Journal of Advanced Scientific Research

Available online through https://sciensage.info

ISSN
0976-9595
Research Article

Optimization of Culture Conditions and Characterization of Pectinase from Isolated Bacteria and Application in Juice Clarification

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Received: 20-05-2024; Accepted: 15-06-2024; Published: 29-06-2024

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ABSTRACT

Microbial pectinase has high demand in different industries. Pectinase-producing bacteria were isolated and purified from soil samples and screened by primary and secondary screening for pectinase production. Out of the 13 bacterial samples, sample 4.1(S4.1) showed consistent enzyme activity. Optimum production of pectinase was high when incubated at 24 hours at 37°C with 2% substrate concentration maintaining pH6. Optimum pectinase activity was observed at temperature 37°C and pH 6. Cu++ was found as an activator and Zn++, Ca++ as inhibitors of pectinases. The values for Km and Vmax were found to be 1.181mg/ml and 471.69 µM/min, respectively. Pectinase was efficiently partially purified by 90% acetone saturation. Specific enzyme activity for a partially purified enzyme with acetone was 96870 U/mg and purification fold was 35.41. The utilization of low-cost agro-industrial wastes as substrates has been preferable in pectinase production. The sweet lemon and lemon fruit peel powder was observed good substrate for pectinase production. Furthermore, the yield and clarity of sweet lemon juice increased as the concentration of pectinase increased, indicating its potential use in juice processing. Experimental results suggest pectinases from S4.1 were of high value and can be utilized for fruit juice clarification.

Keywords: Pectinase, Citrus fruit peel, characterization of pectinase, Partial purification by acetone, Juice clarification.

INTRODUCTION

Enzymes are biological catalysts that help in enhancing the rate of chemical reactions that occur under various physicochemical conditions. All enzymes are proteins in nature. Each has a unique performance function. Enzymes were first identified in the mid-nineteenth century. The first to recognize the technical potential of cultivated enzymes and commercialize primarily using fungal enzymes. In France 20 years later, Boidin and Affront founded the synthesis of bacterial enzymes.[1] Pectinases, which have another name, pectic enzymes, hydrolyze pectic compounds linked by α-1,4-glycosidic bonds and esterified with methyl groups. Heterogenic pectic substances are buildup of pectin, protopectin, and pectinic acids or polygalacturonic acids. Based on their mode of action, pectinases are classified as polygalacturonases that hydrolyze unesterified polygalacturonic acid substances, pectin esterases, pectin lyases that de-esterify pectin into pectate and methanol, and pectate lyases or polymethylgalacturonases that catalyze β–elimination, forming galacturonides. Polygalacturonases, pectin lyases and pectate lyases have been found to show the highest activity among the pectinases. A homology at the sequence level between these pectinolytic enzymes has also been observed. The main natural sources of pectinases are bacteria, fungi, and plants.[2-6]

The study of pectinases for their sustainability and maximum activity is a major area of research around the globe. Pectinases have various applications under varied physiological conditions and thus have wide applications. This is a reason for application studies with pectinases are ongoing in global research fields. Pectinases have been used in several conventional industrial processes, such as textile, plant fiber processing, tea, coffee, and oil extraction and treatment of industrial wastewater containing pectinaceous material.[3, 7-10] The major application of pectinases is in food industries for the production and clarification of wines and juices.[5, 6] Naturally, fruits have a high concentration of pectin, which leads to colloid formation in the juice, creating problems in the processing of clear fruit juices. Cloudiness appearance in fruit juices also leads to a problem in the market. The traditional processes of extracting fruit juice are also not attractive and consume huge amounts of energy. Due to this reason, pectinase has a great role in the production and extraction of fruit juices.[8, 11]

Fungi and yeast contribute about 50% of the enzymes used, 35% from bacteria and the remaining 15% are either of plant or animal origin.[4]

Thus, the present study deals with the isolation of pectinase-producing bacteria with optimization of production conditions. As the acidic pectinases have application in food industry characterization and partial purification of pectinases was carried out.

