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ORIGINAL ARTICLE **OPEN ACCESS**

Purification and characterization of pectinase from *Bacillus* sp. and its application in fruit juice clarification

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ABSTRACT
This study aimed to novel bacterium capable of producing pectinase and optimize various parameters to maximize enzyme production. Using sub merge fermentation, the production of an industrially important pectinase from a bacterial strain was attempted, which was further subjected to purification and characterization. The enzyme was purified by three steps, specifically ammonium sulfate precipitation, ion-exchange chromatography followed by gel filtration chromatography using Sephadex G-75 column with 60.86% yield. The study revealed that maximum pectinase activity occurred at pH 6 and 50°C, with enhanced activity in the presence of Fe^{+2} ions. Given the stability of enzyme from Bacillus sp. in various surfactants and inhibitors, it holds promise for applications in diverse industries requiring pectin degradation. The study also determined the enzyme's K_m and V_{max} values as 166.53 M and 769.24 µM/ml, respectively.
***Keywords**: Pectinase, purification, juice clarification, enzyme*

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INTRODUCTION

Pectic compounds which are abundantly found in almost all the crop-based food items like fruits and vegetable, tea and coffee etc. Pectinase is one of the important enzymes to hydrolyze pectic compounds linked by α-1,4-glycosidic bonds esterified with methyl groups. Although pectinases are ubiquitous in nature but for industrial application pectinases from bacterial origin is more oftenly exploited. Pectinases have been the central issue of research due to its rigorous use in various industry. In food and beverages industry it is widely used in extraction, filtration and clarification of fruit juices to enhance the organoleptic properties of wine and to promote fermentation speed of tea, coffee and vegetable oil [6]. Apart from the food industry, it has also been used in feed industry, as an additive in animal fed to ameliorate the digestibility and nutritional profile. Textile industry which is one of the promising fields and plays an important role in contributing country's economic growth, involves several steps to produce the finished product from raw cotton, which are based on chemical processing and is criticized for the hazards it cost to environment. But the awareness towards eco-friendly approach make its way to use the enzymatic methods for cotton scouring [7]. Moreover the enzymatic degraded product from pectinases has its application for the therapeutic purposes as well. Enzyme degradation of substrate depends upon the various factors like type of micro-organism, fermentation physiological condition such as pH, temperature, incubation time, carbon and nitrogen sources etc. Meanwhile All these parameters have already been optimized for the desired isolate for maximum enzyme production moreover the fermentation broth so achieved under this optimized condition is processed for harvesting the crude enzyme and further processed for the partial purification using ammonium sulfate precipitation method followed by purification using ion-exchange and gel permeation chromatography. Purified enzyme so obtained was characterized to study the effect of pH, temp, metal ion & additives and K_m and V_{max} was identified. In addition to that the purified enzyme so obtained was studied for its juice clarification application also at lab level. Hence, keeping all the industrial application in mind, pectinase is found to be one of the most promising enzymes, and in the current study we have worked on the purification, characterization, and application of purified enzyme.

MATERIAL AND METHODS

Purification of pectinase:

Partial purification of enzyme was completed by ammonium sulphate precipitation followed by dialysis.

100 ml of cell-free extract was centrifuged at 10000 rpm for 15 min. The supernatant was collected and saturated up to 0 -20%, 20-40%, 40-60%, 60-80%, and 80-100% with ammonium sulphate. Next, the content was centrifuged at 12000 rpm for 15 min and the pellet was collected for further analysis. The pellets were suspended in a minimal amount of 20 mM tris buffer, pH 7.0 and dialyzed against the same buffer overnight at 4 ℃. The buffer was continuously stirred using a magnetic stirrer throughout the process. The buffer was changed three times during the process to obtain partial purification [12]. The total protein amount in enzyme samples was detected by the method of Lowry, using bovine serum albumin (BSA) as standard [11]. The dialyzate was loaded on to a DEAE-sephadex ion exchange column (10 x 2.8 cm), which was equilibrated and washed with 20 mM tris buffer, pH 7.5. The enzyme was eluted with a linear gradient of NaCl (0.1 - 1.5 M) at the flow rate of 0.5 ml/min. The enzyme concentrate was using subsequently applied to a Sephadex G-75 column (22 cm x 1.1 cm) equilibrated with 20 mM tris buffer (pH 7.5). The flow rate was consistently set at 10 ml hr^{-1}, and 2 ml fractions were collected, assayed and preserved at 4°C.

