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Research Article

Potential of a Bacillus aerius Pectinase in Fruit Juice Clarification Produced by Submerged Fermentation Using Agri-Residues

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ABSTRACT:

Tropical fruits like banana, papaya, guava etc. are too pulpy and highly pectinaceous that it becomes hard to express juice from them by using conventional methods. Pectinolytic enzymes play an important role in fruit processing industries by degrading fruit pulp pectin and thus increasing yield and production of juice. A pectinase has been produced from newly isolated strain Bacillus aerius under submerged fermentation in production medium supplemented with (citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4$. $7H_2O$), wheat bran (0.5%), pH 6 and at 37 ºC. After optimization of various production parameters, pectinase production was increased to nearly 3 fold. The produced pectinase is stable in neutral to alkaline pH region up to 42 ºC. The suitability of this pectinase for use in juice clarification was investigated. A pectinase dose of 500 µl enzyme with 1 g of fruit pulp exhibited optimum release of reducing sugar and decrease in viscosity at pH 7, after 24 h of treatment at 37 ºC. It was observed that viscosity decreases around 54 % was obtained as compared to control. These results indicated that a pectinase isolated from this strain and produced under given optimized conditions has good application and is effective in releasing sugars from the fruit pulp aiding in the increased clarity and expression of juice from tropical pulpy fruits.

Keywords: Pectinase, fruit juice clarification, pectic substances, Bacillus sp, tropical fruits

[I] INTRODUCTION

Expression of juice from tropical fruits like banana, apple, guava, mangoes etc. has always been a challenge to fruit processing industries because of the presence of high amount of pectic substances in the pulp [1]. It is difficult to extract the juice by pressing or using other mechanical methods as it is highly viscous and is in bound form with pulp resulting in a jellified mass. Pectic substances are the high molecular weight, negatively charged, acidic, complex glycosidic macromolecules (polysaccharides) present in the plant kingdom, as the major components of middle lamella between the cells in the form of calcium pectate [2]. Pectic substances are responsible for the consistency, turbidity and appearance of fruit juices [3]. Enzymes have already been used widely for their various applications in animal

feed [4], baking, food, beverage and pulp paper industry [5].

Pectinase enzymes break down pectic polysaccharides of plant tissues into simpler molecules like galacturonic acids and hence have been used in fruit processing industry for fruit juice clarification. In fruit juice industry, they are used to reduce viscosity which ultimately leads to formation of clear juice and increase the yield of juices by enzymatic liquefaction of pulps thus forming pulpy products by macerating the organized tissue into suspension of intact cells [6]. These enzymes degrade the long and complex molecules in the fruit pulp called pectin that occur as structural polysaccharides and responsible for turbidity in pulp. With the addition of pectinase the viscosity of the fruit juice drops, the press ability of the pulp improves, the jelly structure disintegrates and the fruit juice is easily obtained with higher yields [1] Microbial pectinase account for 25% of the global food enzymes sales [2].

Pectin, the important structural constituent of plant cell walls, is composed essentially of long chains of (1-4)-α-D-polygalacturonate, which are partially methyl esterified. Microbial pectin degradation is accomplished by methylesterases, which remove the methyl groups from pectin and the depolymerases which degrade both pectin and pectate [7]. Filamentous microorganisms are most widely used in submerged and solid-state fermentation for pectinase production because of their ability to colonize the substrate by apical growth and penetration which gives them a considerable ecological advantage over non-motile bacteria and yeast, which are less able to multiply and colonize on low moisture substrate [8].

A lot of literature has been reported for using solid state fermentation (SSF) and submerged fermentation (SmF) for pectinase production [9] but since the SSF parameters are hard to control and optimize leading to low productivity it underlines the requirement for using SmF for pectinase production. In the present investigation, we report high level production of a pectinase using agro-industrial bio-product in submerged fermentation from a newly isolated strain of *Bacillus aerius*, which has been potentially applied for the processing of banana fruit pulp for decreasing the viscosity and cloudiness of the juice.

