



PRODUCTION AND CHARACTERIZATION OF PECTINASE ENZYME FROM *RHIZOPUS ORYZAE*

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ABSTRACT

The research was conducted with the production of pectinase enzyme by fungal fermentation using *Rhizopus oryzae* and characterization of produced enzyme with respect to pH, temperature, incubation time, and substrate specificity. Carbon source was optimized replacing sugar with different concentration of pectin (0.5, 0.75, and 1.0%) during submerged fermentation. The outcomes of the fermentation process showed that media containing carbon source of 1% pectin replaced dextrose at pH 6.0 incubating for 72 hours at 35 °C were the best condition for pectinase production. The maximal activity for pectinase enzyme produced from *Rhizopus oryzae* by fermentation was 3.16 U.mL⁻¹ and it was found at 40 °C and pH 6.5. The produced pectinase enzyme was found thermo stable up to 60 °C for 50 min. The activity of the enzyme was increased with the increasing pectin concentration in the media and maximum activity was found at the pectin concentration of 5 mg.mL⁻¹. The k_m and V_{max} values were found 0.84 mg.mL⁻¹ and 5.294 mg.mL⁻¹ respectively at optimized condition. The outcomes of the research may be useful for further research in low cost production of pectinases from *R.oryzae* for domestic consumption in many industries.

Keywords: pectinase; submerged fermentation; Pectin; enzyme; incubation

INTRODUCTION

Enzyme is an essential tool in juice processes, both in terms of quality improvement and cost saving (Ramadan and Moersel, 2007). Now a day's production of fruit and vegetable juice is almost unthinkable without the use of enzymes (Baumann, 1981). The degradation of plant cell walls by exogenous enzymatic treatment results in easier release of the components contained in cells (Janser, 1997).

The utilization of microbial enzymes has found broad technological application in different industrial processes. Among the various enzymes commercialized many are products of fermentation of filamentous fungi (Piccoli-valle et al., 2001).

Pectinases are the groups of enzymes, which cause degradation of pectin that, are chain molecules with a rhamnogalacturonan backbone, associated with other polymers and carbohydrates. These pectinases have wide applications in fruit juice industry and wine industry. In fruit juice industry, it is used for clarification, where reduction in viscosity is caused which ultimately leads to formation of clear juice. In wine industry pectinases are mainly used for decreasing astringency by solubilizing anthocyanins without leaching out procyanidin polyphenols, and pectinases also increase pigmentation by extracting more anthocyanins (Tucker and Woods, 1991).

Pectinases can be produced by both submerged and solid state fermentation (SSF). Submerged fermentation is cultivation of microorganisms on liquid broth. It requires high volumes of water, continuous agitation and generates lot of effluents. Solid state fermentation incorporates microbial growth and product formation on or within particles of a solid substrate (Mudgett, 1986) under aerobic conditions, in the absence or near absence of free water, and does not generally require aseptic conditions for enzyme production. Many filamentous fungi like *Aspergillus niger*, *Aspergillus awamori*, *Penicillium restrictum*, *Trichoderma viride*, *Mucor piriformis*, and *Yarrowia lipolytica* are used in both submerged as well as solid state fermentation for production of various industrially important products such as citric acid and ethanol.

Fungi can produce both intracellular as well as extracellular enzymes. All fungi are heterotrophic, and rely on carbon compounds synthesized by other living organisms. Small molecules like mono disaccharides, fatty acids and amino acids can easily pass through but for breaking down of larger complex compounds like pectin, fungi secrete extra cellular enzymes. It is well known that as compared to intracellular enzymes, the extra cellular enzymes are easier to be extracted. Intracellular enzymes require more time and costly chemicals for extraction (Hankin and Anagnostakis, 1975).

Rhizopus oryzae is a filamentous fungus which is commonly found in dead organic matter. *Rhizopus sp.*, *Gleosporium kake*, *Ciniotrium diplodella* and *Aspergillus niger* have been reported to produce exo-Polygalacturonases as well as endo Polygalacturonase which release galacturonic acid from the terminal pectin chain (Kawano *et al.*, 1999).