MATERIALS AND METHODS

Isolation, Purification, Preservation of Pectinase-producing Bacteria

Ten soil samples were collected in sterile test tubes from various places of vegetable and fruit dumping areas (specially collected soil from citrus fruit dumping area) from Aurangabad, Maharashtra, India. For isolation of bacteria, the soil sample was inoculated in pectin broth (1% yeast extract, 0.5% sodium chloride, 1% pectin) and incubated at 37°C for 48 hours. The grown culture was streak on pectin agar and incubated at 37°C for 48 hours. By streak plate method, bacterial cultures were purified.[9] For preservation, the bacterial cultures were stored on a pectin agar slant at 4°C.

Screening of Pectinase-Producing Bacteria

Primary screening

A single colony of all 13 bacterial samples was inoculated in freshly prepared pectin broth and incubated for 48 hours at 37°C. Prepared pectin agar plates were spot inoculated by bacterial sample and incubated for 48 hours at 37°C. Flooding plates measured zone of clearance with Gram's iodine.[9, 12, 13]

Secondary screening

Secondary screening is quantitative screening. In this screening the bacteria were selected on the basis of enzyme activity. Single colonies of all bacterial cultures having a maximum zone of clearance were inoculated in freshly prepared pectin broth and incubated for 48 hours at 37°C. About 1-mL of broth was taken and centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was treated as crude enzyme and an enzyme assay was performed.[9]

Pectinase enzyme assay:

The pectinase enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by the di-nitro-salicylic acid reagent (DNSA) method. For enzyme assay, 1-mL of supernatant, 1-mL of 1% pectin substrate and 1-mL of phosphate buffer was incubated at 37°C for 60 minutes. The product formed was measured by adding 1-mL of DNSA and kept in a boiling water bath for 10min. After cooling 8 mL of distilled water was added and absorbance was measured at 540nm. The experiment was performed in triplicate. One enzyme unit is µmole of reducing sugar produced per min of time.[4, 14-17]

Evaluation of bacterial cultures for utilization of agro waste (citrus fruit peel powder) as substrate for pectinase production

Agro waste (citrus fruit peel powder) as a substrate for pectinase production was evaluated.[18] For analysis, three types of broth media consisting of lab pectin, sweet lemon peel powder, and lemon peel powder were prepared. In each broth, inoculated single colonies of S5.1, S5.2, S4.1, S9 were incubated at 37°C for 48 hours. About 1-mL of culture was centrifuged at 10,000 rpm for 10 minutes. The supernatant was treated as a crude enzyme source. An enzyme assay was performed for each substrate with each selected bacterial culture.

Optimization for production condition:

To reduce the production cost of enzyme, it is essential to optimize production conditions in which bacteria can secret maximum enzymes.[6] Thus, to optimize the production conditions modified method.[6,19] was adopted for achieving maximum enzyme production from the bacteria, which holds significant importance for various industrial applications. A comprehensive investigation was conducted across various parameters.

Initially, different incubation periods spanning from 24, 48, 72, 98 and 120 were explored. The influence of pH on enzyme production was evaluated by preparing pectin broths with pH levels ranging from 5, 6, 7 and 8. Optimum incubation temperature was evaluated by incubating inoculum at temperatures 4, 20, 30, 37, and 50°C for 48 hours. The impact of substrate concentration on enzyme production was assessed by preparing pectin broths with varying substrate concentrations ranging from 0.5, 1, 1.5 and 2%.

Production of pectinase

A methodical approach was followed for production of pectinase. To pectin broth single colony of S4.1 was inoculated and underwent a precisely timed incubation period of 24 hours at 37°C. The active culture was then transferred to 100ml of production media (1% yeast extract, 0.5% sodium chloride and 1% pectin). The flasks were placed in a shaking incubator and maintained at a temperature of 37°C for duration of 24 hours. Following the completion of the incubation period, the broths in the flasks were subjected to centrifugation at 10,000 rpm for 10 minutes. The resulting supernatant was collected into sterile buffer bottles under aseptic conditions, representing the crude enzyme. This crude enzyme was then stored at 4°C for subsequent characterization of pectinase and partial purification processes.

