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AI and Biochemistry

Biochemical Evaluation of Properties of *Klebsiella oxytoca* Pectinase Isolated from Some Vegetable Wastes

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Abstract

ABSTRACT

Pectinase is a group of enzymes that catalyze the breakdown of pectin. This study was carried out to investigate the biochemical and physicochemical properties of pectinase isolated from the fermentation of vegetable wastes using *Klebsiella oxytoca*. Pectinase was produced from *Klebsiella oxytoca* in a submerged fermentation system and purified in a three-step purification technique. The biochemical and physicochemical properties of purified pectinase were determined. The purified pectinase exhibited a subunit molecular weight of 46.34 kDa, specific activity of 164.87 mmol/min, and maximum stability at pH 6 and 50˚C. The enhancement of pectinase activity in the presence of Ca^{2+}, Na^{+}, Zn^{2+}, and K^{+} aided the breakdown of pectin, while it was significantly reduced in the presence of Hg^{2+} and Mn^{2+}. Some surfactants reduced the activity of the purified pectinase, while EDTA and ß-mercaptoethanol inhibited the enzyme. The estimated values of the kinetic constants, V_{max} and $K_{m,}$ were 8.07 µmol/ml/min and 2.44 mM, respectively. The vegetable wastes produced by *Klebsiella oxytoca* and enzymes possess characteristic properties that may be suitable for use in industrial processes where pectinase is required.

INTRODUCTION

Vegetable waste is an abundant source of biodegradable material. When it comes to cost and availability, vegetable waste differs from other solid trash. Its distinct color and texture, along with its good water-binding capacity, make it simple to identify, separate, and degrade [1]. Vegetable waste serves as an abundant source of nutrients for microorganisms capable of producing hydrolytic enzymes, particularly pectinases, due to the abundance of plant-derived polysaccharides such as cellulose, hemicellulose, and pectin [2]. Pectinases are enzymes that break down pectin, a key polysaccharide in plant cell walls. By severing specific bonds, pectinases decompose pectin, which is vital for maintaining plant cell walls' structure, texture, and function. Understanding the structure of pectin and the role of pectinases in its degradation is essential for various industrial applications and biological processes [3]. Pectin consists of a D-galacturonic acid monomer in a large proportion, which is joined together by α (1–4) linkages, and some carboxylic groups of galacturonic acids are partially esterified with methanol [4]. Understanding the structure of pectin and the role of pectinases in its degradation is essential for various industrial applications and biological processes [3]. Pectinesterase (EC 3.1.1.11), polygalacturonase (EC 3.2.1.15), galacturan 1,4 α-galactouronidase (EC 3.2.1.67), exo-β-galacturonosidase (EC 3.2.1.82), endopectatelyase (EC 4.2.2.2), exo-pectatelyase (EC 4.2.2.9), and endopectinylase (EC 4.2.2.10) are the seven classes of pectinases according to how they work [5].

Industries use acidic pectinases to extract and clarify fruit juices. Alkaliphilic pectinases, on the other hand, are frequently employed in the degumming of fiber and the treatment of wastewater released by fruit processing facilities. Although alkaliphilic bacteria can also create acidic pectinases, fungi are the primary producers of these enzymes [6]. The market for microbial pectinases is growing daily and makes up about 25% of the world's sales of food and industrial enzymes [7]. Pectinolytic enzymes from microorganisms are being studied as possible biocatalysts for several industrial processes. Pectinolytic enzymes can be used in a variety of industrial sectors when a specific process requires the breakdown of pectin. Various microorganisms were used to manufacture different types of pectinolytic enzymes [8]. Microbial enzymes are acknowledged as effective tools for environmentally

friendly biotechnological activities, which appear to be highly significant in today's society [9]. *Klebsiella oxytoca* is a Gram-negative, rod-shaped bacterium commonly found in soil, water, and the gastrointestinal tracts of humans and animals. It is known for producing industrially important enzymes, including pectinase, which breaks down pectin [10].

Recent work has demonstrated that *K. oxytoca* isolated directly from vegetable waste is capable of producing pectinase, with detailed biochemical and molecular characterization confirming its potential for industrial use [2]. Similarly, polygalacturonase-producing *Klebsiella* and *Staphylococcus* strains have been reported from spoiled fruits and vegetables, underscoring the importance of agro-waste as a natural reservoir for pectinolytic microbes [11]. Furthermore, the pehX gene has been identified as a reliable molecular marker for *K. oxytoca* pectinase activity, enabling precise genetic confirmation of enzyme-producing isolates [12]. The enormous generation of vegetable waste across the main markets in Nigeria requires its conversion to usable products or raw materials for industrial use. Hence, this study investigates the use of *Klebsiella oxytoca* to ferment some vegetable wastes for pectinase production with a view to evaluating the physicochemical properties of the enzyme.

MATERIALS AND METHODS

Collection of vegetable wastes

Some vegetable (carrot, cucumber, cabbage, and green pepper) wastes were collected in a sterilized container from a dumpsite at Shasha Market, Akure, Ondo State, Nigeria. The vegetable wastes were taken to the Department of Biology, Federal University of Technology, Akure (FUTA), for identification, and a portion of the vegetable wastes were taken to the Department of Microbiology, FUTA, for the isolation of microorganisms.

Reagents and chemicals

Ammonium sulphate, bovine serum albumin (BSA), sodium acetate, acetic acid, and sodium chloride were purchased from BDH Chemicals, Pate, England. Monopotassium dihydrogen phosphate, dipotassium monohydrogen phosphate, sodium dodecyl sulphate (SDS), tetramethylethylenediamine, ammonium persulphate (APS), 2-mercaptoethanol, ethylenediamine tetraacetic acid (EDTA), pectin, sodium hydroxide, Bradford reagent, and Sephadex G-150 were purchased from Sigma Chemical Company, St. Louis, MO, USA. Protein standard molecular weight marker (Bio-Rad Laboratories, India). All other chemicals and reagents used were of analytical grade.

