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Pectinase Producing Bacteria Isolation from Halophilic Soil, Water Samples and Partial Purification of the Enzyme

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ABSTRACT

Pectinases are protease enzymes capable of degrading pectin, which is one of the most important polysaccharide found in nature. Pectinase enzymes find uses in paper-pulp industry, textile industry, food industry and other industries where lignocellulosic material is utilized. Commercially pectinase enzymes are obtained from bacterial and fungal culture grown on decaying fruits and vegetables. In the present study, an attempt was made to isolate pectinase producing bacteria growing in halophilic conditions. Soil (sand) and water samples containing high concentrations of salts from beaches of Taiwan and Karwar, Karnataka were collected and screened for pectinase activity using modified M9 media. Among the obtained 16 bacterial strains, strain 15 showed the highest pectinase activity during screening and was identified to be a Bacillus sp. from morphological features and biochemical tests. This strain was selected and its cultural conditions were standardized for maximum production of pectinase enzyme. Maximum enzyme activity was obtained at pH 11, temperature 40°C, and incubation period of 24 hours. To enhance the productivity modification were tried out in culture media related to nitrogen source and carbon source in which the nitrogen source did not have any significance in activity of enzyme but glucose seemed to be the best carbon source for the growth of bacteria. Further the obtained enzyme was partially purified using the salting out method, dialysis followed by desalting, gel filtration chromatography and ion-exchange chromatography.

Keywords : Halophilic Bacteria, Pectinase, Ion Exchange Chromatography, Gel Filtration Chromatography

I. INTRODUCTION

Pectinases are a group of enzymes capable of degrading pectin, a unique polysaccharide present in plant cellwalls [1][2]. They are usually classified into 3 types, pectin esterases, pectin depolymerizing enzymes, and protopectinases [3]. Pectinases find their uses in many industries which use lignocellulosic material for their working. These include paper-pulp industry, textile industry and food industry, especially fruit juice industry and others[4]. Based on applications pectinases can further be classified into two types. Acidic pectinases and alkaline pectinases. Acidic pectinases are used in fruit juice industries and is required at different stages at different concentrations during many stages of processing of fruit juices in industries [3] [Kashyap 2001]. Clarifying juice to produce clear juice is possible with the help of pectinases [5]. Alkaline pectinases find their uses in softening of fibers of fiber corps and fibrous fruits and vegetables. Therefore they are useful in retting and degumming of fibrous

plant material such as jute, flax, hemp, coconut husk etc., [6][7]. Therefore it can be inferred that there is a constant need of pectin enzyme in industrial sectors. They can also be used in treating pectic waste water like the one coming out of paper industry or textile industry etc. and therefore also has a role to play in bioremediation process also. This is because pectinase is one of the key enzyme produced by biodegrading endophytic fungi used in bioremediation [8] .

Generally pectinase enzymes are obtained from bacteria or fungal cultures grown on decaying fruits and vegetables[1]. Recently Yi Guan and his team studied on seven different Bacillus sp. capable of producing pectinases [9]. Though there are a lot of commercially available pectinase enzymes and also organisms producing pectinases, it is always worth trying to have new strains of microbes looking at their environmental and industrial applications. Often many industries stress the importance of looking out for sources of their enzymes in extremophiles including halophilic bacteria. This is because usually enzymes isolated from extremophiles have very high salt tolerance and remain active even at very high pH and temperature and manufacturing process of industries often require such conditions.[10]. The present study was also taken up with this aim. We tried isolate Bacterial strain capable of having very good pectinase activity from soil and water samples collected from different beaches inside the country as well as abroad. Our study is expected to contribute to knowledge source about halophiles and especially the ones capable of producing pectinases and help us understand the pectinase enzyme character in a better way.

II. METHODS AND MATERIAL

Sample collection, Isolation, identification and screening of bacteria with pectinase activity

Soil/sand and water samples were collected from beaches of Karwar-Karnataka,India and Taiwan. A total of 13 samples were collected 6 water samples and 7 soil samples. The collected samples were suitably diluted using 1% saline solution and were prepared for inoculation. Using pour plate method the inoculum were inoculated into modified M9 media (Modified M9 media-Potassium di hydrogen phosphate-3g, di sodium hydrogen phosphate-6g, sodiumchloride-5g, Magnesiumsulphate-0.1g, Agar-20g, Peptone-1g, Pectin-1% dissolved. pH was adjusted to 8.0). The cultures were incubated at 37°C in Bacterial incubator for 18hours to 24hours as per the requirement to observe growth.

