imitate natural biodegradation; microorganisms produce hydrolytic enzymes, such as proteases, lipases and amylases, to decompose complex organic matter into simpler compounds. The manufacture of eco-enzymes, therefore, would be inexpensive and eco-friendly as an alternative to traditional fermentation systems, but would also deal with issues of organic waste management. (Mardhiyah et al., 2022) Microbial α-amylase, in particular, has several advantages over plant and animal ones due to better stability at different temperatures and pH values, which offers a wide range of possibilities for their application in different industrial processes (Sahu et al. 2024). These enzymes are produced under various conditions, such as pH, temperature, organic solvents and salt concentration, via submerged and solid-state fermentation methods (Ali et al. 2025). Such amylase enzymes have been experiencing rising demand, being involved in the hydrolysis of oligosaccharides, polysaccharides and starch to monosugars, as well as being a contributor to carbon-neutral technologies like the processing of cellulosic bioethanol (Hameed et al. 2022).

Citrus maxima peel was used for this investigation because it is a cheap and easily accessible substrate for the synthesis of enzymes and is a common agro-waste produced from citrus processing and household consumption (Diniso et al., 2024). The peel's high sugar, carbohydrate, and organic nutrient content promote microbial fermentation and the synthesis of amylase. Additionally, using *Citrus maxima* peel encourages sustainable waste valorisation within the context of the circular bio economy and lessens environmental contamination brought on by the disposal of citrus waste. (Dobariya et al., 2023)

1.1. Agro–waste utilisation and circular bio-economy

The enzyme production in the valorisation of *Citrus maxima* peel is a twofold benefit, as it reduces waste and provides industries with highly useful biocatalysts. A high-potential and cost-effective method for α-amylase production has been developed through the use of eco-enzymes derived from food waste, specifically *Citrus maxima* (Ashok et al. 2024; Paul et al. 2026). Among agro-wastes, *Citrus maxima* (pomelo) peel represents a significant and underutilised biomass resource. Citrus processing industries generate substantial quantities of peel waste, accounting for approximately 30–50% of the total fruit weight. Globally, citrus waste production is estimated to exceed 15 million tonnes annually, posing serious environmental concerns due to its high moisture content, rapid microbial spoilage, and potential to cause odour and landfill burden (Abd-Elaziz et al., 2020).

In addition, the economic feasibility of eco-enzyme-derived α-amylases was also evaluated in terms of production yields and purification efficiency to assess their competitiveness in the enzyme market (Shad et al. 2023). Microbial α-amylases have wide-ranging applications in industries such as food, fermentation and pharmaceuticals, where they are primarily used to transform starch into oligosaccharides. The development of biotechnology has led to the diversification of α-amylases and increased their applications in medicinal chemistry, analytical chemistry, automatic dishwashing detergents, textile desizing and the pulp and paper industry (Fuadh-Al-Kabir M 2026). In addition, the growing push for sustainable and economical industrial processes has created a demand for obtaining these enzymes from agro-industrial waste, which has the additional benefit of potential valorisation and recovery of value-added products (Paul et al. 2021). The global enzyme market also experienced a valuation of USD 6.9 billion (2021), and it is projected to reach USD 10.6 billion by 2026; α-amylases make up approximately a quarter (25 %) of the market, as it is used in many industrial applications, such as food processing and biosynthesis (Oslan 2019).

The present study focuses on the production, purification, characterisation, and optimisation of eco-enzyme-derived amylase from the waste peel of *Citrus maxima* with a view to maximising its potential application commercially and to promote a circular bioeconomy. In addition, the complete production, purification, and characterisation of all enzymatic properties, such as thermal stability, pH optima, sensitivity to various agents, and substrate specificity, were investigated in detail.

1.2. Research gap

Few studies have investigated the possibility of producing amylase from an eco-enzyme produced from *Citrus maxima* peel waste, despite the widespread usage of microbial amylases. Also, there hasn't been nearly enough research on the purification, characterisation, and optimisation of amylase produced from eco-enzymes for use in environmentally friendly manufacturing processes. In light of the importance of recycling in the circular bio-economy, this research aims to find a way to make amylase from *Citrus maxima* peel waste that is both inexpensive and environmentally safe.

Aim

To synthesize and characterize amylase from the eco-enzyme obtained from *Citrus maxima* peel waste for sustainable industrial applications.

Objective

1. To produce amylase from *Citrus maxima* peel waste via fermentation of the eco-enzyme.
2. To purify and characterize the amylase enzyme utilizing biochemical and molecular techniques.
3. To assess the potential of *Citrus maxima* peel waste as an environmentally benign and cost-effective source of industrial enzyme production.