[II] MATERIALS AND METHODS

2.1. Microorganism

Bacillus aerius was isolated from soil sample collected from dump sites of juice wastes; using 0.1% pectin agar medium at the temperature of 37 ºC. Its ability to produce pectinase was qualitatively confirmed when it formed clear zones of hydrolysis on 0.1% pectin agar plates when flooded with iodine-potassium Iodide solution (1.0 g iodine, 5.0 g potassium iodide, and 330 ml distilled water). The organism was identified as *Bacillus aerius* from the Institute of Microbial Technology (IMTECH), Chandigarh, India on the basis of its morphological, physiological and biochemical characteristics. The culture was maintained and stored at 4 ºC on nutrient agar medium.

2.2. Pectinase production under submerged fermentation

The enzyme production was studied in Erlenmeyer flask (250 ml) containing liquid media having 1% citrus pectin, 0.14% $(NH_4)_2SO_4$, 0.6% K_2HPO_4, 0.20% KH_2PO_4 and 0.01% $MgSO_4.7H_2O$, 0.5 % wheat bran (pH 6.0). The flask was inoculated with 1ml (O.D~0.5) 18 h old culture and then incubated for 24 h at 37 ºC in shaker incubator at 200 rpm. After 24 h, the biomass was separated by centrifugation at 10,000 rpm for 20 min and the supernatant was used as crude extract, and stored at 4 ºC.

2.3. Enzyme Assay

Pectinase activity was determined using citrus pectin as a substrate by measuring the release of reducing sugar during enzyme substrate reaction using method as described by [4]. The concentration of glucose released by enzyme was determined by comparing against standard curve plotted using concentrations of

galacturonic acid ranging from 50-500µg/µl.

2.4. Parametric optimization of pectinase production

- Incubation period: submerged fermentation was carried out for 20 to 40 h.
- Temperature: pectinase production at temperature ranging from 20 to 47 °C.
- pH: Production medium of pH ranging from 5-10 were used for pectinase production.
- Carbon source: Different carbon sources *viz*. Glucose, Maltose, Lactose, Dextrose, Starch, Cellulose, and Fructose were tested separately at 2 % concentration as sole carbon source for pectinase production

After optimization of these parameters, pectinase production was carried out under optimized fermentation conditions for maximum yield of the enzyme to be applied in fruit juice for clarification.

2.5. Optimization of enzyme dosage and other parameters for fruit juice clarification

The optimization of pH, enzyme dosage and treatment time for juice clarification was carried out by treating semi-solid reaction mixture (5 ml of 0.01M phosphate buffer per 1 gm of banana fruit paste) with varying dosage of 100 to 500 µl for variable time interval starting from 19 to 27 h at different pH ranges (6 to 8) at 37 °C. The viscosity was determined by directly observing the absorbance at 540 nm with the help of colorimeter and the reducing sugar was observed by Miller's method [10].

2.6. Partial Purification

After production of enzyme, it was further purified with the help of purification techniques *i.e.* ammonium sulphate precipitation and dialysis. The crude enzyme was purified from the culture supernatant fluid using ammonium sulphate in a 0.1M phosphate buffer of pH 8. For this purpose, various ammonium sulphate concentrations *i.e.* 0-20, 20-30, 30-50 and 50-70% were used for the precipitation of enzymes. The respective levels were mixed in 400 ml of crude enzyme filtrate and kept at 4 °C for one to two hours with continuous and constant stirring. The precipitates were collected and analyzed for pectinase activity. After precipitation, the ammonium sulphate present in the enzyme solution was removed by subjecting the solution to dialysis bag in a 0.01M phosphate buffer of pH 8 at 4 ºC for 24 h.