After initially growing cultures on modified M9 media, colonies with best growth were subcultured on LB agar media using streak plate method. This step allowed us in growing of the isolated microbes in higher quantity. These plates were preserved for further use in later steps of screening procedure.

For the sake of screening of bacteria with pectinase activity, streak cultures were prepared from the above stock plates on modified M9 media[11]. A total of 13 streaks **were** prepared pertaining to 13 different strains of bacteria and screening for the production of pectinase was done using Gram's iodine method. This procedure involves addition of Gram's Iodine on to the culture plate. When pectin is used as the only carbon source in the media it would hydrolyze pectin if it is pectinolytic in nature. Therefore when iodine will not be in a position to form complex with the pectin, that particular area would remain transparent. But where ever pectin is present a yellowish brown color will develop. Therefore if there is appearance of clear zone around the bacterial colonies, then it implies that particular bacteria is capable of producing pectinase enzyme[12]. Using this method a total of 16 different strains was screened.

After initial screening procedure, out of 16 strains of bacteria, 10 showed positive result to pectinase activity. Among these16, 3 strains of bacteria were selected using gel diffusion method. In this method

plates containing modified M9 media was prepared with uniform sized well using sterile technique. To this well 100µl modified M9 broth media containing the above screened three strains i.e., T1, T4, and T15 were inoculated. The plates were incubated in the incubator at 37°C for 24hours[13]

After 24 hours, Gram's iodine was added on to the plates to observe the best clear zone. Bacteria with maximum zone of clearance and also taking into account into the enzyme activity using DNS method T15 strain was taken for further studies.

Identification of the selected bacteria based on morphological, staining and biochemical studies

Morphological observation, Microscopic observations and Grams staining procedure was performed and the results are shown in figure 1,2,3,4 respectively and in table1.

Biochemical tests were performed on the selected bacteria and the results are tabulated in table 2.

Crude enzyme preparation

The selected bacterial culture was grown in broth culture 100ml in 250ml flask in shaker incubator at 37°C. After incubation in required temperature for 24 hours, the media along with the cells are centrifuged at 6000rpm for 10 minutes to get a clear suspension. This suspension was used as crude enzyme extract for further experiments (Khan I.G and Barate D. L 2016).

Pectinase enzyme assay (DNS method)

The enzyme activity was determined using a very simple principle that reducing sugars are released due to activity of pectinase enzyme and by measuring the release of reducing sugars using DNS it is possible to asses enzyme activity [14][15].
Assay was performed by taking 0.5ml of substrate (1%pectin), 1.5ml of buffer of pH 8.0 in a test tube and 0.5ml of enzyme solution(crude enzyme without dilution taken from broth) was added and mixed well. A test tube with only buffer served as blank. The reaction mixture were allowed to stand for 30 minutes at 37°C. Then 1 ml of DNS was added, mixed well and incubated on boiling water bath for 10 minutes. The absorbance was read at 540nm using spectrophotometer. The results of the enzyme assay performed in this manner on different enzyme samples are tabulated in table 3.

$$\textit{Enzyme activity} = \frac{\text{µmoles of glucose liberate X dilution factor}}{\text{volume of enzyme X incubation time}}$$

Optimizing growth conditions for the selected strain of Bacteria

After performing preliminary enzyme assay, the growth conditions of the selected bacterial strain was performed with respect to its physical parameters such as incubation time, optimum pH of the growth medium, and optimum temperature for growth. The results of these studies are represented in fig.7,8 and 9 respectively. Chemical growth requirement parameters such as Nitrogen source (Peptone, Ammonium Nitrate, Gelatin, Sodium Nitrate, Casein, Tryptone) and Carbon source (Starch, Cellulose, Glucose, Maltose, Sucrose, Lactose) were also optimized, the results of which are represented in fig.10 and 11 respectively[16].

