OPTIMIZATION OF PHYSIOLOGICAL PARAMETERS FOR PECTINASE PRODUCTION FROM SOIL ISOLATES AND ITS APPLICATIONS IN FRUIT JUICE CLARIFICATION

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ABSTRACT

Pectinase enzymes are today one of the upcoming enzyme of commercial sector. It has been reported that microbial pectinase account for 25% of global food sales. Primarily, these enzymes are heterogeneous group of enzymes that hydrolyse the pectin substance, present mostly in plants. It has also been used to increase yields and clarify fruit juices. Pectinases are one of the most widely distributed enzymes in bacteria, fungi and plants. The present investigation was undertaken to isolate pectinase producing microorganism and to standardize the condition for cost effective production of enzyme. Total 15 samples were collected from different sites and 47 isolates were obtained. From this 47 different isolates, 12 isolates were efficient producers. Among them FW5 produced maximum zone of clearance of 3.2 mm. FW2, L4, L3,GF13,GF14,GF12,L1 have more pectinolytic potential. GF1, GF10, MW8, G1 were less capable for pectin degradation. All those 12 isolates were then analysed for secondary screening. Enzyme productivity (EU/ml) and range for pectin degradation was calculated. FW2 had maximum capacity as, enzyme yield of it is high (22 EU/ml). L6 and FW5 were efficient for enzyme production and their enzyme yield were 17.1 and 19 EU/ml. All 12 isolates were tentatively identified following Bergey’s Manual of determinative bacteriology. After characterization FW5 isolate was identified as Bacillus sp. FW2 isolate as Erwinia sp. Other isolates could not be identified specifically. Optimization of temperature, pH, pectin concentration and time was done. Application in fruit juice clarification was found.

Key Words: Microbial pectinase production, Enrichment, Isolation, Screening, Pectinase assay

INTRODUCTION

Resources from forest and agriculture residues such as cellulose, lignin and pectin are available abundantly. Thus, they are always in demand for the utilization as an alternative feed stock. 1 A large number of strains of microorganisms such as bacteria, fungi, actinomycetes, yeast have ability to degrade pectin. 2,3 However, Bacterial strain are always preferred over fungal strain because of ease in fermentation and strain improvement which can be carried out easily in bacterial strain to improve the yield. 4 Pectin which is complex polysaccharide it is present abundance in the middle lamella and primary cell walls of plant tissue. 5 Pectin are complex groups of substances and various enzymes are needed to completely degrade it. However these enzymes differ in their mode of action and thus can be classified depending on their mode of specificity and activity. 1 The enzyme types, structure, substrate, classification, physicochemical and biological properties and application were studied. Pectinases have various industrial applications like improving juice yields, scouring of cotton, degumming of plant fibre’s, waste water treatment, vegetable oil extraction so used in various industries as pulp industry, textile industry, food industry etc. The application of enzyme to alter the texture

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or flavour of fruit juice, to increase extraction and clarification and to reduce viscosity has also been described. Several enzymes for food applications are available however, search for novel enzymes with desirable characteristics and low cost is always in demand for fulfilling national need and to increase industrial economy. These micro-organisms can be exploited for production of pectinase which is an industrially important enzyme and have potential applications in industries.

AIMS AND OBJECTIVES
To isolate novel organism for pectinase production, screening its pectinolytic potentiality through conventional methods and to evaluate its industrial application.

MATERIAL AND METHODS
Sample collection
Soil samples were collected from 5 different sites as market waste (Sardar market), lemon farm (Surat), Garden sites, Guava farm and dumped fruit juice waste (Bardoli) with help of sterile spatula in a sterile container and were brought to Microbiology laboratory for further study.

Isolation of microorganisms
One gram of soil samples from each collection site were pooled and homogenized in sterile distilled water and 10-fold serial dilutions were prepared. 0.1 ml of each dilution was spreaded on sterile Mc Beth’s medium plate (K$_2$HPO$_4$ 0.1gm, MgSO$_4$.7H$_2$O 0.1gm, Na$$_2$SO$_4$ 0.2gm, (NH$_4$)$_2$SO$_4$ 0.2gm, CaCO$_3$ 0.2gm, pectin 0.5gm, Agar 3gm, distilled water 100ml). After 48-72 hours incubation clear zone around colonies were observed. Pure cultures were sub cultured onto slant and maintained for identification and enzyme study.

Screening of pectinase producing microorganisms
Plate assay
The isolates were then screened for pectinase production using Mc Beth’s medium. The plates were incubated at room temperature for 48-72 hrs. Following incubation, the plates were observed for the zones of clearance around colonies, which indicate pectinase activity. After incubation plates, were flooded with 1% CTAB solution (Cetyltrimethyl ammonium bromide solution) after 15 min clear zone of hydrolysis shows production of pectinase enzyme. All the positive isolates for pectinase production were then subjected to shake flask study for determination of enzyme activity produced by individual isolates. All the potent isolates were preserved on sterile NA slants under refrigeration for further studies.