Characterization of pectinase

In order to determine the conditions under which the pectinase exhibits the highest enzyme activity, various parameters (pH, temperature, substrate concentration and effect of metal ions) were systematically examined by modified methods.[4, 6] pH affects the ionization of amino acids at the active center of the enzyme.[2] Thus, it is necessary to find out the impact of pH on enzyme activity. The phosphate buffer of pH5, pH6, pH7, and pH8 was utilized for analysis of the effect of pH on pectinase activity.

The influence of temperature on enzyme activity was examined using incubation temperatures 4, 20, 30, 37, and 50°C. Effects of metal ions, such as Ca^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, K^{+} were studied by measuring the steady-state velocity in the reaction solutions containing 1-mM of the metal ions. In this investigation, the impact of various metal ions on enzyme activity was studied by evaluating enzyme activity in the presence of metal ions. The effect of substrate concentration on enzyme activity was investigated using substrate concentrations as 0.5, 1, 1.5, and 2%.

Determination of Km and Vmax

The effect of substrate concentration was used to calculate Km and Vmax of the enzyme. The graph was plotted with the reciprocal of the enzyme activity (1/V) on the Y-axis and the reciprocal of the substrate concentration (1/[S]) on the X-axis to define the Km and Vmax values according to the Lineweaver Burk plot.[14]

Partial purification of pectinase by solvent extraction (acetone) method

The solvent extraction method using acetone as solvent was explored

for partial purification of pectinase. Partial purification was carried out by adding chilled acetone at 60, 70, 80, 90 and 100% saturation. Chilled acetone was added slowly with gentle stirring at 4℃ to the crude enzyme extract. The treated crude enzyme solution was allowed to stand for 20 minutes in the refrigerator and then centrifuged at 10,000 rpm for 15 minutes at 4℃. The pellet was collected and dissolved in phosphate buffer.[4, 6, 12]

The enzyme activity for each saturation was found by performing an enzyme assay. The specific enzyme activity and purification fold was calculated.

Specific enzyme activity = Enzyme unit/concentration of protein in mg

Purification fold = Initial specific enzyme activity / Final specific enzyme activity

Clarification of Juice using pectinase

Sweet lemon juice clarification was evaluated for its pH and clarity. The prepared juice was transferred into a conical flask and incubated in a water bath at 85°C for 5 minutes. Four different enzymatic treatments were used: 20 mL juice without enzyme treatment as control, 20 mL juice was added with 1, 2 and 3 mL of crude pectinase. The pectinase-treated juice was incubated at 37°C for 1-hour. To inactivate enzymatic reaction, it was heated at 90°C for 5 minutes. The treated juice was analyzed for change in pH. The clarity of the juice obtained was determined by measuring the absorbance at a wavelength of 660 nm using a spectrophotometer and distilled water was used as the reference.[6, 12]

Table 1: Zone of clearance

Samples	*Zone of clearance (in mm)*
S1	10
S2.1	10.5
S2.2	6.5
S3.1	6
S3.2	8.3
S4.1	9.5
S4.2	3
S5.1	9
S5.2	17.6
S6	4
S7	26
S9	9.75
S10	10

Table 2 : Secondary screening Enzyme Activity

Samples	*Enzyme activity (U)*
S4.1	285.87 ± 0.063
S5.1	417.05 ± 0.15
S5.2	298.75 ± 0.058
S7	138.3 ± 0.07
S9	141.7 ± 0.067

Table 3: Utilization of agro-waste

Substrate	*S4.1*	*S5.1*	*S5.2*
Pectin	71.9 ± 0.063	44.25 ± 0.050	38.7 ± 0.049
Sweet lemon	69.15 ± 0.056	58.05 ± 0.108	44.22 ± 0.103
Lemon	47 ± 0.031	44.18 ± 0.017	33.15 ± 0.068

RESULTS AND DISCUSSION

Isolation, Purification, Preservation of Pectinase-producing Bacteria

From the ten soil samples collected, 13 bacterial cultures were isolated and purified by the streak plate technique. All the 13 had ability to synthesize extracellular pectinase.