Estimation of Protein concentrations

Protein concentrations of enzyme samples obtained at different levels of purification steps such as crude enzyme, salt precipitation, dialysis, ion-exchange chromatography and gel filtration chromatography were determined using Lowry's method with the Folin phenol reagent [17]. First a standard curve was prepared using BSA as the standard protein (fig. 6) with concentrations ranging from 0-200µg/ml. At later stages this curve was utilized for finding out the concentration of protein concentrations at different

levels of partial purification. After finding the concentration of protein in different samples of enzyme, their specific activity was estimated using the following formula.

$$\textit{Specific activity} = \frac{\text{Enzyme activity in units/ml}}{\text{Protein conc. of sample in mg/ml}}$$

Production and partial purification of the Pectinase enzyme

Enzyme production

For the production of pectinase enzyme from the selected bacterial strain, 200ml of M9 broth with pectin as carbon source and by maintaining the media with all the optimum conditions ,it was autoclaved and allowed to cool. To the broth 3-4 loopful of bacterial culture was inoculated and incubated at optimum temperature for optimum time. After the incubation time, the M9 broth was centrifuged at 6000rpm for 10minutes. The clear supernatant was collected in a beaker for the purification process and the pellet was discarded. The supernatant was used as crude enzyme extract. A small volume of crude was collected in a separate tube for further analysis.

Partial purification of enzyme

Salt precipitation (Ammonium sulphate)

Rest of the collected supernatant from the previous step was subjected to salt precipitation using Ammonium suphate salt. 66.0g of salt was added to nearly 150ml of supernatant in small quantities to give saturation of upto 70%. During the whole process the supernatant was kept in cold conditions and in continuous stirring with the help of magnetic stirrer. This was done for nearly 3-4 hours. After this step the mixture was kept at 4°C overnight to increase precipitation. On the next day salt solution with precipitate is taken into centrifuge tube and centrifuged at 8500rpm for 18 minutes to separate out protein pellet. The supernatant is discarded and the protein pellet is resuspended in 10mM tris-HCl buffer pH-8.0[18].This sample was used as salted out sample. 2ml of salted out sample was taken for estimations and the rest was utilized for further purification.

Dialysis

Dialysis was carried out using cellulose acetate membrane. Membrane activation was done by heat treatment in boiling water for 10 minutes followed by 2% sodium bicarbonate treatment which acts as detergent. This step was followed by boiling water treatment once more and after this the dialysis bags are allowed to cool off naturally and were used for dialysis.

The salt suspension was filled into the activated dialysis bag and tied at the other end strongly as to keep the bag intact. The bag is kept in a beaker containing water and left for de-osmosis overnight. The next day water was changed and fresh water was added by partially immersing the bag into the beaker and kept on a magnetic stirrer with a magnetic bead. For every 30 minutes water was changed and this was continued for 2hours. After this process, in an empty beaker the dialysis bag was punched and sample was collected. This was used as dialysis sample. A small amount is taken was taken for estimations and the rest of it is taken for further purification[19].

Ion exchange chromatography

The next step of purification was by Ion exchange chromatography using DEAE Cellulose as matrix. Dialysis sample from the previous step is used as starting material for this. The Ion exchange column is first cleaned with methanol. The column was filled with water and sonicated for 5minutes. The water was completely eluted from the column and filled with DEAE(Diethyl aminoethyl- anion exchanger) cellulose gel. It was left to settle down and 7 different elusions using Tris-HCL, NaCl and water were

prepared. After the gel settled down elution 1 was added and completely eluted out to equilibrate the column. Dialysis sample was added and the upper layer of the sample was marked on the column for reference. In the successive steps each of the remaining elution was added and collected in their respective tubes.1ml each of these elusions were collected separately and refrigerated in eppendorf tubes. Eppendorf tubes were labeled as elution 1-7[1] .

Assay to determine the highest elution value by Lowry's method.

Protein estimation was carried out for samples (crude, salt precipitation, dialysis) and all the elusions (1-7) to check the highest elution value. The amount of protein was evaluated by using Lowry's method [17].

Gel filtration chromatography

Column was filled with Sephadex G 75 gel. Elusion 4 from the previous step was taken as sample over here and was added to column. On top of this 25ml of phosphate buffer pH-7.0 was added and elusions were collected in eppendorf tubes. The amount of protein was measured using lowry's method.{ Patidar et al. (2017)}

Calculation of fold of purification and percentage yield

Enzyme activity, concentration of protein in enzyme samples at different levels of purification, number of folds of purification and percentage yield has been tabulated in table 3.