Production of pectinase enzyme
The media was composed of 50 ml mineral solution (g/l) (K$_2$HPO$_4$.0.2, MgSO$_4$.0.1,(NH$_4$)$_2$. SO$_4$.0.4, FeSO$_4$.0.08, MnSO$_4$.0.008, ZnSO$_4$. 0.006), 10 gm. wheat bran, 150 ml distilled water). In 250 ml flasks 200 ml of the medium was added and inoculated with $10^6$ cell suspensions. The culture was incubated on a rotary shaker (150 rpm) at 30°C for 3 days (72 hrs). After every 24 hrs incubation inoculums of individual isolate was then transferred into 135 ml of production media (same as inoculums media but different in the wheat bran concentration i.e. 40 gm.)The production media flasks were then incubated at room temperature for 48-72 hours on a rotary shaker at 120 rpm. Following incubation, individual production medium was harvested separately by centrifugation at 3500 rpm for 15 min and cell free supernatant was used for quantitative determination of enzyme activity per ml.

Pectinase assay
Enzyme assay was based on the determination of reducing sugars produced as a result of enzymatic hydrolysis of pectin by Dinitrosalicylic Acid Reagent (DNS) method. For this, 1% pectin solution was prepared in sodium citrate buffer (pH – 6) 0.45 ml of substrate was then mixed with distilled water to make system volume 3 ml, then 0.1 ml of enzyme (cell free supernatant) was added. The system was incubated for 15 min in water bath (65°C) then 2 ml of DNSA reagent was added to mixture and again incubate the system in water bath for 15 min. After it, 7 ml of distilled water was added. Amount of reducing sugar liberated was estimated by taking absorbance at 540 nm. The enzyme and substrate blanks were run parallel. The standard curve was prepared for
reducing sugars with glucose. One unit of pectinase activity was defined as the amount of enzyme required to release 1 μmole of reducing sugar per ml per minute under above assay conditions. The most efficient isolates were then screened on the basis of maximum enzyme units produced. These isolates were then further studied.

**Characterization of selected isolates**

Morphological, cultural and biochemical characteristics of the selected isolates were studied according to standard techniques.

**Optimization of process parameters of isolates for pectinase production**

The pectinase enzyme produced by isolates was assayed for optimization of physiochemical parameters, like temperature, pH, incubation time and substrate concentration. A temperature range of 20-70°C and pH range 5-10 was screened for optimization. Also incubation period of 24 hr, 48 hr, 72 hr, 96 hr, and 120 hr and substrate concentration of 0.2, 0.5, 1, 1.5 and 2 % was studied to determine the optimum incubation period and substrate concentration of enzyme respectively.

**Application in fruit juice clarification**

Label two tubes one as test and other as blank. With a syringe, add 1 ml of pectinase into the first test tube. Finally, add 1 ml of distilled water to the remaining blank tube. Stir the Orange juice well to distribute any suspended particles evenly through it. To each test tube add 10 ml of Orange juice. Agitate or stir the contents of the tubes to mix the enzymes throughout the juice. Put the tubes into the water bath. Observe the tubes and record the appearance of their contents at 5 minute intervals over a half hour period. Further clearing may occur if the tubes are left to stand overnight at room temperature. After incubation filter the solution and autoclave it then allow it to cool.

**RESULTS AND DISCUSSION**

**Isolation and primary screening of microorganisms**

Total 15 samples were collected from different sites and 47 isolates were obtained which were analysed for primary screening by plate assay. From this 47 different isolates, 12 isolates were found as efficient producers. Among them FW5 produced maximum zone of clearance of 3.2 mm. FW2, L4, L3, GF13, GF14, GF12, L1 also showed zone of clearance (Fig. 1(a)) and have more pectinolytic potential. GF1, GF10, MW8, G1 were less capable for pectin degradation. All those 12 isolates were then analysed for secondary screening (Fig. 1(b)).

Fig. 1(a) : Zone of clearance of potential producers
Plate assay: Zone of clearance around colonies

Maximum zone producing bacteria was selected as they are efficient producer and used for the shake flask study to determine enzyme units. 12 isolates were selected according to their zone of clearance (on the basis of their primary screening) and used for the shake flask study. Enzyme productivity (EU/ml) of those different isolates was calculated which is listed in Table 1.

Table 1: Enzyme productivity of potent isolates

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Enzyme unit (EU/ml)</th>
<th>Isolates</th>
<th>Enzyme unit (EU/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>11</td>
<td>L3</td>
<td>12</td>
</tr>
<tr>
<td>GF1</td>
<td>13.9</td>
<td>L4</td>
<td>9</td>
</tr>
<tr>
<td>GF10</td>
<td>15.7</td>
<td>L6</td>
<td>17.1</td>
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<tr>
<td>GF12</td>
<td>10.5</td>
<td>MW8</td>
<td>13</td>
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<tr>
<td>GF13</td>
<td>16.4</td>
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<tr>
<td>GF14</td>
<td>10</td>
<td>FW5</td>
<td>19</td>
</tr>
</tbody>
</table>

All 12 isolates have range of capacity for pectin degradation. FW2 had maximum capacity as, enzyme yield of it is high (22 EU/ml) after that L6 and FW5 were efficient for enzyme production as their enzyme yield were 17.1 and 19 EU/ml (Table 1). Other isolates were also higher producing. All those isolates were then characterized by various biochemical tests.

