EFFECT OF INCUBATION PERIOD AND REACTION CONDITIONS ON PECTINASE ENZYME PRODUCED BY BACTERIAL ISOLATES

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Abstract— The present work has been undertaken for the screening and isolation of pectinase producing bacteria from the fruit and vegetable waste samples (Apple, Orange, Guava, Pears, Banana and Tomato) collected from Mandalay region and Shan state in Myanmar. Screening of pectinase producing bacterial strains was done by spot inoculation on pectinase screening agar medium. Two bacterial isolates were found positive results for pectinolytic activity giving halozone formation and designated as O6 and T2. The quantitative determination of pectinase producing activities was carried out by Miller method using Dinitrosalicylic Acid Reagent in which pectin was used as substrate for pectinase enzyme. One of the major components of pectin, D Galacturonic acid, was used for standard curve to determine the enzyme activity by quantitatively. Pectinase activity was optimized for various parameters like incubation time, temperature, pH and reaction time. Maximum enzyme activity was observed at seven days incubation period and the reaction condition of 40°C and 45°C temperatures and at pH 5. O6 showed better pectinase activity than T2 at all optimum conditions except reaction temperature.

Key Works— Pectin, bacteria, DNS, pectinase, optimization

I. INTRODUCTION

Enzymes are the bio-active compounds that regulate many chemicals changes in living tissues. Pectinase are the heterogeneous group of related enzymes that hydrolyze the pectin substances, present mostly in plants [1]. Pectinase are among the major enzymes required in extraction of fruit and vegetable juices to increase yield; controlling clarity of juices; enzymatic peeling of fruits; improving the texture of fruits and vegetables; wine production; extraction of pigments and food colorings [2]. It is also used in industries for waste water treatment, Textile processing and bioscouring of cotton fibers, Degumming of plant bast fibers Retting of plant fibers, Waste water treatment, Coffee and tea fermentation, Paper and pulp industry, Animal feed, Purification of plant viruses, Oil extraction, Improvement of chromaticity and stability of red wines [4]. This enzyme account for approximately 25% of the world enzyme market [5]. In recent years, interest in its microbial production has increased [6].

The history of pectinases began with an understanding the structure of pectin substances and the mechanism by which pectinolytic enzymes degrade pectic substances [7]. Later, the microbial production of pectinases became prominent for several decades. Evidence showed that pectinases are inducible and they can produce from different carbon sources. In the course of time, numerous reports have appeared on the optimization of fermentation and microbiological parameters and different fermentation strategies for the production of pectinases [8].

Microbial pectinases are the main sources for pectinase production and serve as a preferred source because of rapid growth, limited space required for microbe cultivation, low production cost and more predictable and controllable enzyme content [9]. Many studies have been conducted on the production of pectinases from various microorganisms. Several microorganisms have been used to produce different types of pectinolytic enzymes. Many microorganism viz., bacteria, yeast, fungi could produce pectinases. 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[10]. Among the various pectinase, bacterial extracellular pectinase are the most significant, compared with animal, plants, viruses and fungal extracellular pectinase. Extracellular pectinase produced by Bacillus and Cocci species.

Pectinases could also be used for the scarification of pectin-rich agricultural residues, with the resulting sugars being used for the production of ethanol or platform chemicals. The saccharification of pectin involves a number of enzymes working in conjunction. In the current paper, we use the term “pectinolytic activity” to denote the liberation of reducing sugars from pectin by this mixture of enzymes, which we refer to as a “pectinase complex”. Many researchers having isolated new strains and undertook medium optimization and strain improvement programs. In these programs, it is necessary to assay pectinolytic activity. When the interest is to produce pectinases for the food industry, it is common to determine pectinolytic activity based on measurements of the reduction in viscosity of a pectin solution .The progress towards complete saccharification is best characterized by measuring the liberation of reducing sugars from pectin. The most frequently used method for doing this involves a “single-time-point” assay that requires two steps. The first step is a hydrolysis step, in which the pectinase-containing extract is incubated for a fixed time under selected conditions of pH, temperature and initial pectin concentration. In the second step, the reducing

sugars liberated in the first step are quantified, either by the DNS method or by the Somogyi-Nelson method. The advantage of this assay is that it is simple, uses relatively cheap reagents and can be used to process a relatively large number of samples simultaneously [11].