Determination of molecular weight:

The molecular weight of partial purified and purified enzymes was determined by SDS-PAGE. The SDS-PAGE was performed according to Laemmli method [5] with some modification by using 4% and 12% stacking and separating gels, respectively. The protein samples (20µl) were mixed with sample buffer and then heated for 3 min before loading to the gel. The electrophoresis was carried out in running buffer (0.25 M Tris, 0.192 M glycine, 0.1% SDS, pH 8.3). After electrophoresis, the gel was stained by a solution of 0.15% Coomassie Brilliant Blue (CBB) R-250 in 50% ethanol and 10% glacial acetic acid with constant shaking for 8h to visualize protein band. After staining, the gel was destained with destaining solution containing 40% ethanol and 10% glacial acetic acid till the gel background was clear [6].

Characterization of purified enzymes:

The purified enzymes were analysed to define their characterization by studying pH, temperature, metal ions, surfactant, and enzyme kinetics.

Effect of pH on pectinase:

The optimum pH was determined by measuring the stable velocity in the buffered reaction solution in the pH range of 2.0 – 10.0. The different buffers used were citrate phosphate buffer (2.0 - 4.0), sodium acetate buffer (5.0 -7.0), glycine–NaOH (8.0 - 10.0), all at 100 mM concentration. The pH stability of the purified enzymes was studied by exposing the enzyme to a particular pH for 24 h at 4°C and consequently plotting the percent enzyme activity against the pH at which the enzyme has been exposed [13].

Effect of temperature on pectinase:

The enzyme was in a 100 mM sodium acetate buffer (pH 6) at different temperatures: 30, 40, 50, 60, 70, 80 and 90°C for 30 min (Sudeep et al., 2020). After incubation, the residual activity of the enzyme was measured by the dinitro salicylic acid (DNS) method.

Effect of metal ions on pectinase:

The influence of chemical compounds on the activity of enzyme was evaluated by incubation of the purified enzyme with 20 mM metal ions and reagents including Co^{+2}, Mg^{+2}, K^{+}, Na^{+}, Ca^{+2} and Zn^{+2} [14]. The enzyme activity was determined using DNSA method.

Effect of Additives on pectinase:

The stability of enzymes in the presence of certain surfactants (i.e. Tween 20, Tween 80, mercaptoethanol, EDTA and SDS) was investigated. The test tube used contained 100µL of purified enzyme and 100µL (5 mM) of surfactant the tube was preheated for 30 min at 50°C (Amid et al. 2014). Then, the enzyme activity was determined by performing assay as described above, and the results were compared to those obtained in control tubes incubated without surfactant.

Effect of substrate on the pectinase:

The K_m and V_{max} values were determined by measuring the reaction velocity at different concentrations of the substrate. A first stock solution of pectin which was 5mg/ml concentration was prepared with citrate buffer (pH-6) [8]. Then the stock solution was diluted by an appropriate volume of buffer to make the final concentration and perform the enzyme assay.

Application of pectinase in clarification of fruit juice:

To initiate the fruit processing, a careful collection procedure was followed. Firstly, fresh fruits at their full ripeness, free from any visible blemishes, were procured from a local market in Gujarat. The fruits were carefully washed and rinsed under running water. This process ensured the removal of any external contaminants or debris. Once the fruits were properly cleaned, they were then prepared for further processing. Using a laboratory mixer, the fruits were carefully ground for a duration of 2-3 minutes. This grinding process aimed to achieve a homogenous fruit juice, ensuring that the resulting extract would possess a uniform composition. The extracted fruit juice underwent a pre-treatment process involving