2. Materials and Methods

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Citrus maxima peel residue was employed in the current experimental laboratory-based study to produce and characterise eco-enzyme-derived amylase. Eco-enzyme preparation, enzyme purification, biochemical characterisation, and optimisation of amylase activity under various physicochemical conditions were all included in the investigation.

2.1. Study design

The research was an experimental laboratory-based design that was designed to generate, purify, and characterise amylase from an eco-enzyme derived from *Citrus maxima* peel waste.

2.2. Collection and Preparation of the sample

The waste peel of *Citrus maxima* was collected from the local market, Guwahati, Assam, in a sterile container (Lahmar et al. 2017). The experimental methodology typically involves several key steps, including the preparation of the enzyme by the Bio–enzyme technique. Waste peels of *Citrus maxima* were washed thoroughly under running tap water and were chopped into small pieces. The ingredient ratio for preparing eco enzyme is 1:3:10 eco-enzyme solutions, 15 g Jaggery, 45 g of peels waste, and 150 mL of Distilled Water, to be mixed in an airtight sterile container at (25-30°C) for 3 months' anaerobic fermentation. The principle behind the enzyme preparation is the simple fermentation process (Vama & Cherekar, 2020). An airtight container is used to promote fermentation by minimizing oxygen (Figure 1). Usually, it takes three months for the eco-enzyme to be prepared and filtered (squeezed) through muslin cloth.

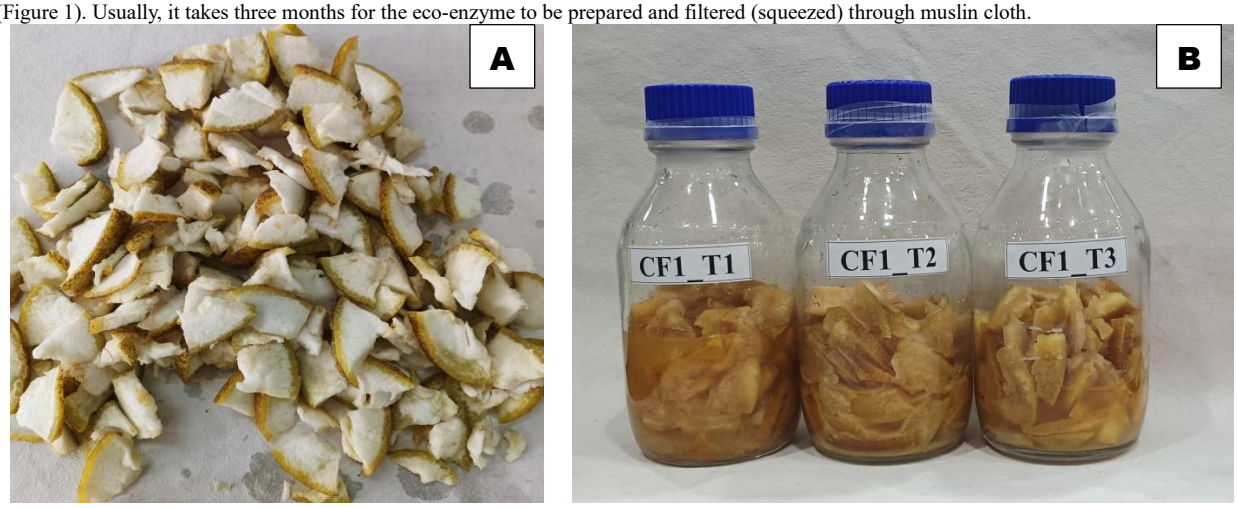

Figure 1: Waste peel of *Citrus maxima* (A), an air-tight container containing jiggery, waste peel, and water (1:3:10) for fermentation (B).

2.3. Qualitative analysis

A. Quick Tube Test:

1 mL test tube containing a clear 1% starch solution was coloured dark blue by adding 1 drop of Lugol's iodine solution. This colour change signifies the presence of starch in the solution. After adding 100 µl of crude enzyme to the test tube and shaking it for a few seconds, I looked for a colour change. The presence of an active enzyme causes starch to hydrolyse, as indicated by the loss of blue colour. The tube that contained 100 µl of water rather than crude enzyme was designated as the blank.