Investigation of Pectinases is a central issue in enzymology due to their wide applications in Pharmaceutical, Food, Agricultural products and Bioremediation process. Pectinases are also used as additives in animal feeds, mainly for poultry and ruminants, to improve digestibility and nutritional value Pectin is a major constituent of cereals, vegetables, Pectin fibers are complex, high molecular weight heterogeneous and acidic structural polysaccharide. D-galacturonic acid is one of the major components of pectin. [12].

New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have been the focus of many studies [13]. The breakdown of biomolecules enzymes depends upon the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations [14]. Microbial enzymes work depends on the type of enzymes application, temperature, concentration, pH and so on. Therefore, pectinase enzymes also differentiated according to their physical and chemical factors [15].

The present study was conducted to optimize the conditions of pH, temperature, reaction time and incubation time for the activity of pectinase from Cocci species.

II. MATERIAL AND METHODS

A. Collections of Samples

For the isolation of pectinase producing bacteria, decaying fruits and vegetables wastes (apple, orange, guava, pear, banana and tomato) were collected in sterile plastic bags from the markets of Mandalay region and Shan state. All samples were stored at 4°C till further investigation. All the chemicals and reagents used for the study were of analytical/microbiological grade and were obtained from commercial vendors.

B. Isolation of the Bacteria

The pectin medium was prepared and sterilized along with required glassware in an autoclave. One gram of fruits and vegetables spoilage samples from each collection site were pooled and homogenized in sterile 100ml of 0.9 % sodium chloride saline solution and 10-fold serial dilutions were prepared. 100µl of each dilution was spreaded on sterile pectin screening agar medium (PSAM) plate containing (% w/v): pectin (1.0), $(NH4)_2HPO_4$ (0.3), KH_2PO_4 (0.2), K_2HPO_4 (0.3), $MgSO_4.7H_2O$ (0.01), Agar (2.5), and the initial pH of the medium was adjusted to 7. Inoculated plates were incubated at 37ºC for 48-72 hours incubation in order to obtain colonial growth. Pure cultures were sub cultured onto slant and maintained for identification and enzyme study.

C. Qualitative Screening for Pectinase Producing Bacteria

The isolates were then screened for pectinase production using pectin screening agar medium. The plates were incubated at 37ºC for 7 days. Following incubation, the plates were observed for the zones of clearance around colonies, which indicate pectinase activity. After incubation plates, were flooded with 50mM potassium iodide solution and incubated for 15 min. After 15 min clear zone of hydrolysis shows production of pectinase enzyme. 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 PSAM slants under refrigeration for further studies.

D. Characterization of selected isolates

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

E. Submerged fermentation conditions for pectinase production

(1) Inoculum Preparation

A loopful of the bacterial strain (Cocci spp) showing the zone of hydrolysis on pectin agar plates was inoculated on liquid medium contained (% w/v): peptone (1.5), yeast extract (0.3), glucose (0.2), NaCl (0.2), K_2HPO_4 (0.12), Na_2CO_3 (0.3), pH 10. Medium (60 ml) contained in 250-ml Erlenmeyer flasks were inoculated and cultivated at 37°C by shaking at 100 rpm for 24 hr.

(2) Pectinase production

The submerged fermentation (SmF) was carried out in 250-ml Erlenmeyer flasks, each with 90 ml of the fermentation (the same medium used in qualitative screening without agar-agar). The sterilized medium was inoculated using 10 ml of a 24h-old of the prepared inoculum culture. Fermentation was carried out on a rotary shaker at 100 rpm for 7days at 37 °C. After 7 days incubation, the culture broth was centrifuged at 6000 rpm for 20 min to remove the cells and debris. The supernatant was designated as the crude enzyme which then used for enzyme assay and characterization [16].