In Bangladesh, this enzyme is not commercially produced and cost to import is very high. At the same time, commercial production of enzymes is not reputed in Bangladesh. Normally enzyme is produced either by fermentation or extracted from plant or animal sources. It involves high cost for the production of enzymes employing any of the processes. Moreover, to get the potential benefits from extracted enzymes; it has to send through series of purification steps and needs special care to stabilize during storage. Due to these constraints, no companies are interested to adopt this technology in food industries of Bangladesh.

Scientific hypothesis

Keeping the above-mentioned points in consideration, the present investigation was undertaken to produce and to characterize pectinase enzymes from the *Rhizopus oryzae* collected from DSMZ, Germany in the form of freeze-dried. The following main objectives were drawn to carry out the investigation:

- To optimize cell growth of *Rhizopus oryzae*
- To optimize medium conditions for enzyme production
- To characterize pectinase enzyme.

MATERIAL AND METHODOLOGY

Materials

Fermentative organism

Rhizopus oryzae (DSM-1185) was collected from DSMZ, Germany in the form of freeze-dried. The culture was activated according to the following method as mentioned by suppliers:

At first the tip of the ampoule was heated in a flame. Two or three drops of water were then placed onto the hot tip to crack the glass. After that the glass tip was carefully strike off with an appropriate tool (e.g. forceps). The insulation material was removed with forceps and taken out the inner vial. The cotton plug of the inner vial was lifted and kept it under sterile condition and the top of the inner vial was then flamed. Added 0.5 mL of medium prepared following the manufacturer's formulation and replaced the plug. Then the pallet was allowed to rehydrate for about

30 minutes. The content was mixed gently with an inoculation loop or a pasteur pipette and transferred the whole amount of the mixture in to a test tube with about 5 mL of potato dextrose liquid medium. The mixture was then transferred in to the vial. 100 µL of the suspension was streaked onto an agar plate. The liquid and agar culture was then incubated under sterile conditions.

Chemicals

Dinitrosalicylic acid (DNS) (purity 99%), potassium mono-hydrogen phosphate (purity 99%), dipotassium hydrogen phosphate (purity 97%), sodium potassium tartrate (purity 99%), D (+) galcturonic acid (purity >95%) were collected from commercial suppliers. All the chemicals were analytical grade with the highest purity.

Instruments

Microscope; Thermo shaker (Hangz hou long Gene Scientific Instruments Co. Ltd. P.R., China); Potable pH meter (HANNA Instruments Inc., Woonsocket, Rhode Island, USA); Centrifuge (cat. No.: CF, 405, 5000 rpm. Gallenkamp, England); Air oven (Memmert, Germany), Incubator (Model: 1H-100, Gallenkamp, England); Water bath (Wytownia Sprzetu Laboratorynego, Poland); Laminar flow (HEPA filter 0.3 micron, area: 3×2×2 ft., Bangladesh); Sterilizer (St. Steel Autoclave, Capacity 30 liter, cat.no. ST 3028, model no. ST××28, Unipath Ltd., Basingstoke, England); Spectrophotometer (Spectronic ins., USA) was used throughout research period.

Growth of *Rhizopus oryzae* on potato dextrose agar

R. oryzae culture was cultivated on the potato dextrose agar as the spores were to be stored for longer period for the utilization of organism in different trials. The Potato dextrose agar (PDA) medium was prepared according to the composition given in Table 1

At first healthy potato tuber was taken and skin was removed. Then the potato was cut into small slices. 200 g sliced potato and 500 mL of water was then boil together for 15 minutes so that the potato tissues could be softened. The prepared pulp was then sieved through a muslin cloth. After that 15 g of agar and 500 mL of water was heated separately. Then both water and agar solutions were mixed and 20 g dextrose was added to it. Thus, 1000 mL potato dextrose agar medium (PDA) was prepared.

The prepared medium was autoclaved at 121 °C for 15 minutes under 1.1 kg.cm⁻² pressures (Asghar *et al.*, 2000). After cooling, the medium was transferred

Table 1 Sporulation medium (PDA) composition for *Rhizopus oryzae*.