Screening of Pectinase-Producing Bacteria

Primary screening

From Fig. 1 and Table 1 the sample maximum zone of clearance was of S7, S5.2, S1, S10, S2.1, S9 and S4.1.

Secondary screening

All 13 bacterial samples were able to synthesize pectinase. Samples S4.1, S5.1, S5.2, S7, S9 were selected for further studies due to consistent pectinase activity. Table 2 shows highest pectinase activity was of S5.1 and subsequently of S5.2, S4.1, S7 and S9.

Evaluation of bacterial cultures for utilization of agrowaste (citrus fruits peels powder) as substrate foe pectinase production:

Table 3 suggests that bacterial cultures S4.1, S5.1, S5.2 and S9 were able to utilize pectin, Sweet lemon as well as lemon fruit peel powder as substrate for pectinase activity. Sweet lemon peel powder was found to be a good substrate for pectinase production. The S4.1 was found to be most efficient for pectinase production when pectin, lemon peel powder, and sweet lemon peel powder was utilized as substrates. For maximum production of pectinase, sweet lemon peel can serve as a good agrowaste.

In this study we have optimized the production conditions for S4.1 and characterized the enzyme from S4.1.

Optimization for production condition

For cost effective production of pectinases, it is important to find out optimal production conditions. The maximum production of enzyme was found to be for 24hr incubation as the highest enzyme activity (177U) observed (Fig. 2). *Klebsiella oxytoca* af-G4, Bacillus subtilis ADI1 and *Chryseobacterium indologenes* were reported to have maximum production of the enzyme in 72 hours incubation,[14, 20, 21] which was higher than the S4.1 used for the production of enzyme. From the selected pH ranges 5, 6, 7 and 8, it was observed from Fig. 3 that pH6 was optimum for the production of pectinase. That is the acidic pH allowed maximum production of enzymes similar results with pH 5 optimum was obtained.[14] At 37℃ optimum enzyme activity of pectinase was measured, suggesting that for enzyme production, incubation temperature could be used as 37℃ (Fig. 4). Fig. 5 shows substrate pectin used in 2% of concentration found to efficient in the