B. Isolation of total protein by Organic Solvent and Inorganic Salt precipitation

Protein was isolated from that filtered by organic precipitate methods using Acetone (50%). The pellet was collected by centrifugation at 10,000 rpm for 15 min at 4º C, and the supernatant was discarded. The pellet was washed using 0.05 M phosphate buffer (pH 7.4) and lyophilised. Similarly, ammonium sulphate precipitation (0-50%) was carried out with a continuous gentle stirring in an ice bath and was stored later overnight at 4º C. The solution was centrifuged at 10000 rpm for 30 min at 4∘C and dissolved in 0.05 M phosphate buffer (pH 7.4) and dialysed against the same buffer for 2 days at 4º C by changing the buffer several times. Dialysis was carried out using dialysis tubing (molecular weight cut-off 12 kDa, Sigma Aldrich, D9277-100FT), and the sample was freeze-dried using a Lyophilizer (Esquare EBT10 N). The protein content was determined by the Bradford method (Bradford 1976), referring to bovine serum albumin as a standard, and α- amylase assay was carried out following the DNSA (3, 5-dinitrosalicylic acid) method optical density was measured at 540 nm against substrate and enzyme. One unit of amylase was defined as the enzyme amount that releases 1 μ mole of maltose per minute.

C. Purification of Amylase

The dialysed product was transferred to a DEAE Sephadex column (1.8 × 16.7 cm) that had been previously rinsed with a 0.05 M phosphate buffer (pH 7.4). Eluted 4 mL of bound proteins at a flow rate of 15 mL/h using a linear gradient of NaCl (0.1–1 M) in the same buffer. Protein content was checked at 280 nm, and determined using the following methods of Bradford similarly amylase activity was checked by the DNSA method, followed by deep freeze dry. Ionised product passed through size exclusion chromatography (Sephadex G100, Sigma Aldrich), 0.05M phosphate buffer pH 7.4, which was earlier pre-equilibrated with Blue dextran (Sigma Aldrich) and Gel filtration standard (Biorad, # 1511901). 3 mL of elute was collected in different tubes (flow rate 12 mL/ hr.), and protein was checked at 280 nm and determined by the Bradford method, followed by the DNSA method for amylase activity.

a) Native PAGE

The 12% Native PAGE was subjected to activity staining by first soaking the gel for 15 minutes in a solution of 1% starch in a 0.05M sodium phosphate buffer (pH 7.4), and then staining it with a solution of 0.04% iodine.

b) SDS – PAGE

The subunit molecular mass of amylase was assessed via 12% SDS–PAGE utilising standard protein markers (Biorad 161-0377) with molecular masses spanning from 2 to 250 kDa. Subsequent to electrophoresis, the gels were stained with a 1% (w/v) solution of Coomassie Brilliant Blue R to elucidate protein bands.

c) Western Blot

Western Blot analysis was done for amylase using amylase alpha polyclonal antibody (PA5-122250). The samples of proteins at different concentrations (20, 15, and 10 µg) were separated on SDS-PAGE and transferred onto PVDF membranes. The primary antibody (1:1000) was added overnight at 4°C in membranes blocked with 5% skimmed milk in TBS-T (Tris buffer saline with 0.1% Tween 20), and then the secondary antibody (HRP-conjugated anti-rabbit) was added (1:2000). The visualisation of protein bands was performed with the help of enhanced chemiluminescence (ECL substrate, Biorad) reagents and visualisation in the ChemiDoc Imaging System, Biorad (Burnette 2015).

2.4. Optimization of different parameters on Amylase activity:

The pH optimum was established by incubating the amylase-substrate reaction for 3 minutes throughout a pH spectrum of 4.0 to 11.0. The temperature optimisation of amylase was assessed by maintaining the reaction mixture for 10 minutes at temperatures ranging from 30 to 80°C, while ensuring a constant pH of 7.4 using a phosphate buffer. pH stability of the enzyme was checked by keeping 1mg/mL of enzyme in a 0.05 M buffer of different pH values (4.0 to 11.0) for 2 hours at 4°C. After this, enzyme activity was measured to see how well it remained active. Thermal stability in different time intervals of amylase was studied by incubating the enzyme at its optimum pH at 40°C and 50°C for different time period shown. Samples were taken every 15 minutes, and the enzyme activity was measured.

a. Effect of pH on Enzyme Activity

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The influence of pH on amylase activity was assessed under standardised assay settings, using starch as the substrate. Enzyme activity was assessed in a 0.05 M sodium phosphate buffer across a pH range of 4 to 11 and at pH 7.4. Relative enzyme activities at each pH were determined by standardising the activity at the optimal pH to 100%.

b. Effect of Temperature on Enzyme Activity

The influence of temperature on amylase activity was assessed under standardised assay settings, using starch as the substrate. The reaction mixture was incubated at temperatures between 30° C and 80° C in sodium phosphate buffer at the optimal pH of 7.4. The relative enzyme activity at each temperature was determined by using the activity at the optimal temperature as the baseline of 100%.