[III] RESULTS

3.1. Microbial strain and its growth conditions

The isolated bacterial strain is a Gram positive, moderate thermophile with minimum, optimum and maximum temperature for growth at 25, 37 and 42 °C, respectively. The strain is an alkalophile being capable of growing at pH values up to 10.0

3.2. Pectinase production under submerged fermentation

Effect of different incubation period on pectinase production using wheat bran as substrate under submerged fermentation condition by *Bacillus aerius*, was tested at different time intervals. The enzyme production started after 20 h and was determined after every 4 h of incubation and showed maximum production of 36 IU/ml at 24 h (**Figure 1**). The effect of temperature on pectinase production by *Bacillus aerius* was examined at various temperatures *viz*. 20 to 47°C for 24 h. The growth of strain was recorded from 27 to 47 °C and it did not grow at temperature above and below this range. The results showed maximum pectinase production (36.3 IU/ml) at 37 °C (**Figure 2**). The bacterium did not produce any pectinase when grown in medium of pH 4 and pectinase production started in a medium of pH 5 and results showed maximum production (41.60 IU/ml), at pH 8.0 in previously optimized conditions (**Figure 3**). Pectinase production after pH 9 shows decrease in enzyme activity. Different carbon sources were tested to determine the ones best suited for pectinase production. The effect of 7 different carbon sources *viz.* glucose, maltose, lactose, dextrose, starch, cellulose, and fructose at 2% concentration, introduced into the modified production medium for pectinase production by

Bacillus aerius under submerged fermentation were studied. Among these sources, fructose supplemented medium had attained the maximum production at 69 IU/ml (**Figure 4**). Further the pectinase production was carried out under optimized conditions (pH, temperature and incubation time), supplemented with fructose as carbon source that resulted in nearly threefold increase in enzyme production as compared to the production under un-optimized conditions.

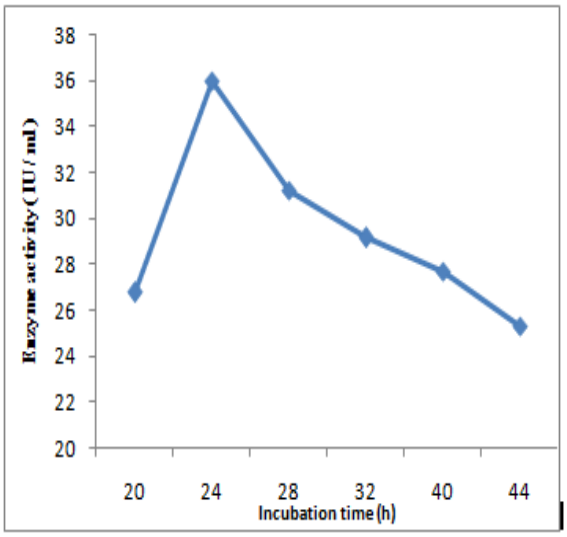

Fig: 1. Effect of incubation time on pectinase production using wheat bran as substrate under submerged fermentation condition by *Bacillus aerius*.

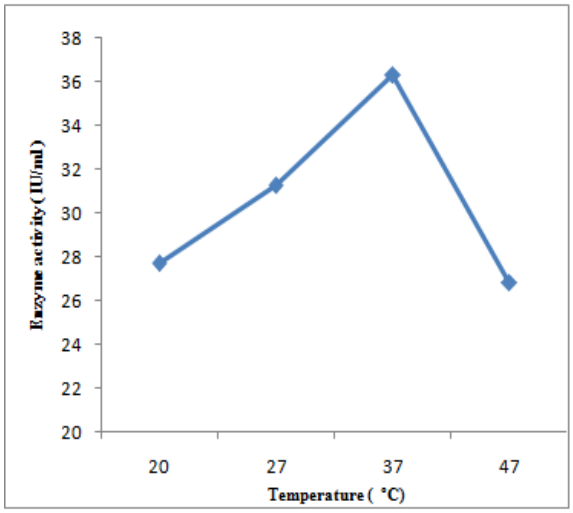

Fig: 2. Effect of temperature on pectinase production using wheat bran as substrate under submerged fermentation condition by *Bacillus aerius.*