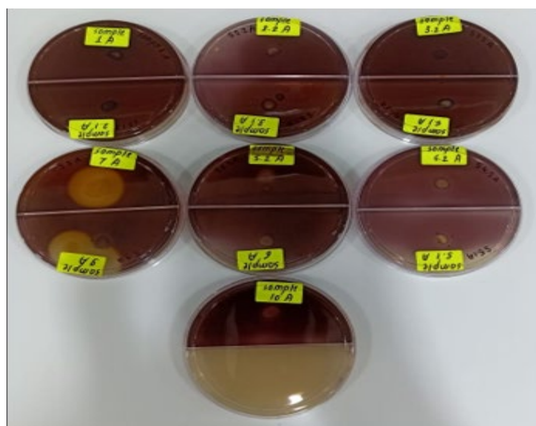
Fig. 1: Zone of clearance

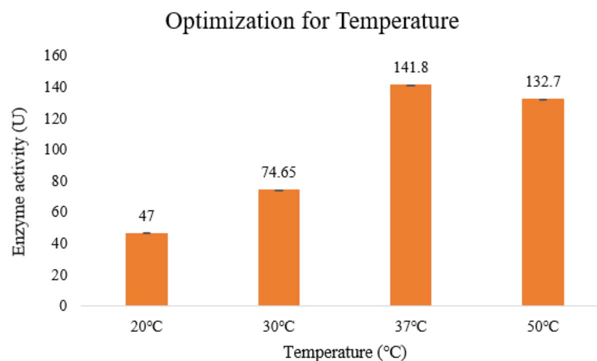

Fig. 4: Optimization for temperature on production of pectinase

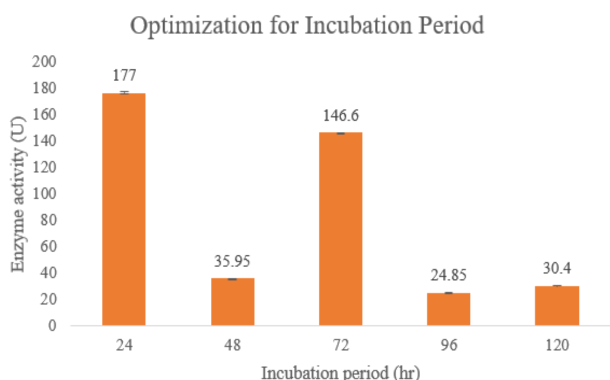

Fig. 2: Optimization for incubation period on produaction of pectinase

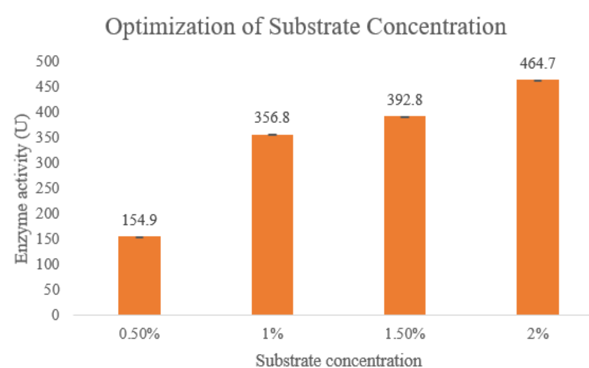

Fig. 5: Optimization for substrate concentration on pectinase production

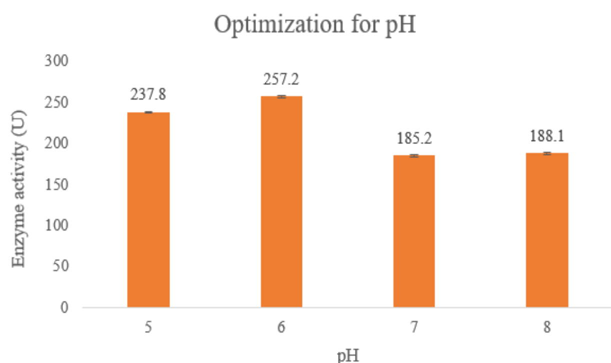

Fig. 3: Optimization for pH on production of pectinase

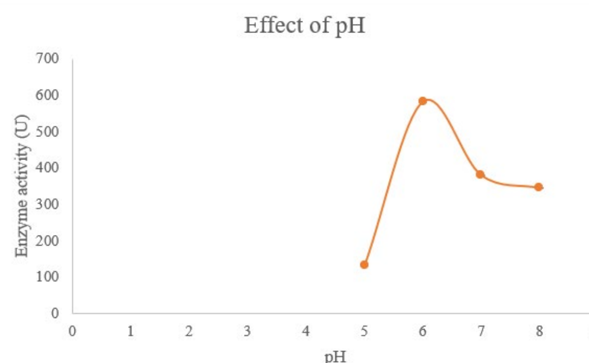

Fig. 6: Effect of pH

production of pectinase as the pectinase activity was highest than substrate concentration 0.5, 1, 1.5%.

Characterization of pectinase

Effect of pH

At pH 6 pectinase was found to have maximum enzyme activity (Fig. 6). Thus, pH 6 was the optimum pH for pectinases from isolated bacteria. Thus pectinases were acidic pectinases with similar results obtained by Alqahtani, Y.S ,2022[2] but varied with Pedrolli et al where neutral pH was observed as optimum. Most of the *Bacillus sp.* have alkaline pH as optimum.[3, 8]

Effect of temperature

From Fig. 7 from the selected incubation temperature 20, 30, 37 and 50°C, pectinase was efficiently hydrolyzing pectin at 37°C. Thus, 37°C was the optimum temperature for pectinase. The enzyme was found to reduce its activity as the temperature was increased up to 50°C. The pectinases from Bacillus subtilis have a high-temperature optimum 50°C, which contradicts the results obtained by Alqahtani, Y.S ,2022 [2].