F. Enzyme Essay

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, to 0.2ml of 1% pectin 2.0ml of sodium citrate buffer of pH 5 and 1.0 ml of enzyme extract were added. The reaction mixture was incubated at 35ºC for 30 min. After 30 min, 1.0ml of this reaction mixture was withdrawn and added to test tubes containing 0.5ml of 1M sodium carbonate solution. To each test tube, 3.0ml of DNS reagent was added and the test tubes were shaken to mix the contents. The test tubes were heated to boiling on

water bath for 10 min. Then these were cooled and 20ml of distilled water was added to contents of each tube and the absorbance was measured at 540 nm using spectrophotometer. The enzyme and the substrate were run parallel. The standard curve was prepared for reducing sugars with galacturonic acid as standard [17].

G. Effect of Incubation Periods

The isolated bacteria O6 and T2 were subjected to different culture conditions to derive the optimum conditions for pectinase activity. Growth and pectinase activity were estimated at regular day's intervals (3, 5, 7, 9, 11, and 13 days). The experiments were carried out (temperature 35°Cc , pH 5 and reaction time 30 min) in 250 ml Erlenmeyer flask containing 100 ml of basal medium.

H. Screening of reaction parameters for the activity of pectinase produced by isolated bacteria

The pectinase enzyme produced by isolates was assayed for optimization of physiochemical parameters, like temperature, pH and reaction time. A temperature range of 20-70°C and pH range 2-8 and reaction time of 15, 20, 25, 30, 35, 40 and 45mins was screened for optimization. One parameter was being screened the other two parameter kept constant (Temperature-35°C, pH-5, Reaction time-30min).

III. RESULTS AND DISCUSSIONS

A. Isolation and Screening of Pectinase Producing Bacteria

Isolation of pectinase producing bacteria was carried out from 6 samples of fruits and vegetables wastes collected from Mandalay region and Shan state in Myanmar (Table 1). 82 bacterial isolates obtained from isolation were screened for pectinase activity by plate assays. 2 isolates (O6 and T2) out of 82 bacteria were found clear zone formation on pectin screening agar media as efficient producer. The results are presented in (Fig 1).Therefore, confirmation could be made for the production of pectinase enzyme from these two bacteria according to method described by E. Venkata Naga raju. et.al [18].

TABLE I: THE NATIVE BACTERIAL ISOLATES AND THEIR SOURCES

Sr. No.	Samples	Number of Isolates
1.	Oranges	36
2.	Guava	7
3.	Pears	8
4.	Apple	16
5.	Banana	6
6.	Mangoes	-
7.	Tomato	9

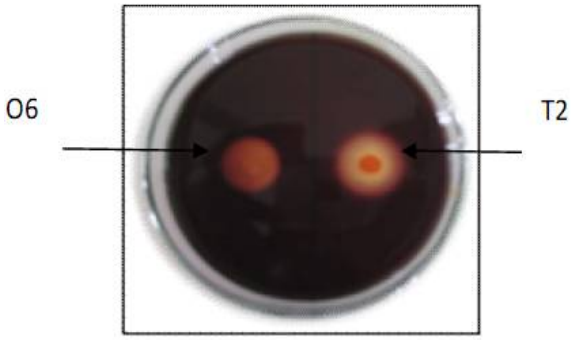

Fig. 1. Clear Zone Formation of Isolated Bacteria, T2 and O6, on Pectinase Screening Agar Media after 7 days Incubation Period

B. Characterization of selected isolates

Morphological, cultural and biochemical characteristics of the selected isolates (O6 and T2) were studied according to standard techniques and the details are presented in (Table 2).The two isolates were tentatively identified following Bergey's Manual of determinative bacteriology and methods stated in Microbiology –Laboratory Manual. The isolates O6 and T2 were positive for Catalase and Starch hydrolysis tests. T2 strain was positive for Methyl red, Citrate utilization, Gelatin hydrolysis, and Voges Paskauer test and O6 strain show negative. These two strains negative results for nitrate reduction test and MacConkey's Plate. Microscopic morphology and colonial morphology of two bacterial strains were analyzed as Gram positive Cocci bacteria. The T2 isolate was positive for glucose, maltose, galactose, and fructose and mannitol fermentation. And O6 was also positive for glucose, lactose, maltose, galactose, fructose except sorbitol. Other isolates even after thorough investigation could not identified specifically.