Preparation of culture

The culture was prepared using a sterile fresh nutrient agar (NA) medium. 2.8 g of NA was weighed and dissolved in 100 mL of distilled water, then autoclaved at 121 °C (15 atm) for 15 min. A petri dish was prepared and labeled. The NA solution was poured into the labeled petri dish. The colonies of microbial growth on the already grown isolate were transferred into a prepared petri dish using the pour plate method under sterile conditions to prevent contamination. The petri dish was placed in a sterile cellophane bag and incubated at 37 °C for 48 h. Pure colonies of microorganisms were constantly transferred to a new culture medium and maintained on nutrient agar for a period of one month.

Identification of microorganisms

Microorganisms were identified at the Microbiology Research Laboratory of the Federal University of Technology, Akure. Morphological and biochemical tests were conducted to identify the organisms. Microorganisms were maintained on NA slants for one month. Microorganisms were constantly transferred from old cultures to new ones.

Screening of bacterial isolates for pectinase production

Bacterial isolates were screened for the production of pectinase. Extensive screening was carried out by measuring pectinase activity and protein concentration. The seven strains were subjected to the same conditions and the same medium. A single colony from the agar slant was aseptically transferred to a prepared 100 ml nutrient broth in a 250 ml Erlenmeyer flask. The flask was incubated for 24 h at 37 °C (150 rpm); this culture served as the seed culture. Five milliliters (5 mL) from the seed culture were transferred to 100 ml of the basal medium and 10 g/L pectin. The medium was autoclaved at 121 °C at 15 atm for 20 min.

The three different strains of bacteria were inoculated into seven different conical flasks containing the medium under aseptic conditions. The cultures were then incubated in a rotary shaker incubator at 37 °C for 36 h at 150 rpm, during which 5 mL of the mixture at 6 h intervals were taken for 36 h and were kept in the freezer. The fractions collected were centrifuged at 10,000 rpm at 4 °C for 20 min. The flask was incubated at 30 °C in a water-bath shaker for 36 h at 150 rpm. The progressive growth of cells was determined by measuring the absorbance of the collected fractions at 600 nm. The fractions were later centrifuged at 3000 rpm for 5 min in a refrigerated centrifuge and preserved at 4 °C.

Production of pectinase by submerged fermentation

The vegetable (cabbage, cucumber, carrot, and green pepper) wastes were homogenized using an electric blender until smooth and the homogenate was kept in the fridge until required for subsequent experiments. Nutrient broth was prepared by filtering NA using Whatman filter paper and autoclaving at 121 °C (15 atm) for 15 min. The sterilized broth was allowed to cool down to room temperature and was inoculated with the *Klebsiella oxytoca*. The culture was incubated in a rotary shaker incubator at 37 °C for 18 h. The process of submerged fermentation was used to produce pectinase [13]. A quantity of 100 g of blended vegetable waste was weighed into conical flasks containing 2 L of the basal medium. The conical flasks were autoclaved at 121 °C (15 atm) for 15 min, after which they were allowed to cool down to room temperature. After sterilization, the conical flasks were inoculated with the seed culture and incubated in a rotary shaker incubator at 37 °C for 36 h. The culture was centrifuged at 10,000 rpm at 4 °C for 20 min using a cold centrifuge. The supernatant was measured and stored in the freezer for further experiments. The supernatant served as the crude enzyme.

Assay of pectinase activity

The pectinase was assayed using 3, 5-dinitrosalicylic acid (DNSA) [13]. The reaction mixture contained 100 µL of 1% pectin, 100 µL of acetate buffer (50 mM, pH 4.5), and 100 µL of enzyme. The reaction mixture was incubated at 45 °C in a water bath for 30 min. 100 µL of DNSA was added to the reaction mixture and boiled for 5 min. After cooling, 1000 µL of distilled water was added to the reaction mixture. The absorbance was measured at 540 nm.

The standard curve was simultaneously prepared for reducing sugars with galacturonic acid. One unit of pectinase activity was defined as the amount of enzyme needed to catalyze 1 mol of the substrate at standard enzyme conditions. The total protein content of the supernatant was determined according to the Bradford assay using bovine serum albumin (BSA) as the standard protein.

Purification and characterization of pectinase

Ammonium Sulphate Precipitation: The crude enzyme solution was brought to 0–60% ammonium sulphate saturation. A quantity of 390 g of solid ammonium sulphate was added to the crude enzyme solution at 4 °C with gentle stirring of the mixture to allow the complete dissolution of ammonium sulphate. The precipitate was thereafter centrifuged at 10,000 rpm at 4 °C for 20 min and the pellet obtained was dissolved in 5 ml of 50 mM acetate buffer (pH 4.5). The enzyme solution was dialyzed against the same buffer for two days with three changes of buffer after 24 h. Pectinase activity and protein concentration were determined according to a standard procedure.

Ion exchange chromatography on DEAE-Sephacel

The dialysate was applied to a DEAE-Sephacel column (1.5 x 40 cm) and equilibrated with 50 mM acetate buffer pH 4.5. After eluting the unbound protein from the column with the starting buffer, a linear gradient of 0.5 M NaCl in 50 mM acetate buffer pH 4.5 was applied to elute the bound protein. Absorbance of the fractions was determined, and peak fractions were tested for pectinase activity. Fractions exhibiting pectinase activity were pooled and concentrated.