pasteurization at a temperature of 85°C for a duration of 3 minutes. This heat treatment was applied to deactivate the natural fruit enzymes present in the pulps. Subsequently, the pasteurized fruit pulps were cooled down to a room temperature. To optimize the enzymatic treatment process, a series of experiments were conducted using 25ml of juice. Additionally, varying incubation times of 30-180 min were tested, while maintaining a constant temperature of 50°C throughout the process [1]. Upon completion of the enzymatic treatment, the enzyme present in the sample was deactivated by subjecting the juice to a heating step at 90°C for 5 minutes using a water bath. This optimization process aimed to identify the most effective enzyme concentration and incubation time for achieving the desired results in the enzymatic treatment of the juice. The clarity of the juice was assessed spectrophotometrically by quantifying the percentage transmittance at a specific wavelength of 660 nm, following the method [14]. Distilled water was employed as a reference blank during the measurements [15]. To determine the juice clarity, the following formula was used:

$$\textbf{Clarity \%} = (\boldsymbol{Untreated – treated\ untreated / Untreated}) * \mathbf{100}$$

RESULT:

Purification of pectinase:

Various techniques were utilized to purify crude enzymes, and these methods play a crucial role in processing enzyme preparations for research and industrial applications. One common purification method involves ammonium sulfate precipitation, where proteins are selectively precipitated based on their solubility at different salt concentrations. Another technique, dialysis, is employed to remove small molecules, ions, and salts from the enzyme solution, further refining its purity. In addition, DEAE-cellulose ion-exchange chromatography is utilized, undertaking advantage of the enzyme's net charge to separate it from other proteins with different charges. Furthermore, Sephadex G-100 is depend on differences in molecular size to separate the enzyme from smaller impurities. Through these purification processes, undesired proteins and contaminants were effectively removed, leading to highly purified enzyme preparations. Detailed results of the enzyme purification can be found in Table 1, providing valuable insights into the efficiency of the purification methods.

Following four purification steps, the pectinase was purified to achieve a 4.13 -fold increase in purity, resulting in a 60.86% yield and a specific activity of 107.56 U/mg protein. It is noteworthy that prior research efforts have successfully purified various pectinase, achieving substantially higher fold increases and enhanced yields compared to the reported results. In 2017, Patidar et al. used DEAE-cellulose column for chromatography to purify pectinase enzyme from A. niger AN07 cultured on dried papaya peels. They achieved a 24.8-fold purification and a 52.6% recovery of the enzyme [9].

Table -1 Purification of pectinase from *Bacillus* sp.

Purification steps	Total activity (U)	Protein concentration (mg)	Specific activity (U/mg)	Fold	Yield (%)
Crude	2.226	0.102	21.865	1.000	100.000
Ammonium sulphate	2.014	0.088	22.888	1.047	90.460
Ion Exchange Chromatography	1.629	0.037	44.027	2.014	73.167
Gel permeation chromatography	1.355	0.015	90.333	4.131	60.861

Thus, compared to previous studies, our results confirm high total enzyme activity and specific activity of purified pectinase excreted from bacterial strain. The purified pectinase was analysed by SDS-PAGE under reducing conditions that showed a single band on SDS-PAGE. Thus, the size of pectinase determined by SDS-PAGE was found to be about 48 kDa. Similarly, the size of previously reported purified pectinase was falls within the range of 43-68 kDa when isolated from different sources. Pectinase production by ***B. amyloliquefaciens*** **TKU050** was found 58 kDa from the wheat bran culture medium [6]. In 2019, Koshy and De reported ***B. tequilensis*** SALBT had 35 kDa [1], and B. licheniformis had 38 kDa [16].

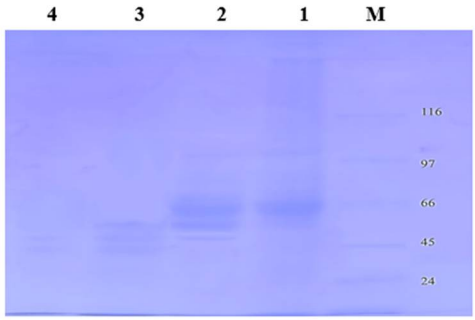


Figure-1: SDS-PAGE profile of *Bacillus spp.* pectinase. Lane M, molecular weight marker; lane (1) crude pectinase; lane (2) ammonium sulphate concentrated and dialysed protein sample; lane (3) partially purified protein sample after DEAE-cellulose ion exchange chromatography; lane (4) purified pectinase after Gel permeation chromatography.