Effect of metal ions

The metal ions have various types of effects on enzyme activity like

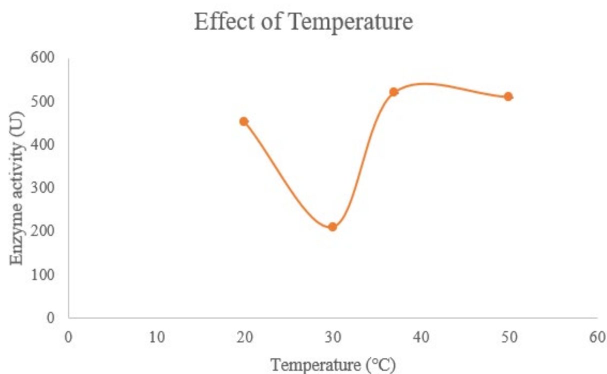

Fig. 7:Effect of temperature

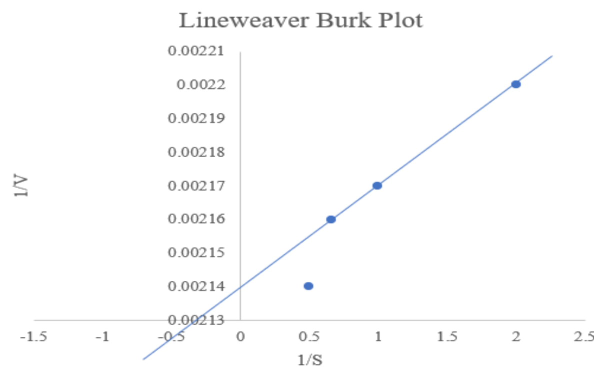

Fig. 10: Lineweaver burk plot

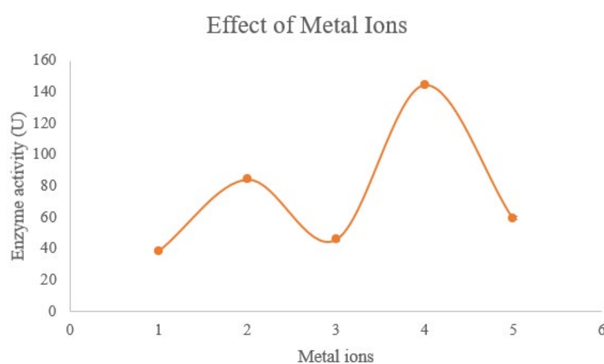

Fig. 8: Effect of metal ions

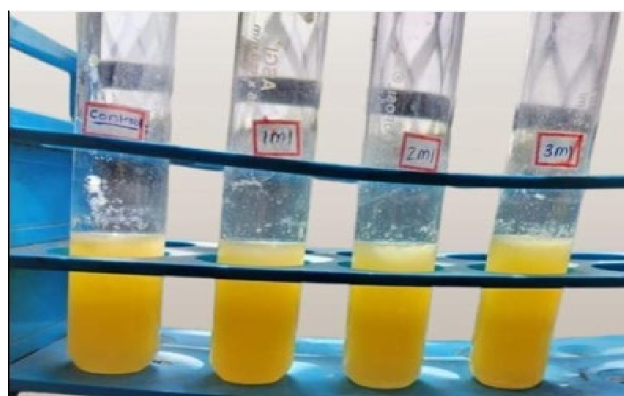

Fig. 11: Clariffication of juice using pectinase

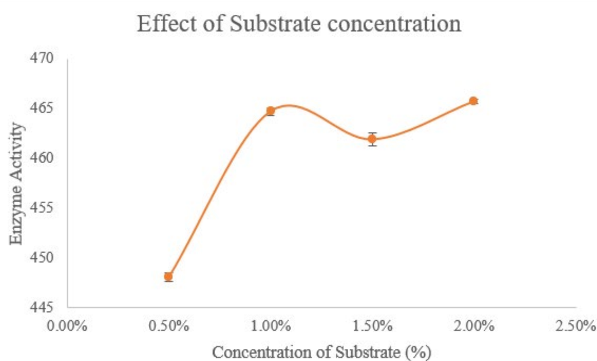

Fig. 9: Effect of Substrate concentration

inhibitors and activators. From Fig.8 Ca^{++}, Co^{++}, K^{+}, and Zn^{++} metal ions were inhibiting the activity of enzyme. Cu^{++} was enhancing the activity suggesting copper ions as inducers for the pectinases. Similar observations for *Bacillus subtilis* [2] and *Aspergillus niger* pectinases were reported for inhibition by Ca^{++}, Zn^{++}.[4]

Effect of substrate concentration

From Fig. 9, substrate pectin was used in 1% of concentration pectinase as the pectinase activity was optimum because substrate concentrations 1.5 and 2% was not observed for significant change in enzyme activity. Enzyme saturation was at 1% of pectin concentration.

Determination of Km and Vmax

The values for Km and Vmax were found to be 1.181 µmole and 471.69 µM/min, respectively with the substrate concentrations 0.5, 1, 1.5 and 2% (Fig. 10). The values Km were lower than those reported by D.N. GaneshPrasad 2022 [14] for *Klebsiella oxytoca* and high Vmax suggesting isolated pectinase have high affinity for substrate with high velocity.

Partial purification of pectinase by solvent extraction (acetone) method:

The purification process enhances the purity and concentration of the enzyme, improving its suitability for industrial use. Calculated specific enzyme activity and purification fold provide measures of purification efficiency. Partial purification of pectinases was standardized by analyzing various percent saturation by chilled acetone (60, 70, 80, 90 and 100%). The 90% of chilled acetone saturation was found to be optimum. The specific enzyme activity was enhanced after partial purification enzyme by 90% acetone and the purification fold was 35.41 (Table 4), which was high than reported by Alqahtani, Y.S, 2022 [2] for *Bacillus subtilis*.