Gel filtration on Sephadex G-100

The concentrated enzyme solution was loaded on Sephadex G-100 column (2.5 × 70 cm; flow rate: 20 mL/h) previously equilibrated with 50 mM acetate buffer at pH 4.5. Absorbance of the fractions collected was determined at 280 nm, and peak fractions were tested for pectinase activity, while fractions exhibiting pectinase activity were pooled together and concentrated. The concentrated aliquot enzyme was used for SDS-PAGE and characterization.

Estimation of subunit molecular weight

SDS-PAGE was carried out using 10% acrylamide-resolving gel [14] using the Bio-Rad electrophoresis system (Bio-Rad, UK). The electrophoresis pack was run at a voltage of 80 V and 21 mA using the running buffer. After complete electrophoresis movement, the gel was stained with Coomassie brilliant blue for 24 h, after which it was destained with destaining solution (530 mL of distilled water, 400 mL of methanol, and 70 mL of acetic acid).

Determination pH optimum and stability

The effect of pH on the activity of purified pectinase was determined using various buffer systems consisting 0.05 M of acetate buffer (pH 3.05.0), phosphate buffer (pH 6.0-8.0), Tris-HCl buffer (pH 9.0-11.0), and incubated at 37 °C for 30 min. Pectinase assay was determined according to the method previously described. The effect of pH on pectinase stability was also carried out by incubating the purified enzyme solution between pH 3-9 for 2 h. Meanwhile, aliquot enzyme was withdrawn at 0 min, followed by subsequent removal at 20-minute intervals. Pectinase activity was determined according to the standard assay procedure earlier described.

Determination of optimum temperature and thermal stability

The temperature profile of the purified pectinase was determined in the temperature range of 30 to 80 °C. Pectinase activity was determined according to the standard assay procedure. The pectinase thermal stability was also determined by incubating the purified enzyme at 40–80 °C for 2 h. Initial activity was recorded at 0 min, while the subsequent activities were observed at 20-minute intervals. Pectinase activity was determined according to the standard assay procedure.

Effect of metal ions on enzyme activity

The effect of metal ions, including Ca^{2+}, Zn^{2+}, K^{+}, Mn^{2+}, Fe^{2+}, Hg^{2+}, Mg^{2+}, and Na^{+}, was studied on the purified enzyme at a concentration of 5 mM. Pectinase activity was determined according to the standard assay procedure.

Effect of inhibitors on the activity of the enzyme

The effect of inhibitors, including 5% EDTA, iodoacetic acid, β-mercaptoethanol, hydrogen peroxide, and sodium hypochlorite, was studied on the purified enzyme. Pectinase activity was determined according to the standard assay procedure.

Effect of surfactants on purified pectinase

The effect of surfactants, including 5% sodium dodecyl sulphate (SDS), Tween-20, urea, and Triton X-100 was studied on the purified enzyme. Pectinase activity was determined according to the standard assay procedure.

Effect of organic solvents on purified pectinase

The effect of organic solvents, including 5% formaldehyde, methanol, toluene, butanol, acetone, ethanol, hexane, carbon tetrachloride, ethyl acetate, and petroleum ether, was studied on the purified enzyme. Pectinase activity was determined according to the standard assay procedure.

Measurement of kinetic parameters

The kinetic constants, K_m and V_{max} of the purified enzyme were determined by the method of Lineweaver and Burk (1934) using pectin as substrate at various concentrations (2–10 mg/ml) in 50 mM acetate buffer (pH 4.5).

RESULTS

Partial identification of the isolate

Results showed that the isolate was a pink-pigmented, Gram-negative, non-spore-forming rod. The bacterium was catalase, indole, urease, VP, nitrate reduction, and starch hydrolysis positive, while oxidase, MR, citrate, gelatin, and casein tests were negative (**Table 1**). A rather broad range of results for carbohydrate fermentation supported a tentative identification as *Klebsiella oxytoca*.

Purification and characterization of pectinase

The summary of the purification of pectinase from *K. oxytoca* is presented in **Table 2**. The purified pectinase after ammonium sulphate precipitation yielded a specific activity of 13.03 mmol/min/mg. The dialysate was applied on DEAE Sephacel followed by gel-filtration chromatography on Sephadex G-100, produced a homogenous enzyme having a 43.00% activity recovery, specific activity of 164.87 mmol/min/mg and 22.94 purification fold.

The homogeneity was confirmed by denaturing polyacrylamide gel electrophoresis, which gives a band of protein on the slab gel and has an estimated subunit molecular weight 46.34 kDa (**Fig. 1**). The biochemical characteristics of the purified pectinase from the fermentation of vegetable wastes by *K. oxytoca* include:

Table 1. Biochemical and morphological identification of the organism.

Tests	Results
Color	Pink
Gram reaction	-
Cellular Morphology	Rods
Catalase Test	+
Oxidase Test	-
Indole Test	+
Motility Test	-
MR	-
VP	+
Urease activity	+
Citrate activity	-
Starch Hydrolysis	+
Gelatin Hydrolysis	-
Casein Hydrolysis	-
NO_3 Reduction	+
Spore Test	-
Glucose	+
Sucrose	+
Lactose	+
Raffinose	+
Arabinose	+
Galactose	+
Salicin	+
Sorbitol	+
Inositol	+
Maltose	+
Mannitol	+
Fructose	+
Xylose	+
Probable Organism	*Klebsiella oxytoca*

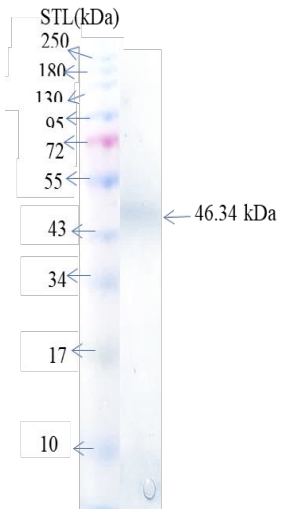

Fig. 1 Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate. STD, standard molecular weight; a-250 kDa, b-180 kDa, c-130 kDa, d-95 kDa, e-72 kDa, f-55 kDa, g-43 kDa, h-34 kDa, i-24 kDa, j-17 kDa, and pp, purified pectinase- 46.34±3.62 kDa.