Effect of pH:

In experimental, we accurately explored the impact of pH variation on pectinase activity. This was completed by reaction mixture consisting of citrus pectin and pectinase to a range of pH values covering from 2.0 to 10.0 The purpose of pH study was to determine the enzyme activities under different acidic and alkaline conditions, on its optimal working environment. Pectinase production by isolate was observed to rise with the pH increase up to 5.0. However, beyond at pH 7.0, there was a consistent decrease in enzyme activity. This trend indicated that the prime pH for optimal enzyme production was 6.0. Specifically, at this optimum pH, pectinase production reached 2.275 U. Conversely, at pH 10.0, the production values were significantly lower [1-3, 15, 17, 11].

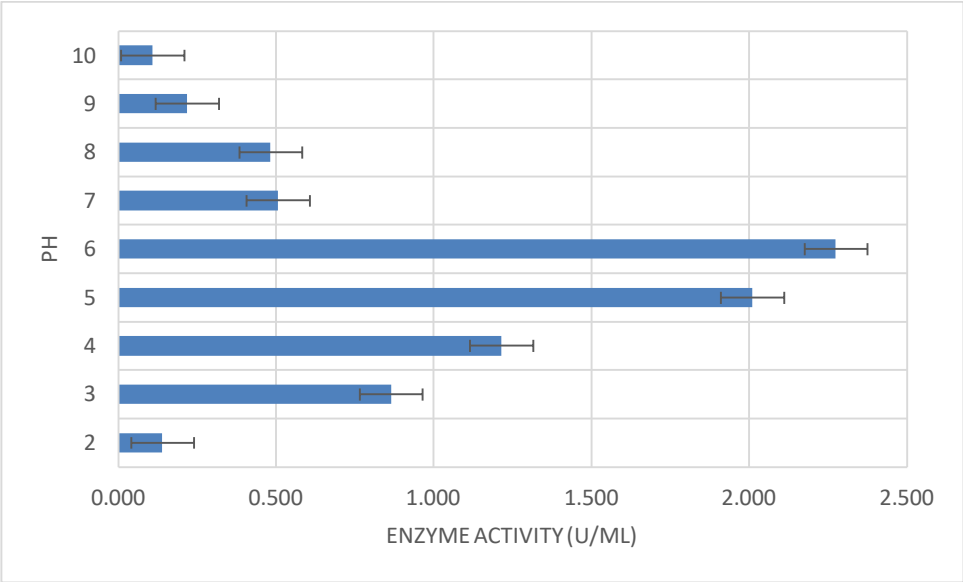


Figure 2: Effect of pH on activity of pectinase.

Effect of Temperature:

The effect of temperature on production of both the enzymes was tested in the range of 30–100 °C. The highest production of pectinase (3.12 U) but declined at higher temperatures (Fig. 3). Hence, the optimum temperature for production of the enzymes was taken as 50 °C. The lower enzyme production at below and above the optimal temperatures could be due partly to reduced growth of the bacterial strain and a decrease in the moisture content of the substrate, respectively.