TABLE II: THE NATIVE BACTERIAL ISOLATES AND THEIR SOURCES

Sr. No.	Morphological and Biochemical Tests	Bacterial Isolates	
		T_2	O_6
1.	Morphology	Cocci	Cocci
2.	Colony Color	Brown	Pale Yellow
3.	Shape of the Colony	Circular	Circular
4.	Elevation of the Colony	Raised	Raised
5.	Visual Characteristics	Translucent	Transluc ent
6.	Colony Surface	Smooth	Smooth
7.	Gram Staining	+	+
8.	Motility	-	-
9.	Catalase Test	-	-
10.	Citrate Utilization	+	-

10.	Citrate Utilization	+	-
11.	Nitrate Utilization	-	-
12.	Starch Hydrolysis	+	+
13.	Gelatin Hydrolysis	+	-
14.	Methyl Red Test	+	-
15.	Voges Proskauer Test	+	-
16.	Mac Conkey's Plate	-	-
17.	Mannitol Salt Agar	-	-
18.	Pectinase Activity	+	+
19.	Protease Activity	+	-

C. Effect of Incubation Period on pectinase activity

To determine the effect of incubation period T2 and O6 incubated at different periods of 3, 5, 7, 9, 11 and 13days. (Fig. 2) Activity of pectinase enzyme increased with increasing incubation days up to 7days. However, it decreased beyond 7days. The maximum production occurs on 7th day's incubation period. Production of pectinase by T2 was 0.396mg/ml and by O6 it was 0.450mg/ml after 7days incubation period. Above this period the pectinase enzyme activity started to decrease. This is because, the cells may reach the decline phase and displayed low pectinase synthesis [19].

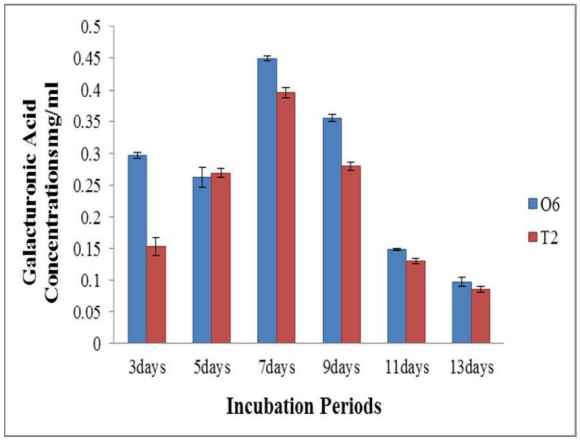

Fig. 2. Effect of incubation period on pectinase production

D. Screening of Reaction parameters

For optimization 2 isolates were selected. Different pH, temperature and reaction time for enzyme from O6 and T2 were carried and activity of pectinase was calculated by performing enzyme assay.

(1) Effect of pH on pectinase activity

To determine the effect of pH on pectinase activity of O6 and T2 were determined at different pH ranging from 2 to 8(Fig. 3). It was found that pectinase enzyme activity increased with increasing pH up to 5 and then it gradually decreased at pH higher than 5. The maximum enzyme activity of two isolates (O6 and T2) occurs at pH 5 with the glacturonic acid concentration of 0.426mg/ml and 0.378mg/ml respectively. Most enzymes function efficiently over a narrow pH range. A change in pH above or below this range reduces the rate of enzyme reaction considerably (123HelpMe.com).