c. Thermal stability in different time intervals

Thermal stability in different time intervals of amylase was studied by incubating the enzyme in its sodium phosphate buffer at the optimum pH 7.4 at 40° C and 50° C for different time periods. Samples were taken every 15 minutes, and the remaining (residual) enzyme activity was measured. Relative enzyme activity at each temperature was calculated by taking the activity at 0 min for both 40° C and 50° C as 100%.

d. Substrate Concentration

Optimization of substrate concentration on amylase activity was determined using different (0.5 to 2.5 %) soluble starch prepared in 0.05 M phosphate buffer (pH 7.4). Reaction mixtures comprising varying concentrations of starch solution (0.5 to 2.5%) and purified enzyme were incubated for 3 minutes, after which the reaction was terminated by the addition of DNSA reagent and subsequent heating in a boiling water bath. Absorbance was quantified at 540 nm after cooling. The released reducing sugars were quantified using a maltose standard curve. Each assay was done in triplicate. Relative enzyme activity at each substrate concentration was calculated by taking the activity at the optimum substrate concentration as 100%.

3. Results and Discussion

In the present study, the production, purification and characterization of amylase enzyme produced from eco-enzyme extracted from *Citrus maxima* peel waste was successfully demonstrated. The presence of amylase activity was verified through the hydrolysis of starch through qualitative analysis. The enzyme was further purified using various techniques like solvent precipitation, ammonium sulphate precipitation, ion-exchange chromatography and gel filtration chromatography. The amylase purified was independently confirmed by molecular characterization by SDS-PAGE, zymogram and western blot, with an approximate molecular weight of 50 kDa. The optimum concentration of the enzyme was found to be 1% starch and optimum pH 7.4 at a temperature of 40 °C.

3.1. Qualitative analysis of amylase activity

The eco-enzyme filtrate derived from *Citrus maxima* peel waste was confirmed to contain amylase activity by the qualitative starch–iodine assay. The enzyme's effective starch hydrolysis was demonstrated by the rapid decolonization of the blue starch–iodine complex in the crude enzyme extract. The presence of soluble enzymatic compounds was indicated by the brownish filtrate that was obtained following fermentation.

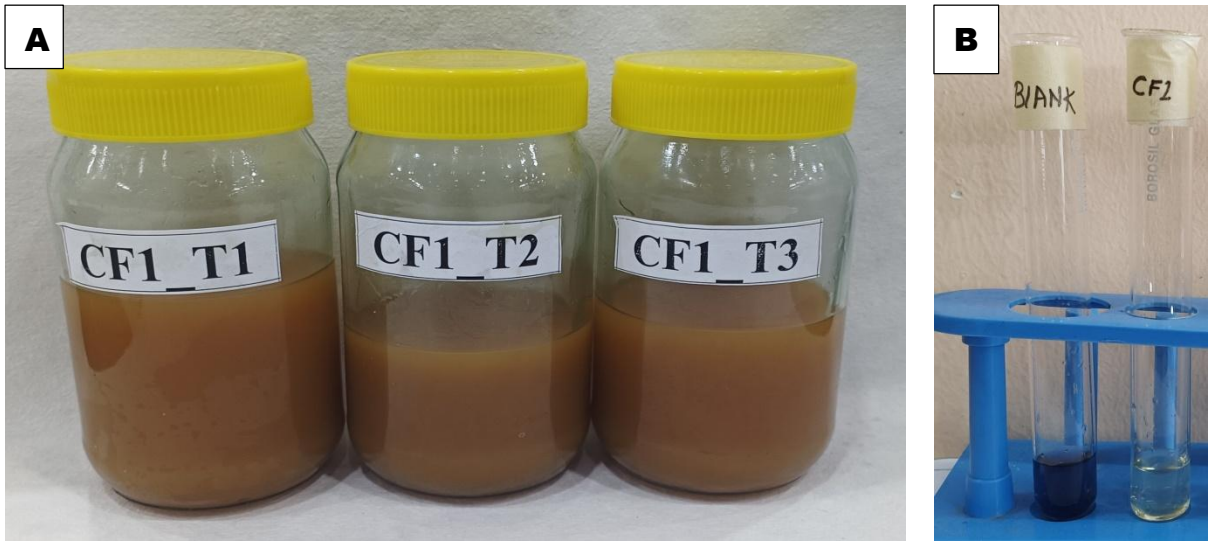

Figure 2: (A), Container containing Crude Eco Enzyme after filtration (B). Colourless indicates the presence of amylase in the crude after filtration

Figure 2 shows the qualitative assay result of crude eco-enzyme extract derived from the fermentation of *Citrus maxima* peel for amylase activity. The brownish filtrated crude enzyme (Figure 2A), typical of fermentation supernatants, presented a soluble organic substance. The amylase activity was identified with the iodine–starch test, starch hydrolysis method.