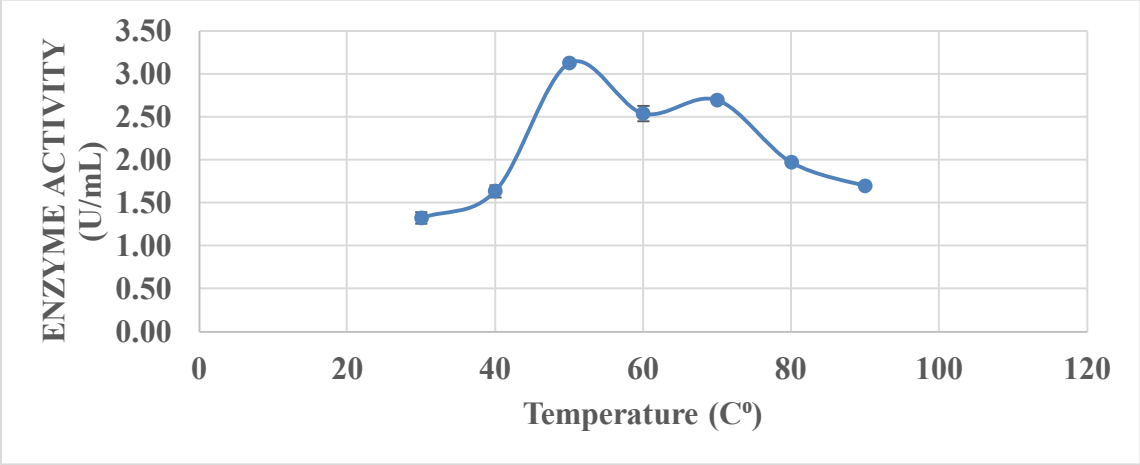


Figure 3: Effect of temperature on activity of pectinase

In consonance with the present observation, the optimal temperature for pectinolytic enzyme production by ***Bacillus* sp.** has been reported in the range of 30–50 °C in most cases. For pectinase production, the optimum temperature was reported as 40 °C for ***B. clausii*** [2-5]. In contrast, the optimal temperature for Pectinase production by ***B. borstelensis*** was recorded at 50°C [6]. Pectinase produced by *Fusarium* MTCC2079 in solid-state fermentation was generated at a temperature of 50°C [18]. The impact of cultivation temperature on enzyme activity was evident, with 50°C identified as the optimal temperature for pectinase activity. However, substantial enzyme activity, ranging from 90% to 95% relative activity, was noted at both 45°C and 55°C. Beyond the optimum temperature, there was a noticeable decrease in enzyme activity [3].

Effect of metals:

Certain metal ions can act as cofactors or coenzymes, essential for the catalytic activity of pectinase. In such cases, the presence of these ions may enhance the enzyme's activity. Some metal ions can stabilize the enzyme structure, leading to increased stability and activity. Optimal concentrations of certain ions may enhance activity, but excessive concentrations could lead to inhibition. Addition of metal salts viz. Co^{+2}, Mg^{+2}, Na^{+}, Ca^{+2}, Zn^{+2}, Fe^{+2} and K^{+} on the production of pectinase revealed a slightly higher production in the presence of Fe^{+2} (2.43U) as compared to control (2.25U), which was devoid of any metal salt. In the presence of other metal salts, enzyme titer was almost the same as in the control [12-18].

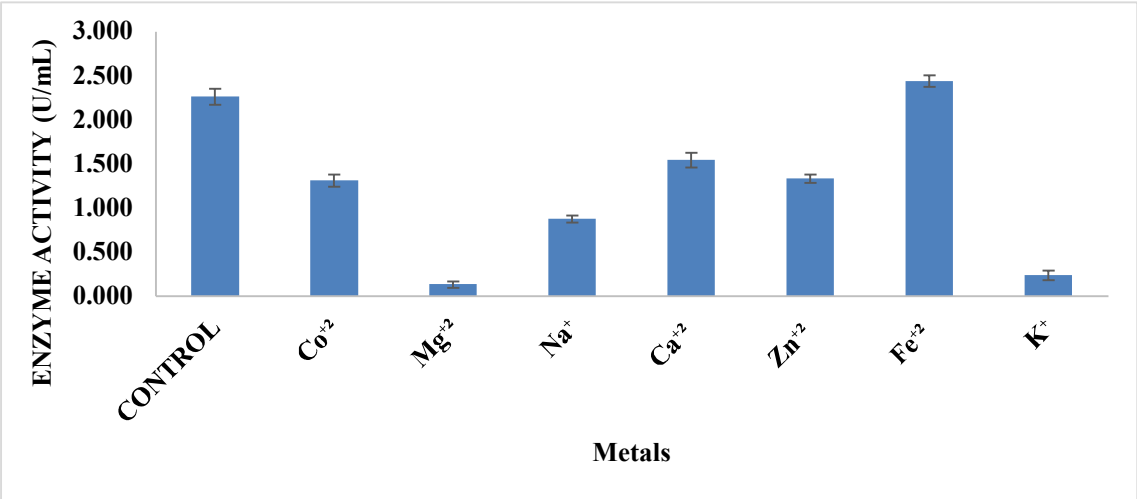


Figure 4: Effects of metal ions on pectinase

Effect of Surfactants and inhibitors on enzyme:

Among the test surfactant and inhibitors, tween-20 and tween -80 were not found to enhance the pectinase activity. It was observed that the presence of mercaptoethanol, SDS and EDTA in the enzyme-substrate decreased pectinase activity significantly [18]. Likewise, the pectinase derived from Aspergillus repens was significantly inhibited by EDTA , as documented in a study by [2].