Clarification of Juice using pectinase

From Table 5 and Fig. 11 it is observed that the turbidity of sweet lemon juice with the addition of pectinases was significantly different from sweet lemon juice without pectinase treatment. The enzyme treatment effectively clarified the juice, as evidenced by a change in

Table 4:Partial purification of pectinase

	Enzyme activity (U)	Specific enzyme activity (U/mg)	Purification fold
Crude enzyme	492.37	2735.41	-
90% saturation	1936.2	96870	35.41

Table 5: Clarification of juice using pectinase

Pectinase	pH	Turbidity (Absorbance at 660 nm)
Control	5.5	2.7
1-mL	4	2.29
2 mL	5	2.19
3 mL	5	1.89

pH and clarity compared to control. The method offers a potential application for pectinase in food processing, improving product quality.

CONCLUSION

In the present investigation, pectinase production, characterization, and evaluation were conducted to assess its applicability, particularly in the juice industry. The produced pectinase exhibited desirable characteristics such as optimal activity at acidic pH6, and temperature 37°C with Km and Vmax values as 1.181 mg/mL and 471.69 µM/min, respectively. Pectinases from S4.1 were able to reduce turbidity efficiently.

The findings suggest that the pectinase derived from the isolated bacteria S4.1 holds significant promise for juice clarification applications. Its ability to effectively clarify juice, coupled with its favorable pH and temperature requirements and high affinity for substrate, makes it a valuable asset for the juice industry.

Overall, this study highlights the potential of the pectinase enzyme as a cost-effective and environmentally friendly solution for improving the quality and clarity of fruit juices.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

SOURCE OF FUNDING

None declared

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HOW TO CITE THIS ARTICLE: Chaudhari A, Dahale I, Pungle R, Jadhav M. Optimization of Culture Conditions and Characterization of Pectinase from Isolated Bacteria and Application in Juice Clarification. *J Adv Sci Res*. 2024;15(6): 36-41 **DOI:** 10.55218/JASR.2024150606