In the quick tube test, a few drops of iodine were added to test tubes containing 1 mL of 1% starch to produce a dark blue solution. Following the addition of 100 µl of crude enzyme of the eco enzyme solution, the colour quickly changed from dark blue to colourless, indicating significant starch hydrolysis activity and presence of amylase enzyme in the eco enzyme solution (Figure 2B).

Similar findings have been reported in earlier studies showing that hydrolytic enzymes (e.g., including amylases) can be produced by microbial fermentations of organic waste materials. The rapid decolorisation demonstrates high enzymatic efficiency, and the potent, potentially useful product from eco-enzymes could be used as a set of low-cost and sustainable enzymes of industrial importance. These preliminary qualitative confirmations can assist with further quantitative and purification tests for enzyme characterisation.

3.2. Purification of Amylase

The purification of amylase from the eco-enzyme obtained via fermentation of the waste peel of *Citrus maxima* was conducted using various methods, which showed a gradual increase in % of total activity and also specific enzyme activity. The protein content and enzyme activity for the crude extract were 558.97 mg and 1097.99 U, respectively; whereas its specific activity was estimated at 1.96 U/mg. Precipitation of protein using organic solvent at varied concentrations (0–50%) increased the specific activity to 2.55 U/mg with a recovery rate of 88.93%, suggesting the removal of non-proteinaceous impurities. However, the specific activity of the subsequent salt precipitate (2.37 U/mg) was slightly lower, indicating partial loss or denaturation of enzyme molecules; this is a general trend during salting-out processes (Table 1).

Further purification involved DEAE-Sephadex ion-exchange chromatography (Figure 3A), which increased the specific activity to 11.33 U/mg and provided a total of about 5.77-fold with a recovery percentage of only 32.06 % (Table 1). This is in agreement with previous reports, which show that chromatography using ionic exchange with charged protein molecules provides high enzyme purity.

A subsequent gel filtration chromatography (Sephadex G-100) also further purified the enzyme according to molecular size (Figure 3B) and resulted in a final specific activity of 16.39 U/mg and an overall purification fold of 8.34, with a recovery of enzyme is 10.14 % (Table 1), which reflects the inevitable balance between purity and yield during in multi-step purification process.

The current study successfully produces, purifies and characterises amylase from eco-enzyme using *Citrus maxima* peel and provides an opportunity to use it as a potential, environmentally friendly, cost-effective biocatalyst in the future. The qualitative iodine–starch assay indicates the presence of amylase. This finding

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supports previous results that showed microbial fermentation of agro-waste substrates produced active hydrolytic enzymes, most notably amylases. The application of eco-enzymes as enzyme reservoirs enhances their importance in circular bio-economy-oriented strategies. An increase in specific activity after DEAE-Sephadex chromatography has been reported earlier that charged proteins could be separated by ion exchange chromatography effectively to obtain a pure form of the enzyme. Similar purification folds have been described for microbial amylases, which indicate that the developed procedure is efficient and comparable with conventional methods. Vama and Makarand 2020 have identified that the Eco-enzyme is a fermented solution that is composed of bioactive metabolites and enzymes, including amylase, protease, and lipase, and is generated from brown sugar, water, and citrus peels. It supports waste recycling and reuse while providing eco-friendly, economical, and multipurpose applications in gardening and housekeeping

Table 1: Purification of amylase from eco-enzyme using waste peel of *Citrus maxima*

Step	Protein (mg)	Amylase (U)	Specific activity	Fold of purification	Recovery (%)
Crude	558.96	1097.99	1.96	1	100
Organic ppt. (50%)	382.34	976.52	2.55	1.30	88.93
Salt ppt. (0-50%)	372.95	883.74	2.36	1.20	80.48
DEAE Sephadex	31.07	352.11	11.33	5.77	32.06
Sephadex- G100	6.79	111.42	16.39	8.34	10.14