Characterization of most efficient isolates

Morphological, cultural and biochemical characteristics of the selected isolates were studied according to standard techniques. All 12 isolates were tentatively identified following Bergey’s Manual of determinative bacteriology and methods stated in Microbiology –Laboratory Manual.11-13 The FW5 isolate was positive for gelatine liquefaction, casein hydrolysis, catalase test, starch hydrolysis, Ammonia production and denitrification activities. It has good growth at 37°C. Based on morphological and biochemical characteristics, FW5 isolate was tentatively identified as Bacillus sp. The FW2 isolate was positive for lactose fermentation, citrate utilization and indole production test. Based on morphological and characteristics, FW2 isolate may be as Erwinia sp. Other isolates even after thorough investigation could not identified specifically.
Optimization of biochemical parameters

For optimization 4 isolates were selected. Different temperature, pH, incubation period, and pectin concentration of FW2, FW5, L6, and GF13 was carried and productivity of pectinase was calculated by performing enzyme assay.

Effect of temperature on pectinase production

Different temperatures as 20°C, 30°C, 37°C, 45°C, 55°C and 65°C were used to determine optimum temperature for enzyme.

Enzyme production increase with increase in temperature up to optimal and then decrease (Fig. 2). The maximum production occur at 37°C in FW2, L6 and GF13 isolates and produce 27.1 EU/ml, 21 EU/ml, 15.6 EU/ml respectively at these temperature but in case of FW5 it reaches maximum at 30°C and produce 25.1 EU/ml at 30°C.

Effect of pH on pectinase production

To determine the effect of pH on pectinase production FW2, FW5, L6 and GF13 were incubated at different pH ranging from 5 to 9.

Enzyme production increase with increase in pH till pH 6 and in some case at pH 7 then it decreases (Fig. 3). The maximum production occur at pH 7 in FW2, L6, GF13 producing 27.1, 24.2, 22.56 EU/ml respectively. Isolates FW5 shows maximum
production at pH 6 which is 23.9 EU/ml respectively. The isolates were active within the range of 6 to 8.

**Effect of incubation period on pectinase production**

To determine the effect of incubation period FW2, FW5, L6 and GF13 incubated at different time of 24, 48, 72, 96 and 120 hrs. (Fig. 4) Enzyme production increase with increase in time duration up to 96 hrs then it decreases. The maximum production occurs on 4th days (96 hrs) incubation period. Production of pectinase by FW2 was 25.2 EU/ml, by FW5 was 19 EU/ml, by L6 it was 18.1 EU/ml and by GF13 it was 15.3 EU/ml after 96 hrs incubation period.

![Fig. 4: Effect of incubation period on pectinase production](image)

**Effect of substrate concentration on pectinase productivity**

To determine the effect of substrate concentration for pectinase production FW2, FW5, L6 and GF13 were incubated at different concentration of pectin as 0.1%, 0.2%, 0.5%, 1.0%, 1.5% (Fig. 5) Enzyme production increases with increase in concentration.

![Fig. 5: Effect of substrate concentration on pectinase production](image)
till 0.5% then it remains constant. The maximum production occurs at 0.5% concentration. FW2 produced 22 EU/ml, FW2, L6, GF13 produce 21.6, 21 and 15.2 EU/ml of enzyme respectively.

**Application in food juice clarification**

Pectinase from FW2 in orange juice shows pectinolytic activities by clarifying it as compared to control tube (Fig. 6).

**CONCLUSION**

Commercial enzyme production has being increased from past decayed and is on demand in the world market. Microorganisms serve as a major source of enzyme. Even majority of industrial enzymes are of microbial origin. Thus attempt of present study is to isolate and screen for potential producers of pectinase from microorganisms. Total 12 isolates were identified as the efficient producers among them screening FW5 were found as efficient producers and was preliminary identified as *Bacillus*. Isolates were grown for different temperature, pH, time and substrate concentration because it is essential to maximize optimum condition for production. Studies revealed that FW5 require temperature of 30°C, pH 6, incubation period of 96 hr and 0.5% of substrate concentration for maximum production. Substrate is the main factor for maximum production. An optimum substrate provides all necessary constituents for optimum production. Thus alternation of the substrate may further leads to maximum production. FW5 was also screened for its application in fruit juice clarification. Environmental conditions stimulate organisms to produce maximum enzyme. Thus different factors were studied even though still more investigation is needed for allowing continuous production at optimum condition by specific isolate. Present work was to determine optimum condition for production of enzyme. In order to obtain high and commercial yields of enzyme, it is essential to optimize the fermentation medium used for growth and enzyme production. Optimal parameters of the pectinases enzyme biosynthesis from microbial origin varied greatly, with the variation of the producing strain, environmental and nutritional factors.

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