Ingredient	Composition
Sliced potato	200g
Dextrose	20g
Agar	15g
Distilled water	1000mL

Table 2 Liquid medium (PD) composition for *Rhizopus oryzae*.

Ingredient	Composition
Sliced potato	200g
Dextrose	20g
Distilled water	1000mL

aseptically to pre-sterilized petri dishes. Then streak 100µl of the mother culture of the organism onto an agar plate or petri dishes and incubated at 30 °C for 3 days to allow the spores to germinate.

Determination of cell growth by dry cell wt. methods

Seven (7) test tubes were taken containing 5 mL of liquid medium. The liquid medium was prepared according to the composition given in Table 2.

By using inoculation loop the culture from the sporulation medium was transferred to the test tubes under aseptic conditions. Then incubated these test tubes at 37 °C in an incubator for 9 days and observed the wt. gain of biomass of the *R. oryzae* for every 24 hr over 9 days by the following method:

At first a filter paper was taken and placed in an air oven (at 65 ±5 °C) for 1 hr. Then the wt. (W₁) of the filter paper was taken. After incubation the biomass was filtrated through the filter paper. Then the filter paper with biomass was placed in an air oven (at 65 ±5 °C) for 24 hr. After drying, the filter paper was removed from the oven and cooled in desiccators. The filter paper was removed from desiccators and weighed (W₂) soon after reaching room temperature. The gain in weight was taken as the cell growth of the *R. oryzae*. The readings were taken after incubating the test tube for every 24 hr over 9 days and the cell growth were calculated as follows:

$$\text{Dry cell wt. (cell growth, g/L)} = (W_2 - W_1)/5 * 1000$$

Enzyme production

The fungal mother culture was used to produce pectinase enzyme using liquid medium containing dextrose 2%, citrus pectin 1% at pH 6. Fermentation was carried out in a test tube containing 5 mL of liquid medium with 100µl mother culture of *R. oryzae* and incubated at 30 °C for 7 days (Angayarkanni et al., 2002). The biomass was separated by filtration. The supernatant was used to evaluate the polygalacturonase enzyme activity.

Optimization

Medium carbon source (dextrose) replaced with pectin

Pectin was used separately at different concentrations (0.5, 0.75 and 1.0%) in the medium. The fermentation was carried out for a period of 120 hours keeping other components and conditions were the same as described before.

pH

Pectinase biosynthesis was carried out by growing fungus at different pH media (5.0, 5.5, 6.0, 6.5, and 7.0) to find out the optimum pH level for enzyme production.

Temperatures

For optimization of temperature, the production of pectinase was performed at different temperatures (30.0, 32.5, 35.0, and 37.5 °C) using the same media.

Incubation time

To find out the optimum time required for maximum pectinase activity, samples were harvested at different time intervals of 24, 48, 72, 96, and 120 hours and pectinase activity were determined.

Sample harvesting

After specific interval of incubation time, the biomass from the experimental test tubes was separated by filtering through Whatman filter paper no.1. The filtrate was centrifuged at 5,000 rpm for 15 minutes in the centrifuge (cat. no.: CF, 405, 5000 rpm. Gallenkamp, England) to remove the spores and mycelia of the organism. The supernatant was carefully collected and stored at refrigerated temperature in sterilized test tubes.

Enzyme activity

Polygalacturonase hydrolyzes the polymer of pectin into the galacturonic acid monomers. The free galacturonic acid units produced as a result of polygalacturonase activity reacts with 3-5 dinitrosalicylic acid (DNS) reagent and form a colored complex. The degree of change of color was measured by spectrophotometer at wavelength of 550 nm. Greater the amount of galacturonic acid produced, darker the color of the enzyme- galacturonic acid complex formed and more the light absorbed.

Preparation of dinitrosalicylic acid (DNS) solution

Different ingredients used for the preparation of DNS solution are as follows:

- 1) Distilled water 1416 mL
- 2) 3.5 Dinitrosalicylic Acid 10.6 g
- 3) NaOH 19.5 g

The above ingredients were dissolved gently in water bath at 80 °C until a clear solution was obtained. Then the following chemicals were added.