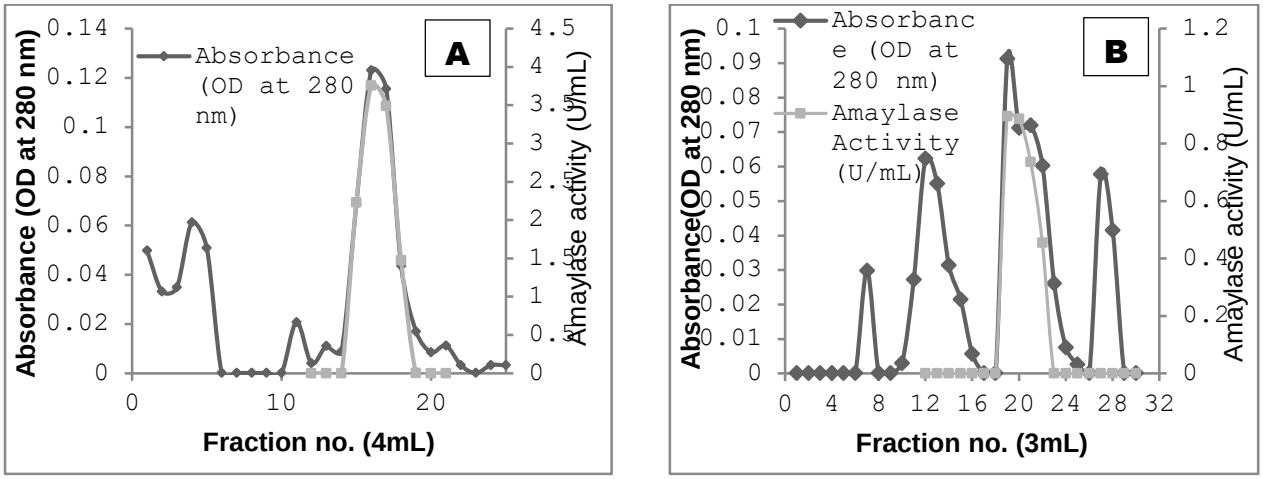

Figure 3: (A) Purification of amylase from Eco enzyme. DEAE sephadex column chromatography, (B) Sephadex G-100 gel chromatography.

3.3. Molecular Determination of Amylase:

Figure 4A illustrates the molecular weight of purified amylase obtained from eco-enzyme produced from *Citrus maxima* peel, which was determined by applying Sephadex G-100 gel filtration chromatography. A calibration curve of standard proteins derived by plotting log molecular weight versus Ve/Vo exhibited linearity ($y = -1.1413x + 7.1146$). The enzyme was estimated to be of about 50 kDa molecular weight on this standard curve, which is within the normal range for microbial α-amylases (45–60 kDa)

SDS-PAGE analysis of amylase (Figures 4B and 4C), where crude and partially purified samples showed several bands confirming protein heterogeneity. A single band (~50 kDa) shows the purity of amylase using DEAE-Sephadex and Sephadex G-100.These results between gel filtration and SDS-PAGE suggest that the enzyme is likely a purified amylase.

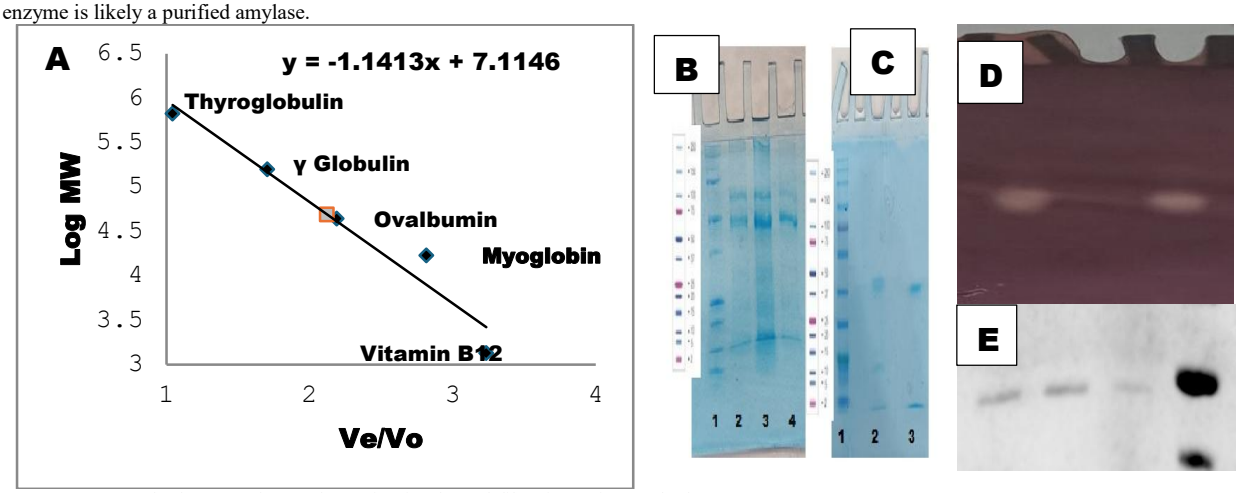

Figure 4: Standard curve of MW determination by gel filtration using sephadex G-100 (A), **SDS-PAGE analysis of amylase purification (B)** Lane 1: Protein MW marker; Lane 2: Crude extract; Lane 3: Salt ppt.; Lane 4: Organic ppt. **(C)** Lane 1: Protein MW marker;; Lane 2: DEAE-Sephadex; Lane 3: Sephadex G-100. **(D) Zymogram analysis of purified amylase,** Clear hydrolytic bands indicating amylase activity due to starch degradation in Zymogram. **(E)**Western blot analysis showing a distinct immunoreactivity band corresponding to ~50 kDa, confirming the presence and purity of amylase enzyme.