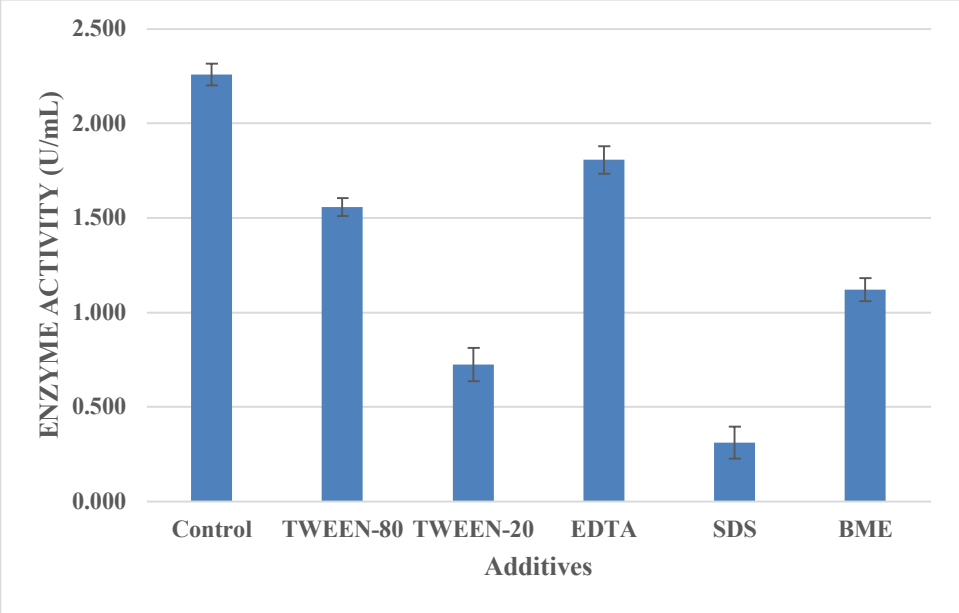
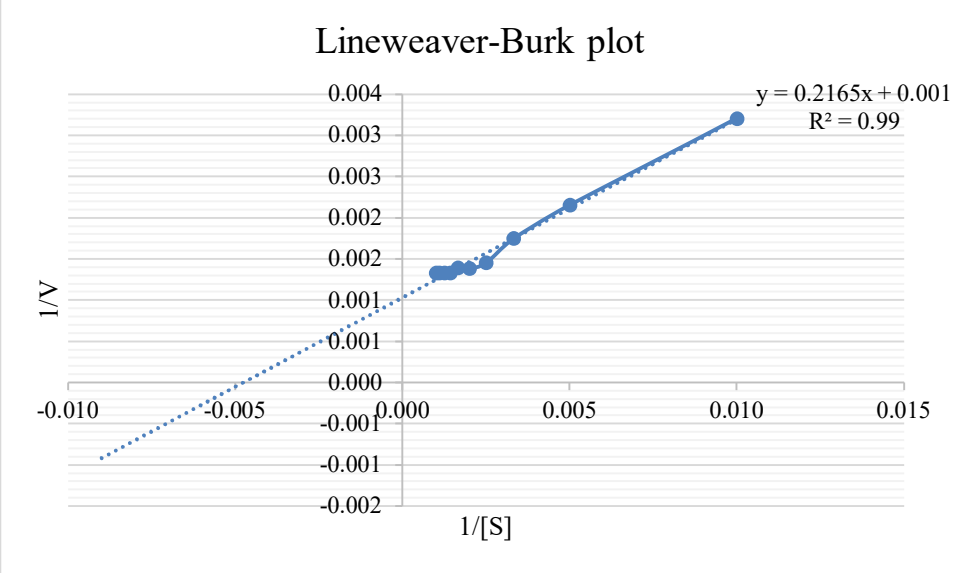