- 4). Rochelle salt (sodium potassium tartrate) 300g

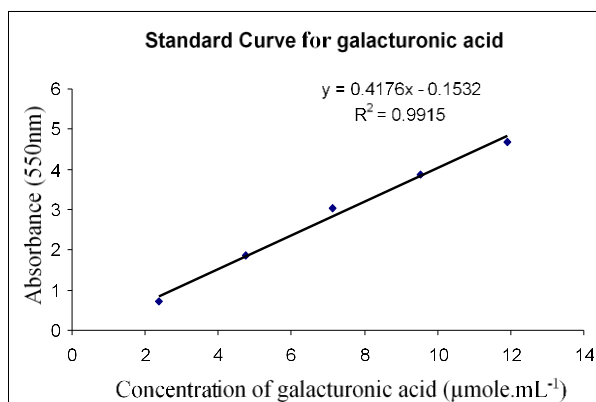


Figure 1 Standard curve for galacturonic acid.

- 5) Phenol (melted at 60 °C) 7.5 mL
- 6) Sodium meta-bisulphate 8.3g

After dissolving the above ingredients, the solution was filtered through Whatman filter paper 1 and stored at room temperature in an amber colored bottle to avoid photo oxidation.

Standard curve for galacturonic acid (GA)

Five solutions having concentration of 0.05 mg.mL⁻¹, 0.10 mg.mL⁻¹, 0.15 mg.mL⁻¹, 0.20 mg.mL⁻¹ and 0.25 mg.mL⁻¹ of galacturonic acid were prepared in distilled water. Each concentration of 500 µL was added in a vial along with 500 µL DNS solution. The vials were kept in boiling water for 3 minutes and cooled. A blank sample was also prepared (500 µL each of distilled water and DNS solution). Absorbance was determined at 550 nm and a graph was drawn by plotting absorbance against concentration (Figure 1).

Polygalacturonase (PG) enzyme assay

The filtrate was assayed for pectinase activity; determined at 50 °C using 0.1% (w/v) pectin as substrate at pH 5.0. Reducing substances were measured using DNS method (Miller, 1959; Carmona et al., 1998). Enzyme activity was expressed as U.mL⁻¹.

Unit of activity

According to the International Union of Biochemistry, one international unit of pectinase (1 U) corresponds to the amount of enzyme required to release 1 micromole of reducing substance (GA) in 1 minute. Enzyme activity was expressed as U.mL⁻¹.

Estimation of activity

The polygalacturonase activity was determined by measuring the amount of reducing substances liberated from citrus pectin. The reaction mixture consisted of substrate buffer (0.1 g citrus pectin dissolved in 100 mL of 0.1 M phosphate buffer, pH 5) 450 µL and enzyme solution 50 µL. This mixture was incubated at 50 °C for 30 min under shaking condition (800 rpm) in the thermo shaker. Later, 500 µL DNS reagent was added to the vial, kept in boiling water for 5 minutes and cooled in ice water. A blank was also prepared in the same way as mentioned earlier. The absorbance was read at 550 nm using spectrophotometer. One unit of enzyme activity (U) was defined as 1 µmole of galacturonic acid released per min

(Silva et al., 2002).

Calculation of enzyme activity

The enzyme activity was calculated by the following formula

$$\text{Activity} = \frac{\text{Dilution} \times \text{Absorbance}}{\text{Time of incubation (min)}} \times \text{Standard Factor}$$

$$\text{Standard factor} = \frac{\text{Concentration of standard solution (\mu\text{mole/mL})}}{\text{Absorbance at 550 nm}}$$

Enzyme characterization

Characterization of pectinase was performed with respect to temperature, pH and substrates following the method described by Coral et al. (2002).

Optimum temperature

For the estimation of optimum temperature, the enzyme assay was carried out at pH 6.5 in six different temperatures of 30 °C, 35 °C, 40 °C, 45 °C, 50 °C, and 55 °C.