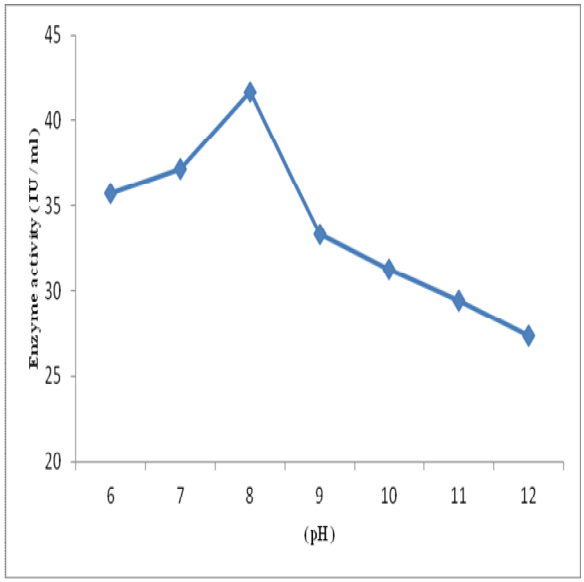

Fig: 3. Effect of pH on pectinase production using wheat bran as substrate under submerged fermentation condition by *Bacillus aerius.*

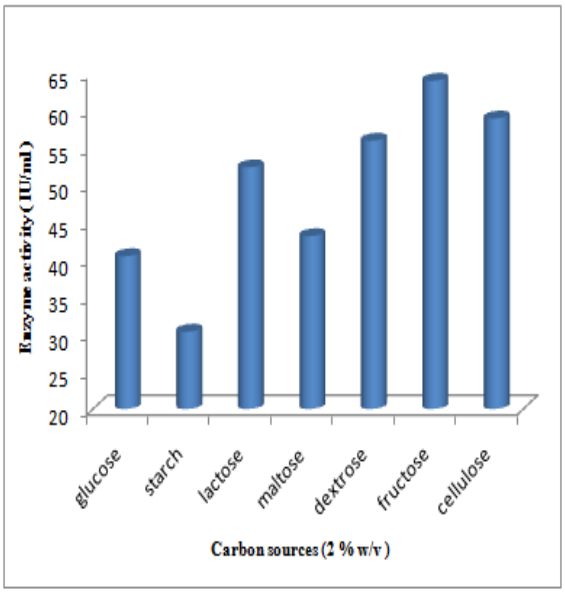

Fig: 4. Effect of various carbon sources on pectinase production using wheat bran as substrate under submerged fermentation condition by *Bacillus aerius.*

3.3. Partial purification

The precipitates from four cut- offs (0-20, 20-30, 30-50 and 50-70%) were obtained from ammonium sulphate precipitation and further dialyzed against 0.01 M phosphate buffer (pH 8).Out of these four precipitates, precipitate obtained from 50-70% cut off showed specific activity of 510 IU/ml, which was 2.7 fold higher than the crude extract.

3.4. Optimization of various parameters for pectinase used in fruit juice clarification

The pectinase produced by *Bacillus aerius* reported better specific activity with optimized parameters, which promoted us to test the suitability of enzyme in fruit juice clarification. To obtain best results from enzymatic use; pH conditions, enzyme dosage and treatment time were optimized. The results showed that maximum reducing sugar was released at pH 7 as compared to pH 6 and 8 (**Table 1**)**.** It was observed that with increase in level of enzyme dosage from 100 µl to 500 µl, the release of reducing sugars was enhanced (**Table 2**). After 24 h no further increase in reducing sugar was observed, therefore, only 24 h treatment time was taken (**Table 3**). The maximum reducing sugar released was found to be 87.80 mg/ml; at pH 7 with 500 µl enzyme dosage, at 24 h of treatment time. Also, there was 54% decrease in viscosity of the banana pulp juice treated with the partially purified enzyme as compared with the control.

pH	Absorbance OD(control) (540 nm)	Absorbance OD (540 nm)	Reducing sugar (control) (mg/ml)	Reducing sugar released (mg/ml)
6	0.88	0.46	0.60	36.08
7	0.89	0.32	0.50	44.88
8	0.87	0.44	0.46	24.42

Table: 1. Effect of pH on juice clarification

Enzyme Dosage (µl)	Absorbance (Control) OD	Absorbance (with enzyme) OD	Reducing sugar released (Control) mg/ml	Reducing sugar released (mg/ml)
100	0.89	0.46	0.45	10.34
200	0.88	0.34	0.47	44.89
300	0.89	0.30	0.48	55.66
400	0.92	0.25	0.50	85.58
500	0.94	0.19	0.52	87.78