3.4. Zymogram analysis of amylase:

Zymogram analysis of amylase identified with a clear hydrolytic zone appeared against the background (staining by iodine) of each crude, purified amylase (Figure 4D). Clear resolved activities bands indicate that the purified enzyme was catalytically active after chromatographic purification the identity of purify amylase enzyme were further confirmed by Western blot analysis (Figure 4E).

3.4.1. pH Stability:

The highest purified amylase activity (100%) of the eco enzyme derived from *Citrus maxima* was observed at pH 7.4, while at pH 4.0, the enzyme exhibited low relative activity (70.68 ± 3.06 %), indicating that acidic conditions are not suitable for its catalytic performance. A gradual increase in activity was observed at pH 5.0 (76.60 ± 2.13 %), pH 6.0 (83.58 ± 1.23 %), and neutral pH 7.0 (88.58 ± 0.69 %). Similarly, enzyme activity declined markedly, with values of 83.40 ± 1.157

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% at pH 8.0 and 61.57 ± 0.87 % at pH 9.0, and a sharp reduction was observed at highly alkaline pH, where activity decreased to 29.95 ± 0.94 % at pH 10.0 and further to 20.16 ± 0.76 % at pH 11.0 (Figure 5A).

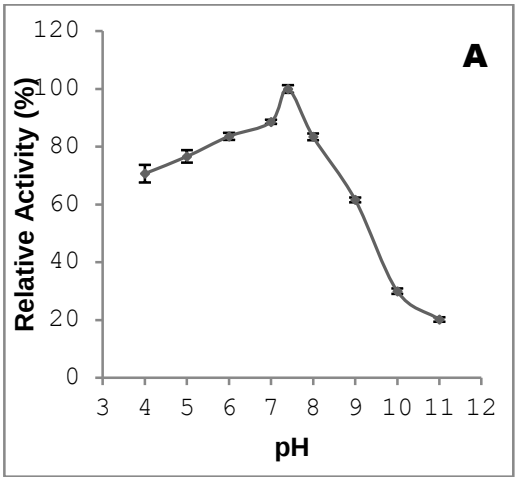
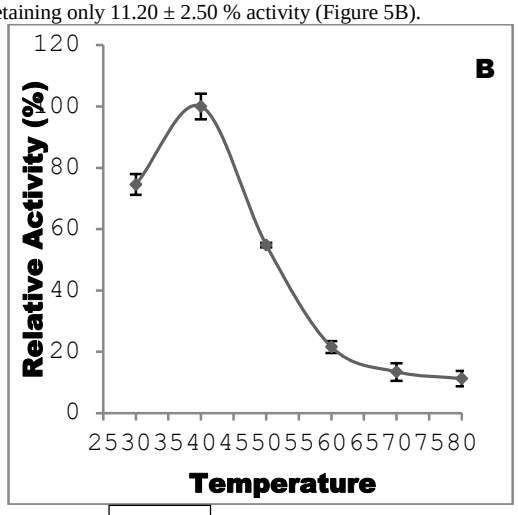


Figure 5A:

3.4.2. Thermal stability:

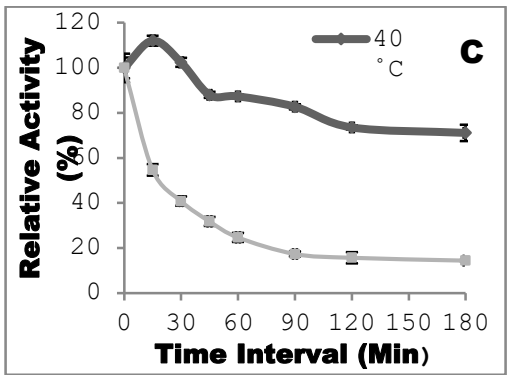
The effect of temperature on purified amylase activity of the eco-enzyme derived from *Citrus maxima* was examined over a range of 30 to 80 ° C for 10 min pre-incubation. The enzyme showed highest relative activity (100%) at 40 ° C. A decrease in activity was observed at 30°C, where the enzyme retained relative activities (74.56 ± 3.36 %), indicating the thermal stability. A sharp decline in enzyme activity occurred at higher temperatures. At 50°C, relative activity decreased markedly to 54.79 ± 0.74 %. Further increases in temperature led to progressive loss of activity, with 21.54 ± 1.88 % at 60°C and 13.40 ± 2.86 % at 70°C. At 80°C, the enzyme showed severe thermal inactivation, retaining only 11.20 ± 2.50 % activity (Figure 5B).