Optimum pH

The best pH for enzyme activity was determined by carrying out the enzyme assay at optimum temperature in different pH levels (5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0).

Heat stability

The heat stability of enzyme was determined by subjecting it to different temperatures of 40 °C, 50 °C, 60 °C, 70 °C and 80 °C for 15 min. The percentage of original activity retained after heat treatment (80 °C for 15 min) was calculated.

Optimum incubation time

For estimation of optimum incubation time, the enzyme assay was carried out at optimum pH and temperature in different incubation time (15, 30, 45, 60, 75, and 90 min.).

K_m and V_{max} value

The reaction speed V_{max} and Michaelis constant K_m were

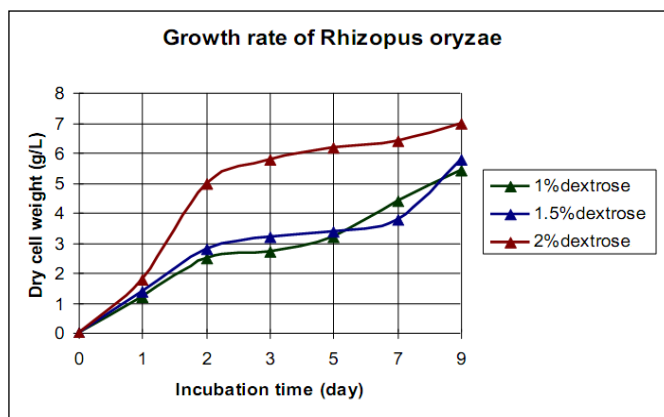


Figure 2 Growth curve of R.oryzae.

Table 3 Rate of change of cell mass growth (g/L/d).

At day	Rate of change of cell growth (g/L/d) at		
	1% dextrose	1.5% dextrose	2% dextrose
1	1.2	1.4	1.8
2	1.3	1.4	3.2
3	0.2	0.4	0.8
5	0.25	0.1	0.2
7	0.6	0.2	0.1
9	0.5	1.0	0.3

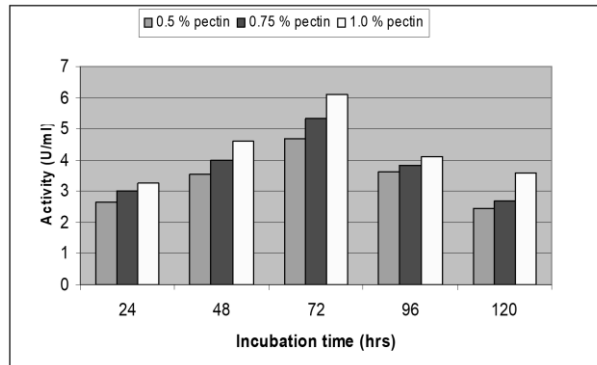


Figure 3 Effect of incubation period for pectinase production in different pectin substitute media.

determined for the enzyme by varying the substrate concentration from 0.25 – 5.0 mg.mL⁻¹ and plotting substrate/velocity as function of substrate concentration (Line-Weaver & Burk, 1934).

Substrate specificity

Pectin is the main substrate of pectinase enzymes. To assess the substrate specificity of extracted pectinase enzymes, starch, CMC in addition of pectin were incubated at optimum pH and temperature. Activities exhibited in different substrates will be used as a measure of substrate specificity of extracted enzymes after cell separation from fermented media.

RESULTS AND DISCUSSION

Growth of *Rhizopus oryzae* on potato dextrose

The prepared potato dextrose agar (PDA) medium was transferred aseptically to pre-sterilized Petri dishes. Then 100µl of mother culture of the organism was streaked onto an agar plate or petri dishes and incubated at 30 °C for 3 days to allow the spores to germinate. From the morphological study of *R. oryzae* and microscopic view of *R. oryzae* it was confirmed that it was the fungus of *R. oryzae*.