Figure 5: Effects of additives on pectinase

Michaelis-Menten Constant (K_m) and (Vmax) Values:



(Fig-6 Lineweaver Burk plot for pectinase)

The enzyme Michaelis-Menten Constant (K_m) and maximum reaction rate (V_{max}) were determined by measuring the reaction velocity at various concentrations of the substrate, Citrus Pectin. Non-regression analysis was employed to analyze the relationship between reaction velocity and substrate concentration. The obtained regression coefficient (R^2) was 0.999, indicating a strong positive correlation between Citrus Pectin concentrations and enzyme activity readings, as illustrated in Figure 6. Through this non-regression analysis, the values for K_m and V_{max} were established as 166.53M and 769.24 µM/ml, respectively [11].

Application of pectinase in the food industry for clarification of fruit juice:

Preliminary experiments were performed to determine the optimum condition like enzyme concentration and incubation time for maximum yield and clarity of fruit juice. For the optimization of the enzyme treatment, 25 gm pulp of grapes and mango were weighed, treated with different concentrations, and were incubated at a temperature of 50°C for different incubation times.

Table-2 Studies related to fruit juice and pulp clarification by pectinase.

Enzyme concentration ml/25gm pulp	Fruit pulp	Treated juice clarity (%)		
		6hr	4hr	2hr
1	Mango	58.5 ± 0.40	55.73 ± 0.24	54.33 ± 0.24
	Grapes	60.4 ± 0.29	57.7 ± 0.16	55.16 ± 0.12
2	Mango	60.8 ± 0.08	57.1 ± 0.08	55.9 ± 0.08
	Grapes	62.6 ± 0.12	60.7 ± 0.08	57.6 ± 0.12
3	Mango	64.3 ± 0.08	58.8 ± 0.12	56.5 ± 0.08
	Grapes	65.4 ± 0.08	64.4 ± 0.09	58.3 ± 0.12
4	Mango	64.5 ± 0.16	58.0 ± 0.04	56.2 ± 0.08
	Grapes	65.9 ± 0.04	64.7 ± 0.04	58.7 ± 0.04

Increasing enzyme concentration and Increasing time, the treated juice showed an increase in the clarity and yield. The maximum juice clarity of grapes pulp was obtained at incubation time of 6hr and pectinase enzyme concentration of 4 ml pulp in fruit [12]. The pectinase from *Bacillus subtilis* 15A B-92 demonstrated superior clarity, turbidity reduction, and viscosity reduction, making it a potential choice for industrial juice clarification. In a prior study, *Penicillium occitanis* pectinase achieved a 98% reduction in viscosity in lemon juice at various enzyme concentrations [8].

CONCLUSION

In conclusion, this study focused on the purification and characterization of pectinase from ***Bacillus* sp.**, with the aim of exploring its potential application in fruit juice clarification. The enzyme purification process involved three main steps: ammonium sulfate precipitation, ion-exchange chromatography, and gel filtration chromatography. The purified enzyme exhibited a 60.86 % yield and a specific activity of 107.56 U/mg protein, indicating a successful purification process. The characterization of the enzyme revealed that it displayed maximum activity at pH 6 and 50°C, with enhanced activity in the presence of Fe+2 ions. The enzyme demonstrated stability in various surfactants and inhibitors, making it a promising candidate for applications in diverse industries requiring pectin degradation. The Km and Vmax values of the enzyme were determined as 166.53 M and 769.24 µM/ml, respectively, providing insights into the enzyme's substrate affinity and catalytic efficiency. The study further explored the potential application of the purified pectinase in fruit juice clarification. The enzyme showed effectiveness in enhancing the clarity of mango and grape juice, with optimization experiments revealing that increasing enzyme concentration and incubation time resulted in improved juice clarity. Overall, the findings of this study contribute valuable information about the purification, characterization, and potential industrial applications of pectinase from Bacillus sp. The enzyme's stability, substrate affinity, and effectiveness in juice clarification highlight its significance in various industries, including the food and beverage sector. Further research and scale-up studies can be conducted to explore the enzyme's practical applications in industrial processes.

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