Number of folds of purification and percentage yield is calculated using the following formulas.

$$\textit{Fold of purification} = \frac{\text{Specific activity of sample}}{\text{Specific activity of crude sample}}$$

$$\textit{Percentage yield} = \frac{\text{Enzyme activity of sample}}{\text{Enzyme activity of crude sample}}$$

III. RESULTS AND DISCUSSION

A total of 13 different samples of soil and water were collected from which 16 different strains of bacteria were isolated using modified M9 media. Modified M9 media specifically has got only Pectin as the sole carbon source which made this media almost a selective media. This was done to rule out isolation of non-desirable micorbes. So what was obtained was only around 10 different strains of bacteria showing positive pectinolytic activity. screening was made more stringent and assays utilizing streak plate method and Gram's iodine assay 3 strains were selected (fig.2 & fig.3). At this stage gel diffusion method was done to be sure that the selected stains indeed have good pectinolytic activity. Best part of this method is that it gave us a rough idea about quantity of pectinase enzyme each of these strains of bacteria produced through their zone of clearance (fig.4.). In this assay it was found that strain T4 and T5 showed almost same sized zone of clearance. At this stage DNS assay for finding maximum pectinolytic activity and in this it was found that one particular strain T15 showed excellent pectinolytic activity compared to other two strains. This bacterium was selected for further studies. Bacterial morphological studies, colony characteristics and other physical parameters were studied and are shown in fig.5. and table 1. It was found that the isolated bacterium after screening was a gram positive Bacillus *sp.* The bacterium classification was confirmed after a series of biochemical tests performed and the results of these are tabulated in table 2. At later stages growth parameters for this particular bacterium was standardized and it was found that optimum temperature was 40°C, pH-11, and incubation time was 24 hours most suited for this particular bacterium (fig7,8,9). Optimization of carbon and nitrogen sources were also tried and it was found that glucose served as the best carbon

source while changes in nitrogen source did not have any significant effect on pectinase production (fig 10 and 11).

The selected strain of bacterium was used for production and pectinase enzyme and it was partially purified using different methods such as salting out followed by dialysis and desalting, ion exchange chromatography and gel filtration chromatography.

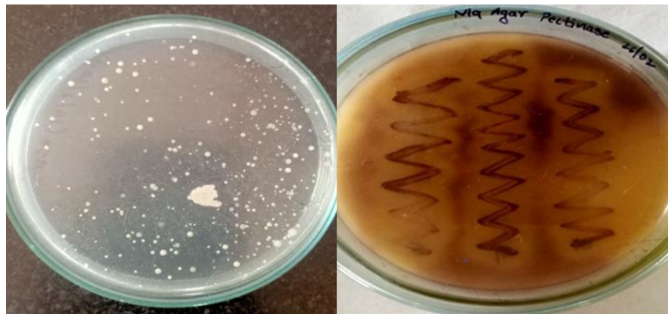

Fig. 1 Plate containing primary bacterial isolate (Representative plate) Fig. 2 Streaking plate method of screening (Representative plate)

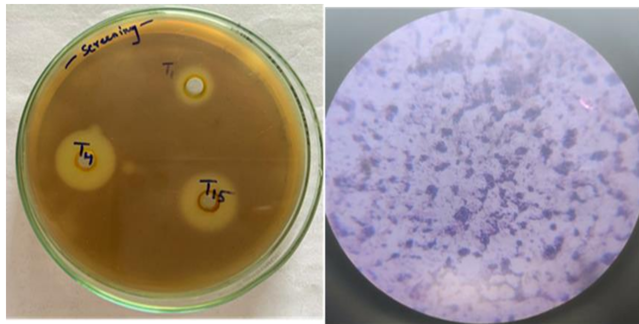

Fig. 3 Well diffusion method of Screening Fig. 4 Microscopic view of Gram stained Bacterial cells

Morphology	Result
Colour	Creamy white
Shape	Circular
Elevation	Umbonate
Optical	Opaque
Texture	Smooth
km Staining	Positive
Cell shape	Rods