Optimization of growth rate of *Rhizopus oryzae*

The dextrose concentration of the culture medium is important for the growth of *R. oryzae*. In the present study the various concentrations of dextrose (1.0%, 1.5% and 2.0%) were used to optimize the growth rate of *R. oryzae*. During fermentation, it was observed that at 2% dextrose solution *R. oryzae* showed rapid growth and higher cell mass than that of at 1.5% and 1.0 % respectively. [Sentence deleted]. It is interesting that the growth rate of *R. oryzae* in fermentation media containing 1% and 1.5% dextrose showed almost the same trend throughout the fermentation period (Figure 2).

According to Table 3, the highest cell growth rate was found at day 2 irrespective of dextrose concentrations. After then cell growth rate drastically reduced and it remained almost unchanged up to day 7.

It means that the three media containing 1%, 1.5% and 2% dextrose respectively were reached in log phase within two days of incubation period and from day 2 to 7 the phase seems to be stationary. In log phase the rate of multiplication is rapid because of sufficient nutrient and favorable growth conditions whereas in stationary phase the fungus was hardly multiplied and the number remained almost static due to cease of nutrient. It is noticeable that the continuation of fermentation (after day 7) resulted in further increase of the dry cell mass. Though the following after stationary phase the fungus goes to die, the unusual increase of dry cell at day 9 might due to waste accumulation and unwanted metabolites in the fermentation media.

Production of pectinase

Pectinase enzyme exhibits extracellular behavior during metabolism of many funguses. In case of extracellular enzymes, they are excreted outside the cell of the microorganism into the media during fermentation. Due to extracellular nature of fungus, *R. oryzae* was used for the pectinase biosynthesis and various conditions including concentration of pectin in the medium, incubation temperature, pH of medium and incubation time were optimized.

Optimization of incubation time and pectin concentration in the medium

Various concentrations of pectin at the rate of 0.5%, 0.75% and 1.0% were added to the production medium and the enzyme production in the submerge fermentation (SmF) was studied for different period of incubation (24h, 48h, 72h, 96h and 120h) at control temperature of 35 °C

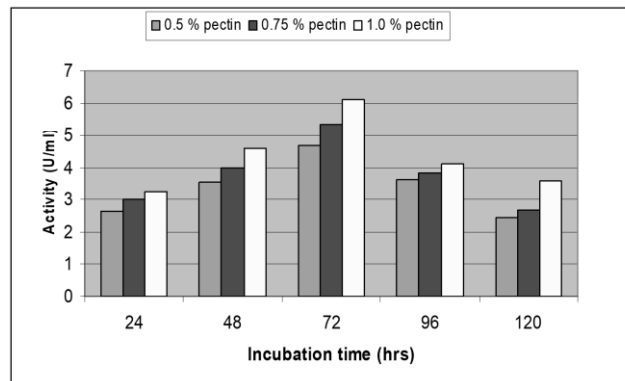


Figure 4 Effect of pH on pectinase production.

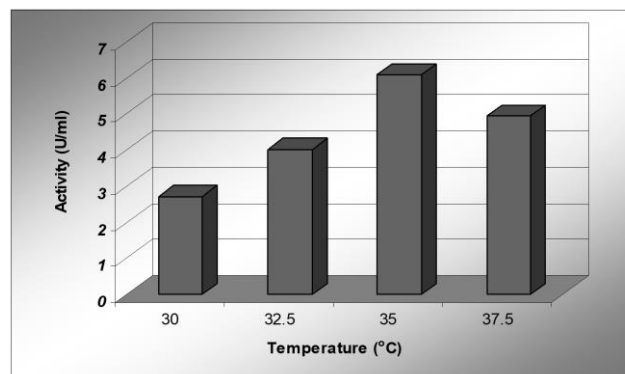


Figure 5 Effect of temperature on pectinase production.

and at pH 6.0. As shown in Figure 3, the maximum pectinase production was found in 72 hrs (4.70 U.mL⁻¹, 5.34 U.mL⁻¹ and 6.09 U.mL⁻¹) irrespective of pectin substitution in the fermentation medium. In comparison to the entire fermentation time, pectinase production in terms of volumetric activity was increasing gradually with increasing incubation time up to 72 hrs and afterwards it was decreasing following the same pattern.