Activity and stability of purified pectinase to changes in pH

Optimum activity of the purified pectinase was obtained at pH 6 (**Fig. 2**). However, the enzyme demonstrated relative activities of 94.6% and 89.9% at pH 7.0 and 8.0, respectively. The purified pectinase attained its maximum stability at pH 6, 7, and 8 for 120 min, with a gradual loss of activity after 100 min to attain residual activity of 94.3-78.6%. Extremely high acidity and alkalinity reduced the activity of the purified pectinase, with an observed decline in residual activity to below 40% (**Fig. 3**).

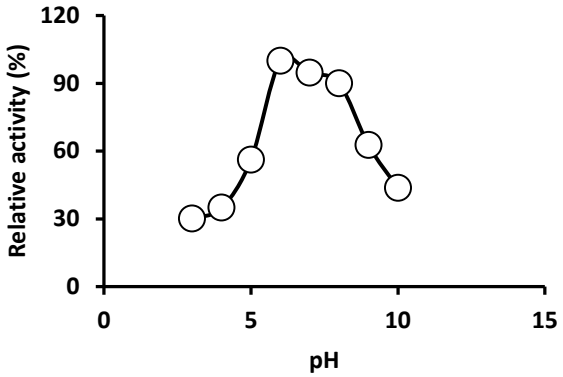

Fig. 2 Effect of pH on the activity of pectinase from *Klebsiella oxytoca*.

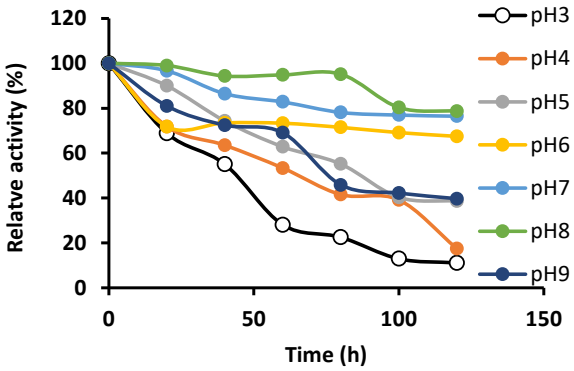

Fig. 3 Effect of pH on the stability of pectinase from *Klebsiella oxytoca*.

Activity and stability of purified pectinase to changes in temperature

The optimum temperature of the purified pectinase was obtained at 50 °C, followed by a significant decrease in the relative activity of and 35% at 60°C and 70°C, respectively (**Fig. 4**). The enzyme retained 96% and 93% residual activity after 40 min of incubation at 50 ºC. Residual activities of 89.8, 72, and 56.9% were obtained at 40, 60, and 70°C, respectively. The enzyme showed a significant loss of stability as the temperature increased (**Fig. 5**).

Table 2. Purification table of pectinase from *Klebsiella oxytoca* 605 AMP- ammonium sulphate precipitation, IEX-Ion exchange chromatography, GFC-gel filtration 606 chromatography.

Step	volume (mL)	Enzyme activity (mmol/min/mL)	Protein concentration (mg/ml)	Total activity (mmol/min)	Total protein (mg)	Specific activity (mmol/min/mg)	Yield (%)	Fold
Crude	750.0±22.3	5.46±0.91	0.76±0.02	4095±26.8	570±4.6	7.18±1.5	100.00±0. 0	1.00±0.0
AMP.	85.5±4.5	7.17±0.4	0.55±0.01	613.03±11.2	47.05±1.2	13.03±0.8	14.97±0.3	1.81±0.01
IEX	51.8±1.6	14.29±0.5	0.35±0.03	740.22±8.4	18.13±1.0	40.82±0.7	18.07±0.1	5.68±0.02
GFC	44.5±1.1	39.57±0.7	0.24±0.01	1760.86±17.9	10.68±0.7	164.87±0.4	43.00±0.3	22.94±0.0 1

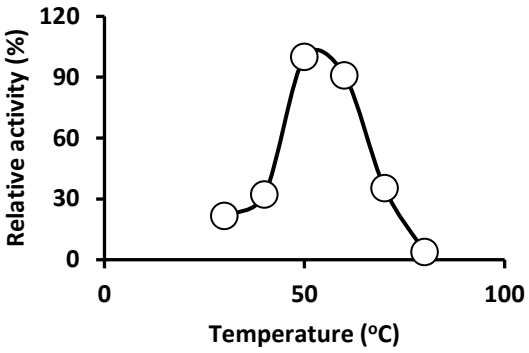

Fig. 4. Effect of temperature on the activity of pectinase from *Klebsiella oxytoca*.

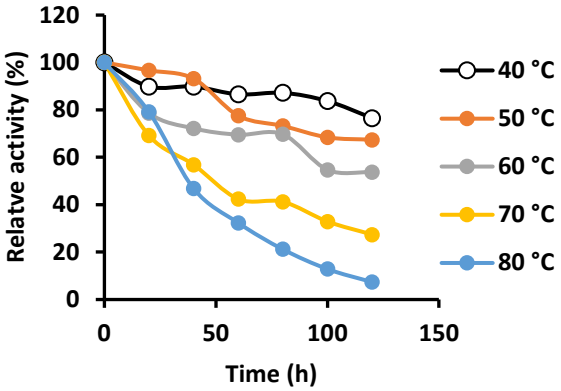

Fig. 5. Effect of temperature on the stability of pectinase from *Klebsiella oxytoca*.