Table 1

Table 2: Biochemical tests performed and result

SL NO	Biochemical tests	Results
1	Indole test	Negative
2	Methyl red test	Positive
3	Voges-proskauer test	Negative
4	Citrate utilization test	Negative
5	Gelatin liquification test	Positive
6	H_2S production test	Negative
7	Casein hydrolysis test	Positive
8	Starch hydrolysis test	Positive
9	Cellulose hydrolysis test	Positive
10	Lipid hydrolysis test	Positive
11	TSI (Triple sugar iron) test	Positive
12	Urease test	Positive
13	Nitrate reduction test	Negative
14	Glucose fermentation test	Positive
15	Lactose fermentation test	Positive
16	Sucrose fermentation test	Positive
17	Mannitol fermentation test	Positive
18	Oxidase test	Positive
19	Catalase test	Positive

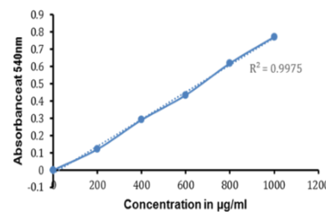

Fig.5 Glucose standard curve

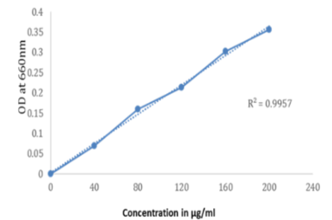

Fig.6 Protein standard curve

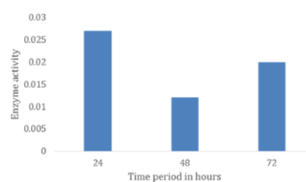

Fig. 7 Optimization of incubation time

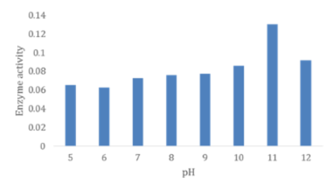

Fig.8 Optimization of pH

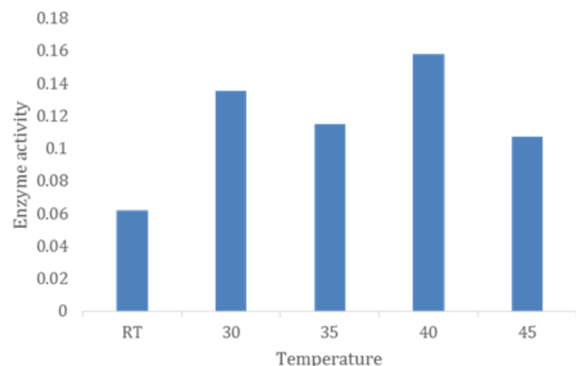

Fig. 9 Optimization of Temperature

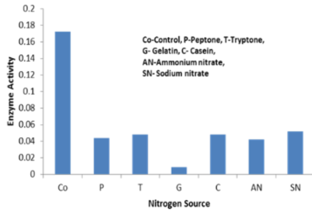

Fig. 10 Optimization of Nitrogen source

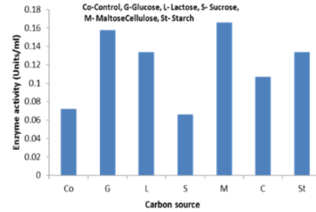

Fig. 11 Optimization of Carbon source

Sample	Enzyme Activity (units/ml)	Protein amount (mg/ml)	Specific activity (Units/mg)	Fold purification	Percentage Yield(%)
Crude	0.3814	5.7043	0.066	1	100
Salting out	0.1653	2.0811	0.079	1.196	43.34
Dialysis	0.0788	0.7652	0.102	1.54	20.66
Ion-exchange	0.0915	0.2318	0.394	5.969	23.99
Gel filtration	0.1246	0.1739	0.716	10.848	32.669

Table3: Enzyme activity at different levels of purification

At every step of purification it is seen that percentage yield is decreasing (100% to 32.669%) and specific activity of the enzyme is increasing (0.066Units/mg – 0.716Units/mg). showing that purification steps have worked properly. It is also reflected in number of fold purification of the enzyme (Table 3).

IV. CONCLUSION

The obtained strain of bacteria can serve as a very good source of pectinase enzyme which could be of industrial importance, since pectinases have a wide range of application in food and beverage industries. Not only that, it can also be utilized in bioremediation projects. A quantitative and qualitative comparative analysis of now commercially available pectinase enzyme and the enzyme produced by our strain of bacteria is necessary to come to conclusion about the superiority of the enzyme for industrial and commercial usage.

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