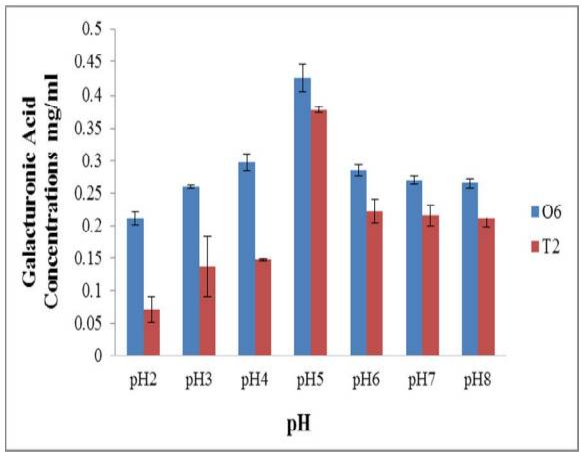

Fig. 3.Effect of pH on pectinase production

(2) Effect of temperature on pectinase activity

Different temperatures as 20°C, 25°C, 30°C, 35°C, 40°C, 45°C, 50°C, 55°C, 60°C, 65°C and 70°C were used to determine optimum reaction temperature for enzyme. Enzyme production increase with increase in temperature up to optimal and then decrease (Fig. 4). In the present study, the effect of temperature on pectinase enzyme activity of isolates (O6 and T2) by SmF revealed that 40°C and 45°C was optimum enzyme activity with the concentration of 0.467mg /ml and 0.528mg/ml respectively and at the tested higher temperatures, the enzyme activity decreased which might be due to growth reduction and enzyme inactivation or suppression of cell viability. These enzymes made from protein and are very specific. Temperature can regulate enzyme activity between high and low. While higher temperatures do increase the activity of enzymes and the rate of reactions, but enzymes are still proteins, and as with all proteins, generally temperatures above 40 degrees Celsius (104 degrees Fahrenheit) will start to break them down [20].

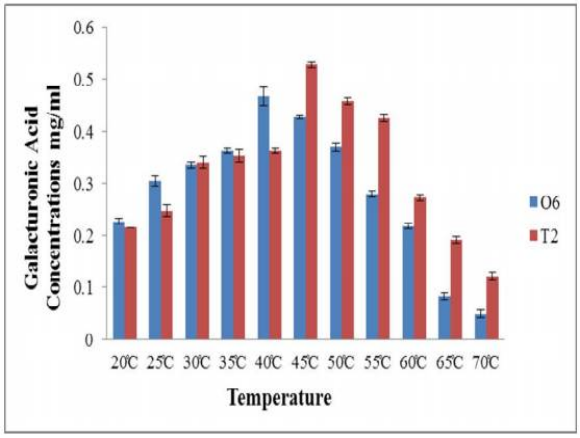

Fig. 4. Effect of temperature on pectinase production

(3) Effect of reaction time on pectinase production

For determination of optimum reaction time, enzyme assay was carried out at different reaction time ranging from 15 to 45 minutes at constant temperature and pH. Enzyme activity increases with increase in reaction time until 35min then the reaction decline. The maximum production of reaction time occurs for 35mins and pectinase activity of T2 was 0.398mg/ml and O6 was 0.526mg/ml respectively (Fig 5). All enzymes have optimum reaction time for showing its maximum catalytic activity.

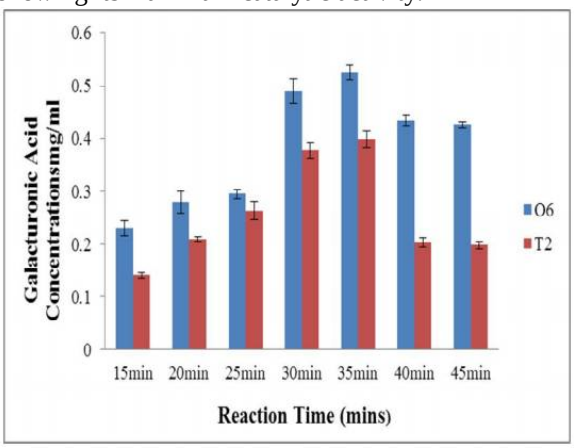

Fig. 5. Effect of reaction time on pectinase production

CONCLUSIONS

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. Results of the study show that potent two bacterial strains (O6 and T2) were able to grow on (PSAM) media and gaved transparent halozones showing that they have pectinase enzyme activity and was preliminary identified as Cocci spp. Isolates were culture at different incubation periods and the various reaction conditions such as pH, and reaction time because it is essential to maximize optimum condition for production. Studies revealed that the best enzyme activity of O6 was occurred at temperature of 40°C, pH 5, and incubation period of 7days and 35min of reaction time. For T2, it is required that the temperature of 45°C, pH 5, incubation period of 7days and 35min of reaction time for maximum production. It is pointed out that the conditions for maximum enzyme activities of two isolates were nearly the same except the reaction temperature. To conclude, this isolates may be further scaled up for juice production after careful investigations.

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