5: B

3.4.3. Time Interval:

Purified amylases were stable at 40◦C beyond 45 min and activity remain (87.97 ± 1.32 %) of their initial activity (100 %) (Figure 5C). At 50◦C after 15 min of enzyme incubation, activity decrease to 54.66 ± 2.56 % of the initial activity (100 %) (Figure 3C). The higher levels of activity retention and thermal stability enhance the applicability of *Pergularia tomentosa* L. across several practical fields; nonetheless, differences in process behavior may be ascribed to the distinct genetic makeup of each species. This thermal stability may be attributed to the presence of secondary and tertiary connections among the catalytic proteins, which enhance the integrity of the enzyme structure and its resistance to thermal treatment. Thermal stability was significantly influenced by the incubation duration (time) reported by (Lahmar et al. 2017).



5: C

3.4.4. Substrate Concentration:

Purified amylase activity on substrate concentration was maximum (100 ±0.2 %) at 1.0% of starch solution (Figure 5D) (Lahmar et al. 2017). This concentration

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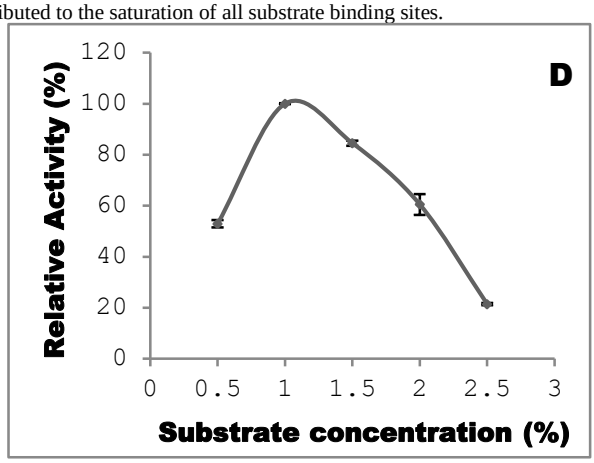
was also often employed to test for amylase in earlier studies. Enzyme activity (100 ±0.2 %) was increasing with rising substrate concentration from 0.5% to 1% of starch, and then enzyme activity again decreased (100 ± 0.2 % to 21.44 ± 0.39 %) with the increasing substrate concentration from 1% to 2.5% of starch. The decrease in amylase activity can be attributed to the saturation of all substrate binding sites.



5: D

Figure 5. Optimization of amylase activity under diff l conditions. (A) Effect of pH on enzyme activity. (B) Effect of temperature (C) Thermal stability profile of amylase at 40 °C and 50 °C over time. (D) Effect of substrate concentration on enzyme activity.

In case of optimization of pH stability, amylase was more active at optimal pH 7.4, which is consistent with some previous reports of neutral to slightly alkaline optima for bacterial amylases. Acidic and highly alkaline conditions both significantly reduced the activity, indicating structural instability of the enzyme outside its optimum pH. The temperature profile disclosed that the highest activity was at 40°C, and a significant decline in activity was observed at higher temperatures, which suggested moderate thermos ability. Mesophilic microbial amylases have been found to behave similarly. Studies on substrate concentrations showed the highest activity with 1% starch; an increase in concentrations brought about a decline in enzyme activity due to substrate saturation (Lahmar et al., 2017).

4. Conclusion:

The study concluded that production, purification and characterization of amylase from eco-enzyme which are the peels of *Citrus maxima* fruit waste which has been studied effectively. The purified enzyme exhibited maximum activity at pH 7.4, temperature 40°C and starch concentration of 1% with the overall purification fold of 8.34 and specific activity of 16.39 U/mg. The purified amylase was molecularly confirmed and was found to have an approximate molecular weight of 50 kDa. The findings reinforce that *Citrus maxima* peel waste can be used as a low-cost, eco-friendly and sustainable substrate for enzyme production while supporting agro-waste valorization and the circular bio-economy strategies. Further studies are required to enhance its commercial viability by implementing it into industry and scaling up to production level.

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Authors Contribution

Rima Paul: Writing original draft, Methodology, Investigation, Software, Data curation. Nayan Talukdar: Writing– review & editing, Supervision. Jyotchna Gogoi: Writing-review & editing, Validation, Supervision, Formal analysis.

Data Availability Statement

All the data is available with the authors and shall be provided upon request.

Declaration of Conflicting Interests

The authors declared no conflicts of interest with respect to the research, authorship, and publication of this article.

Ethical Approval

Not applicable.

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