Metal ions on the activity of pectinase

Pectinase activity was enhanced in the presence of Ca^{2+} and Na^{+}, causing a significant increase in enzyme activity of 119.9% and 95%, No significant changes in activity was observed with K^{+}, Mg^{2+} and Zn^{2+} having relative activities of 90.1%, 83.1 and 82.9%, respectively (**Fig. 6**). However, pectinase activity was reduced in the presence of Mn^{2+} and Hg^{2+} with relative activity 41.6% and 35.6%, respectively.

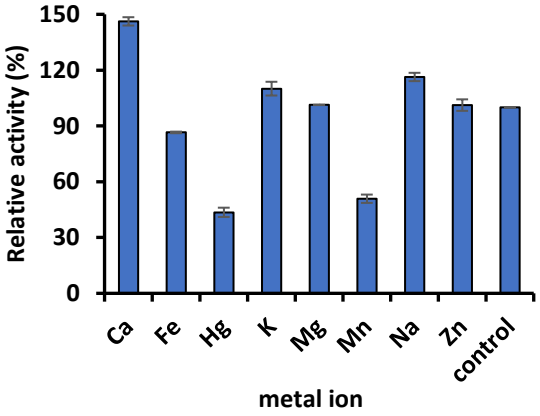

Fig. 6. Effect of metallic ions on the activity of pectinase from *Klebsiella oxytoca*.

Inhibitors of the activity of pectinase

The enzyme activity was considerably inhibited in the presence of β-mercaptoethanol, EDTA, and indo-acetic acid, with relative activities of 40, 41.8, and 42.8%, respectively. However, sodium hypochlorite and hydrogen peroxide cause a slight inhibition to the enzyme activity with 51.5% and 58.5%, respectively (**Fig. 7**).

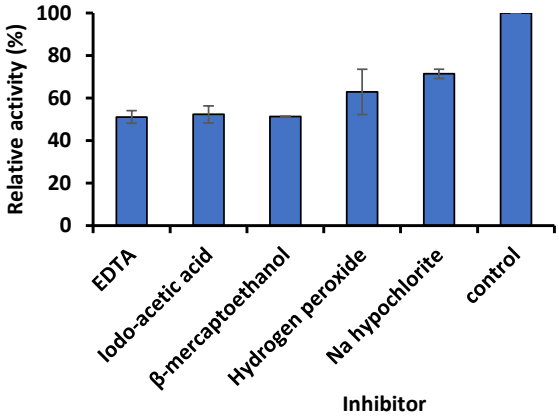

Fig. 7 Effect of inhibitors on the activity of pectinase from *Klebsiella oxytoca*.

Surfactants on the activity of pectinase

The enzyme activity was inhibited in the presence of SDS, urea, and Triton X-100 with relative activities of 55.3%, 54.3% and 56.9%, respectively. However, Tween-80 has a great inhibition of enzyme activity with 49.8%, respectively (**Fig. 8**).

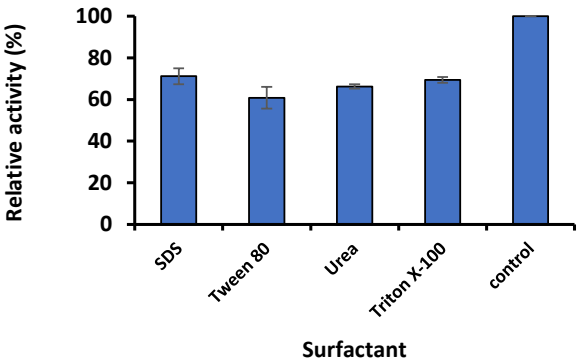

Fig. 8 Effect of surfactants on the activity of pectinase from *Klebsiella oxytoca*.

Organic solvents on the activity of pectinase

The enzyme activity was observed to be greatly inhibited in the presence of methanol, formaldehyde, ethyl acetate, and toluene, with 46.6%, 42%, 41.8%, and 49.8% relative activity, respectively. Similarly, hexane, acetone, ethanol, carbon tetrachloride, butanol, and petroleum slightly inhibited pectinase activity with relative activity of 67.5, 64.7, 54.3, 55.9, 58.5, and 51.9%, respectively (**Fig. 9**).

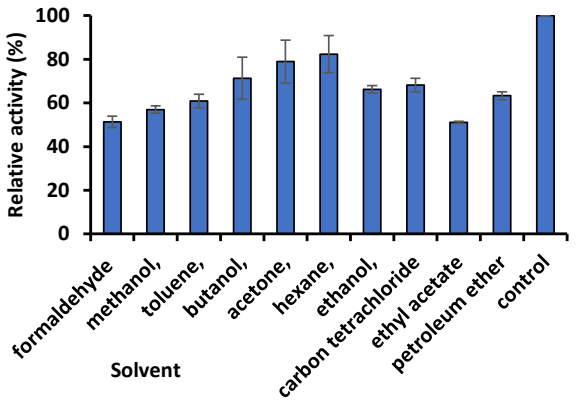

Fig. 9. Effect of organic solvents on the activity of pectinase from *Klebsiella oxytoca*.

Kinetic parameters

K_m and V_{max} values were estimated to be 2.44 mM and 8.07 µmol/min/mL, respectively (**Fig. 10**).