Table: 2. Effect of enzyme dosage on juice clarification

Incubation time (h)	Absorbance (Control) OD	Absorbance (with enzyme) OD	Reducing sugar released (Control) mg/ml	Reducing sugar released (mg/ml)
15	0.97	0.52	0.48	54.6
18	0.93	0.47	0.49	65.3
21	0.95	0.34	0.51	71.2
24	0.96	0.17	0.58	87.80

Table: 3. Effect of treatment time on juice clarification

[IV] DISCUSSION

The pectinase produced by this study requires mild conditions of pH 7 and optimum temperature of 37 °C, which reduces the cost of production; as compared to the pectinase obtained from other Bacillus sp. like *Bacillus sp. RK9* at pH 10 [11]; *Bacillus sp.*NT-33 at pH 10.5 and optimum temperature 75 °C [12]; *Bacillus No. P-4-N* at pH 10-10.5 and temperature 65 °C [13] and *Bacillus sp.* DT 7 at pH 8 and optimum temperature 60 °C [14]. Submerged fermentation (SmF) and solid state fermentation (SSF) have been successfully used for pectinase production by fungi [15,16] and by bacteria [14, 17, 18, 19, 20]. Submerged fermentation is a well-developed and

technically easier system than SSF used on industrial scale to produce a large variety of microbial metabolites on large scale [21]. In one previous study, similar work was performed by [22] in which the results showed highest productive yield of juice in bananas as eight times higher than the control, but the production and isolation techniques used for enzyme were very costly and difficult to achieve. In present study, a decrease of 54% viscosity in enzyme treated banana pulp and release of 87.80 mg/ml reducing sugars after 24 h of enzyme treatment at pH 7 with 500 µl enzyme dosage is reported. The high yield of pectinase from *Bacillus aerius* along with its alkalophilic and moderate thermophilic properties suggest that this strain is better pectinase producer than the earlier reported microbes. In addition to these properties, some additional features like shorter period of incubation for pectinase production, lesser amount of cheap and easily available carbon sources like wheat bran, pectin, and fructose in the growth medium indicate the potential of this organism to be used at commercial level for maceration, liquefaction and extraction of fruit juice industries. This is the first report on the production of pectinase from *Bacillus aerius.*

[V] CONCLUSION

Improvement in process economics and realistic cost estimates are the important factors that play a major role in the commercial success of any technology. Currently, in the fruit juice industry, research is directed toward the discovery of enzymes that are more robust with respect to pH and temperature kinetics. In this present study, *Bacillus aerius*, a bacterium isolated from soil, produced good amount of pectinase activity after 24 h incubation in production medium at 37 °C, pH 8.0 and agitation speed 200 rpm. Maximum enzyme production (69 IU/ml) was found with fructose as carbon source. Pectinase production under optimized conditions has shown 2.7 fold increase in the enzyme productivity as compared to the normal conditions. Pectinase produced from *Bacillus aerius* has wide range of pH and alkaline stability, which are well suited for the fruit juice industry. Submerged fermentation process can be easily optimized and controlled, which makes the scaling up of the process very convenient leading to high production of enzyme. Weighing the potent application of pectinase in fruit processing industry, more emphasis is to be laid on screening the novel bacterial strains producing high yield of pectinase. Production cost can be further minimized by using waste products of fruit processing industries and hence finding a way to their recycling. Further studies may lead to developing novel enzyme systems which are capable of converting the biomass directly into enzymes with higher productivity.

FINANCIAL DISCLOSURE

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REFERENCES

[1] Tapre, A. R, (2014), Pectinases: Enzymes for fruit processing industry, Int Food Res J. Vol-21, issue 2, pg 447-453.

[2] Jayani, R. S, et al., (2005), Microbial Pectinolytic enzymes a review, Process Biochem. Vol-40, pg 2931-2944.