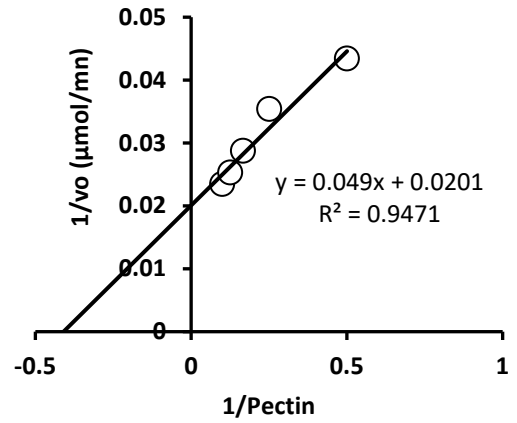

Fig. 10. Double reciprocal curve showing V_{max} and K_m of pectinase from *Klebsiella oxytoca*.

DISCUSSION

Pectinases are extensively used in various industries, and microorganisms are preferred for pectinase production due to their easy cultivation and maintenance [15]. Vegetable waste such as cucumber, carrot, green pepper, and cabbage, which are rich in pectin, may serve as ideal substrates for growing pectinolytic microbes. This study used such vegetable wastes to isolate microorganisms capable of producing pectinase. While pectinases are naturally found in plants, microbial sources are favored for large-scale production due to higher yields and simpler cultivation [16]. Utilizing vegetable waste for isolating pectinase-producing microbes may reduce waste and promote sustainable, eco-friendly industrial processes.

Our results support previous findings that vegetable wastes can serve as effective substrates for isolating *K. oxytoca* with strong pectinolytic activity. [2] Similarly, the biochemical and molecular characterization of *K. oxytoca* from vegetable waste, highlights its potential in biotechnological applications. The detection of polygalacturonase activity in our isolate is also in agreement with [11], who showed that both Klebsiella and Staphylococcus species from decomposed fruits and vegetables possessed strong pectin-degrading capabilities. Moreover, the presence of the pehX gene in our isolate, as demonstrated previously [12], provides molecular confirmation that links pectinase production to a specific genetic marker in *K. oxytoca.*

The biochemical and morphological identification of the isolate from vegetable waste revealed that it was *Klebsiella oxytoca*. The isolate exhibited a pink color and was identified as a Gram-negative rod. It was catalase-positive and oxidase-negative, typical of *K. oxytoca*. The ability to reduce nitrates to nitrites was also confirmed. These findings are consistent with known characteristics of *Klebsiella oxytoca* and are supported by similar studies in the literature [17, 18, 19, 20]. The purification of pectinase with activity recovery of 43.00%, purification fold of 22.94, and a specific activity of 164.87 U/mg as achieved in this study indicates a moderate recovery and a significant improvement in purification, though the specific activity remains relatively low compared to some other studies.

Comparatively, [26] reported on *Aspergillus niger* pectinase, finding a similar specific activity of 9.90 U/mg but with a much lower purification fold of 1.06 and a high yield of 89.5%. In contrast, [5] studied pectinase from *Pichia pastoris* and found a remarkably high specific activity of 2026.03 U/mg, a purification fold of 6.77, and a yield of 58.16%. [22] examined pectinase from *Bacillus subtilis* and reported a specific activity of 143.77 U/mg, a purification fold of 11.14, and a yield of 24.07%. This study shows a balanced approach with a reasonable purification fold and specific activity, though the yield was lower than in some other studies. [7] provided specific activity of 61.54 U/mg, an exceptionally high purification fold of 75.98, but an extremely low yield of 0.01%. The high purification fold indicates a very effective purification process, yet the yield suggests that a significant amount of enzyme was lost during purification.

Molecular weight of the pectinase was estimated to be 46.34 kDa. This finding is consistent with several other studies, but variations exist across different microbial sources. For instance, [22, 23, 24] reported molecular weights of 38 kDa, 58 kDa, 33 kDa for pectinase derived from citrus peel, *Bacillus amyloliquefaciens, and Bacillus subtilis,* respectively. Lower molecular weight could be attributed to the specific properties of the pectinase extracted from plant-based sources, which might differ structurally from those of microbial origins. A significantly higher molecular weight of 70 kDa for pectinase from *Aspergillus fumigatus* [20]. This substantial difference might be due to the presence of additional subunits or different quaternary structures, which can affect the enzyme's overall functionality and its interaction with substrates.

In this study, the optimum pH of purified pectinase from *K. oxytoca* indicated a catalytically active enzyme in a slightly acidic reaction environment. This pH is consistent with several other studies, [25, 21, 5, 26, 27] reported that pectinase from *Geotrichum candidum, Aspergillus niger Aspergillus niger*, *flax retting liquor*, and *Pichia pastoris* exhibited optimum activity at pH 5. The slightly lower pH range for these enzymes may reflect the natural pH environments where these fungi or microorganisms thrive, which could influence enzyme structure and activity. [22] reported optimum pH of 9 for pectinase from *Folia microbiologica*, this variation shows that some pectinases are adapted to more basic environments. [28], found pectinase from *citrus peel* to be most active at pH 8. Furthermore, the stability of purified pectinase at pH 8, maintaining 94.378.6% of its activity after 120 minutes of incubation, was consistent with [23], who found that pectinase from *citrus peel* retained 99% of its activity at pH 6-10. [27] reported that pectinase from

Aspergillus niger maintained 75% of its activity at pH 4-6 for 2 hours. This is beneficial for many biotechnological applications, particularly those requiring a neutral to acidic pH range. [7] reported that pectinase from *Aspergillus niger* was stable in the pH range of 3.5-4.5 for up to 100 minutes, indicating that it is more effective in slightly acidic conditions. This suggests that while *Aspergillus niger* pectinase performs well in acidic environments, its use may be limited in more neutral or alkaline processes.

The purified pectinase exhibited an optimal temperature at 50°C, with a significant decline in activity at higher temperatures. This indicates that the enzyme is most efficient at moderate to high temperatures, but its activity is compromised as the temperature increases further. These thermal properties are key for optimizing enzyme use in industrial processes. When comparing the thermal properties of pectinase across different sources, a range of optimal temperatures has been reported. [25, 29, 21] reported that pectinase from exhibited an optimal temperature of 35 °C, 30 °C, 40 °C, *Geotrichum candidum, Folia microbiologica* and *Aspergillus niger* respectively which are lower than the 50°C reported in the current study. [30] reported that pectinase from *Mucor circinelloides* exhibited optimal activity at 50°C, further reinforcing the idea that a significant number of pectinases from fungal and other microbial sources tend to show optimal activity around 50 °C, making them ideal candidates for processes in industries that involve moderate to higher temperatures, such as in juice clarification or biofuel production. It was found that the pectinase retained 96% and 93% residual activity after 40 minutes of incubation at 50 °C, indicating strong thermal stability at this temperature.

Comparatively, [23] observed that pectinase from *citrus peel* retained 90% activity when incubated at 40°C, 50°C, and 60 °C for 10 minutes but only 51% activity after 60 minutes at these temperatures. This indicates that while the enzyme is initially stable at higher temperatures, its activity decreases significantly with prolonged exposure, suggesting a potential limitation for processes that require sustained enzyme activity at elevated temperatures. [27] reported that pectinase from *Aspergillus niger* was thermally stable at 30°C and 40°C for 2 hours, maintaining activity above 80%. This high stability at moderate temperatures makes it suitable for processes that require long-term enzyme activity at these temperatures. However, it may not be as effective for higher temperature applications.

A previous study [31] identified pomegranate peel as the most suitable substrate and achieved maximal activity under optimized conditions (pH 6, 40 °C, 96 h), the current study demonstrates that mixed vegetable wastes can equally serve as efficient substrates for pectinase production. Both studies highlight the adaptability of *K. oxytoca* to diverse ecological niches, whether insect gut or plant waste, confirming its versatility as a source of robust enzymes. Importantly, both enzymes displayed optimum activity in slightly acidic conditions (pH 6) and moderate temperature ranges (40–50 °C), underscoring their suitability for industrial applications such as juice clarification and textile processing.

The specific activity obtained in this study (164.87 mmol/min/mg) compares favorably with the activity reported by [30], further validating the biotechnological relevance of *K. oxytoca* pectinase. Taken together, these findings strengthen the argument that valorization of agro-wastes - whether fruit residues like pomegranate peel or vegetable waste streams - provides a sustainable and low-cost route to enzyme production. Future studies may also consider the effect of extended fermentation periods, as shown by the bee-gut isolate, to further enhance yields from vegetable-based fermentations. The purified pectinase from *Klebsiella oxytoca* exhibited notable changes in enzyme activity in response to different metal ions. Specifically, the enzyme activity was greatly enhanced by the presence of Ca^{2+} and Na^{+}, suggesting that these metal ions play a crucial role in stabilizing the enzyme or improving its catalytic efficiency. In contrast, the presence of Mn^{2+}, and Hg^{2+} significantly inhibited pectinase activity. These metal ions likely interfere with the enzyme's active site or overall structure, leading to reduced functionality. The findings of this study align with various reports on the influence of metal ions on pectinase activity from different sources. For instance, [21] found that the activity of *Aspergillus niger* pectinase was enhanced by Mg^{2+}, Zn^{2+}, Cu^{2+}, Co, and Fe^{2+}. [23] reported that pectinase from citrus peel was significantly enhanced by Ca^{2+}, similar to the current study's findings for *Klebsiella oxytoca*.

However, other metal ions like Hg^{2+}, Ag^{+}, Co^{2+}, Ni, Mn^{2+}, and Zn^{2+} strongly inhibited its activity. This highlights that while some metal ions like Ca^{2+} are generally beneficial, others can have detrimental effects depending on the enzyme's source and structure. [5] observed that pectinase from *Pichia pastoris* was enhanced by a wide range of metal ions, including Na^{+}, K^{+}, Li^{+}, Ca^{2+}, Mg^{2+}, Cu^{2+}, Fe^{2+}, and Zn^{2+}, but was inhibited by Co, Mn^{2+}, and Pb^{2+}. This broad enhancement suggests a high degree of metal ion tolerance and potential utility in diverse industrial processes. However, the inhibition by specific ions like Co and Pb^{2+} indicates the need for careful consideration of the metal ion environment in practical applications. [24] reported that pectinase from *Bacillus amyloliquefaciens* was enhanced by Mg^{2+} and Ca^{2+} but inhibited by Zn^{2+}, Fe^{2+}, Mn^{2+}, Cu^{2+}, and Ba^{2+}. This mixed response underscores the complexity of metal ion interactions with pectinases and the importance of identifying optimal conditions for enzyme activity in industrial processes. [30] reported that the activity of *Mucor circinelloides* pectinase was inhibited by K^{+}, Mg^{2+}, Ba^{2+}, and Ni^{2+}. This contrasts with the current study's findings on the enhancing effects of K^{+} and Mg^{2+} on *Klebsiella oxytoca* pectinase, indicating that metal ion effects can be highly species-specific.