[3] Bonnin, E, et al., (2003), Mode of action of *Fusarium manilforma* endopolygalacturonase to words acetylated pectic, Carbohydrates Polymers. Vol-52, pg 381-388.

[4] Rathee, P, et al., (2014), Xylanase production under submerged fermentation by isolated *Bacillus tequilensis* strain and its potential application in animal feed, Int J Appl, Physi Bio-Chem Res. Vol-4, issue 4, pg 1-8.

[5] Kirk, O, et al., (2002), Industrial enzyme applications, Curr Opinion Biotechnol. Vol-13, pg 345–351.

[6] Tucker, G. A, Woods L. F. J, (1991), Enzymes in production of Beverages and Fruit juices, Enzymes in Food Processing, Blackie, New York. pg 201-203.

[7] Whitaker, J. R, (1990), Microbial Pectinolytic Enzymes. In: Microbial Enzymes and Biotechnology, Fogarty, W.M and C.T. Kelly (Eds.). 2nd Edn., Elsevier Science Ltd., London. pg 133-176.

[8] Smith, J. E, Aidoo, K. E, (1988), Growth of fungi on Solid Substrates, Physiology of Industrial Fungi, Blackwell, Oxford, England. pg 249-269.

[9] Solis, P. S, et al., (1996), Production of pectinases by *Aspergillus niger* in solid state fermentation at high glucose concentrations, World J Microbiol Biotechnol. Vol-12, pg 275-260.

[10] Miller, G. L, (1959), Use of dinitrosalcyclic acid reagent for the determination of reducing sugars, J Anal Chem. Vol-31, pg 426-429.

[11] Fogarty, M. V, Kelly, C. T, (1983), Pectic enzymes. In: Fogarty, M.W. (Ed.), Microbial Enzymes and Biotechnology, Applied Science Publishers, London. pg 131-182.

[12] Cao J, et al., (1992), Screening of pectinase producer from alkalophilic bacteria and study on its potential application in degumming of ramie, Enz Microbiol Technol. Vol-14, issue 12, pg 1013-1016.

[13] Horikoshi, K, (1990), Enzymes of alkalophiles. In: Fogarty, W.M., Kelly, C.T. (Eds.), Microbial Enzymes and Biotechnology, second ed. Elsevier Applied Science, London. pg 275-294.

[14] Kashyap, D. R, et al., (2000), Production purification and characterization of pectinase from a *Bacillus* sp DT7, World J Microbiol Biotechnol. Vol-16, pg 277-282.

[15] Dinu, D, et al., (2007), Enzymes with new biochemical properties in the pectinolytic complex produced by *Aspergillus niger* MIUG 16, J Biotechnol. Vol- 131, pg 128-137.

[16] Castilho, L. R, et al., (2000), Production and extraction of pectinases obtained by solid state fermentation of agroindustrial residues with *Aspergillus niger*, Bioresour Technol. Vol- 71, issue 1, pg 45-50.

[17] Jacob, N, et al., (2008), Purification and partial characterization of polygalacturonase from *Streptomyces lydicus*, Bioresour Technol. Vol-9, pg 6697-701.

[18] Klug-Santner, B. G, et al., (2006), Purification and characterization of a new bioscouring pectate lyase from *Bacillus pumilus* BK2, J Biotechnol. Vol- 121, pg 390-401.

[19] Soriano, M, et al., (2006), Pectinolytic systems of two aerobic sporogenous bacterial strains with high activity on pectin, Curr Microbiol. Vol-50, pg 114-8.

[20] Soares, M. M. C. N, et al., (2001), Pectinolytic enzyme production by *Bacillus* species and their potential application on juice extraction, J Microbiol Biotechnol. Vol-17, pg 79-82.

[21] Sunnotel, O, Nigam, P, (2002), Pectinolytic activity of bacteria isolated from soil and two fungal strain during submerged fermentation, World J Microbiol Biotechnol. Vol-18, pg 835-839.

[22] Nadaroglu, H, et al., (2010), Production of a Novel Pectin Lyase from *Bacillus pumilus* (P9), Romanian Biotechnological Letters. Vol- 15, issue 2, pg 5167-5176.