The enzyme activity was inhibited by SDS, urea, and Triton X-100, with relative activities of 55.3%, 54.3%, and 56.9%, respectively. Among these inhibitors, Tween-80 had the greatest inhibitory effect, reducing enzyme activity to 49.8%. This suggests that these substances interfere with the enzyme's functionality, possibly by disrupting the enzyme structure or affecting the enzyme-substrate interaction. [23] reported that the activity of pectinase from citrus peel was inhibited by SDS, aligning with the current study's findings. SDS is known to be a strong denaturant, which can disrupt the hydrophobic interactions within the enzyme, leading to loss of activity. [24] found that pectinase activity from *Bacillus amyloliquefaciens* was also inhibited by SDS.

This consistency across different studies indicates that SDS commonly acts as an inhibitor for various pectinases, likely due to its denaturing properties. Interestingly, [32] reported that *Aspergillus niger* pectinase activity was enhanced by SDS, which contrasts with the inhibitory effects observed in other studies. This suggests that the response to SDS can vary significantly depending on the source of the pectinase and its specific structural properties. Regarding TritonX100, [24] reported that pectinase activity from *Bacillus amyloliquefaciens* was enhanced by TritonX-100 and Tween-40, but inhibited by

Tween-20. This indicates that non-ionic surfactants can have differing effects on enzyme activity, which may depend on the enzyme's source and the specific type of surfactant used. In the current study, Triton X-100 inhibited pectinase activity, highlighting the variability in enzyme responses to different surfactants. Finally, [32] also reported that *Aspergillus niger* pectinase was inhibited by SDS, supporting the findings from [23,24], although this contrasts with their earlier report of enhancement.

Methanol, formaldehyde, ethyl acetate, and toluene with the relative activities of 46.6%, 42%, 41.8%, and 49.8%, respectively, inhibited the purified pectinase activity. Additionally, other solvents such as hexane, acetone, ethanol, carbon tetrachloride, butanol, and petroleum ether slightly inhibited pectinase activity, with relative activities of 67.5%, 64.7%, 54.3%, 55.9%, 58.5%, and 51.9%, respectively. These findings indicate that certain organic solvents can significantly reduce pectinase activity, potentially due to their effects on the enzyme's structural integrity or active site environment. [5] reported that pectinase from *Pichia pastoris* was enhanced by methanol, ethanol, DMSO, propanol, and hexane but inhibited by chloroform and acetone. This contrasts with the current study's finding of methanol as an inhibitor, highlighting the variability in enzyme response to organic solvents based on the enzyme's source and specific structure.

The enhancement by ethanol and hexane reported by [5] aligns with their observations, although acetone was found to be an inhibitor in both studies. [22] observed that pectinase activity from *Bacillus subtilis* strain was enhanced by acetone, butanol, and ethanol but inhibited by acetonitrile, DMSO, and isopropanol. The enhancement by acetone and butanol contrasts with the current study's findings, where these solvents slightly inhibited pectinase activity. This discrepancy may be attributed to differences in enzyme sources, as different pectinases can have varied structural features affecting their interaction with organic solvents.

The enzyme activity was considerably inhibited by β-mercaptoethanol, EDTA, and indo-acetic acid, with relative activities of 42.0%, 41.8%, and 42.8%, respectively. Additionally, sodium hypochlorite and hydrogen peroxide caused slight inhibition, with relative activities of 51.5% and 58.5%, respectively. These results suggest that these chemicals interfere with the enzyme's activity, potentially through chelation of essential metal ions or disruption of the enzyme's structure. [23] reported that the activity of pectinase from citrus peel was inhibited by EDTA. EDTA is known to chelate divalent metal ions, which are often essential cofactors for enzymatic activity, leading to inhibition. Karaolan and Erden-Karao [33] similarly found that pectinase from *Pichia pastoris* was inhibited by EDTA.

This consistency suggests that EDTA commonly acts as an inhibitor across different pectinase sources due to its strong chelating properties. In contrast, [24] reported that pectinase activity from *Bacillus amyloliquefaciens* was enhanced by EDTA. This discrepancy could be due to differences in the enzyme's requirement for metal cofactors or the presence of other stabilizing factors in the enzyme from *Bacillus amyloliquefaciens*. [32] observed that *Aspergillus niger* pectinase activity was enhanced by both EDTA and β-mercaptoethanol. β-mercaptoethanol is a reducing agent that can stabilize enzymes by preventing the formation of disulfide bonds. This suggests that the structural requirements and stability of pectinase from different sources can vary significantly, leading to different responses to these chemicals.

In the current study, the V_{max} and K_m for pectinase from *Klebsiella oxytoca* were estimated to be 8.07 µmol/ml/min and 2.44 mM, respectively. These values provide insight into the enzyme's catalytic capability and its affinity for the substrate. [23] reported a K_m of 1.64 g/L and a V_{max} of 232.56 mol/L/min for pectinase from citrus peel. The significantly higher V_{max} indicates that this enzyme can process substrates at a much faster rate compared to the pectinase from *Klebsiella oxytoca*. However, the lower K_m suggests a higher affinity for the substrate. [5] found K_m and V_{max} values of 6.9 mg/mL and 67.57 µmol/mg/min for pectinase from *Pichia pastoris*.

The relatively high K_m indicates a lower substrate affinity, while the V_{max} is considerably high, showing efficient substrate conversion when saturation is achieved. [32] reported a K_m of 3.89 mg/mL and a V_{max} of 1701 U/mg for *Aspergillus niger* pectinase. This enzyme exhibits a high catalytic rate as reflected by its V_{max}, although its K_m value suggests it has a moderate affinity for the substrate compared to other sources. [20] documented K_m and V_{max} values of 7.19 mg/mL and 252.80 µmol/min/mg for pectinase from *Aspergillus fumigatus*. The high V_{max} indicates a robust enzymatic activity, while the K_m shows a relatively low substrate affinity.

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