This might due to the fungi would have entered in to its exponential phase within this period. According to Figure 2, though the log phase was shown up to day 2, it was observed in most of the cases of fermentation that the highest yield of enzymes was achieved at the end log phase and/or just starting of the stationary phase.

Thereafter the fungus started to die so that the metabolism was also stopped and enzyme production started to decrease. Excess waste accumulation and unwanted metabolites production are also responsible to decrease enzyme production in case of fermentation of extra-cellular enzyme producing microorganism.

The results of current work are in close conformity to the findings of Phutela et al. (2005). They reported that the maximal production of pectinase (415 U.g⁻¹) and PG (473 U.g⁻¹) was obtained after 48 and 72 h of incubation,

respectively. Further incubation resulted in the decline in enzyme activities up to 120 h.

In case of pectin substituted media at specific incubation time, pectinase activity increased with increasing pectin concentration. At 72 hrs incubation time, the highest activity (6.09) was found at 1% pectin substituted media and the lowest (4.70) was found at 0.5% pectin substituted media. The activity of pectinase at 0.75% substituted media was 5.34 U.mL⁻¹. At incubation time of 120 hrs, pectin substations at the rate of 0.5% and 0.75% have minor effect. But at incubation time 96 hrs the change in pectinase activity at different pectin solution were observed much close. The phenomenon in case of incubation time 24 hrs was almost the same. Likewise 72 hrs incubation, significant good variation in pectinase activity was observed at incubation time 48 hrs.

After separation of cells from fermentation media, the clarified solution was used as pectinase samples and it was observed that the solution with higher activity showed more turbid which resulted in higher absorbance reading in DNS solution. This is due to the higher concentration of protein as pectinase enzyme, which was secreted during metabolism of pectin in fermentation.

Table 4 Effect of temperature on the pectinase activity.

Temperature (°C)	Absorbance at 550nm	Volumetric activity (U.mL ⁻¹)
30 °C	1.76	2.80
35 °C	1.83	2.92
40 °C	1.90	3.03
45 °C	1.89	3.01
50 °C	1.77	2.85
55 °C	1.71	2.72

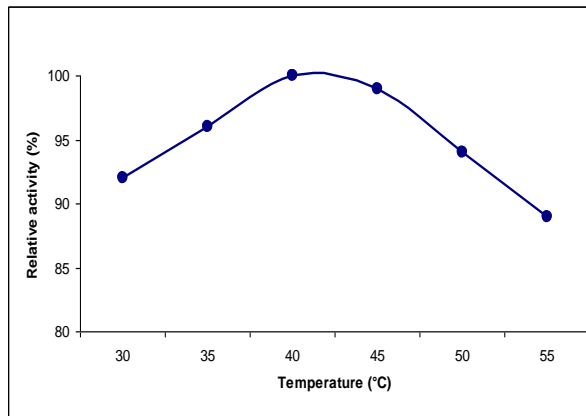


Figure 6 The relative activity of pectinase at pH 6.5 under different temperatures.

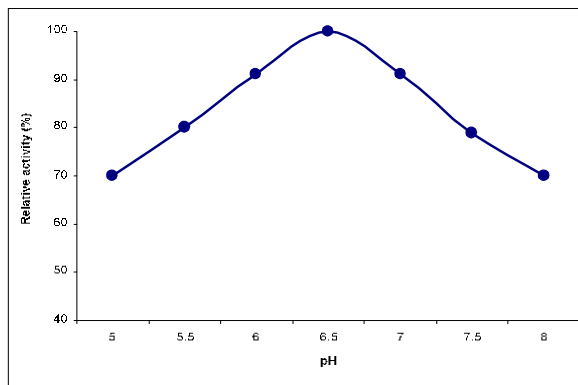


Figure 7 The relative activity of pectinase at 40 °C under different pH levels.

Effect of pH of cultivated media on pectinase production

The pH of the cultivation medium is an important factor in the production of pectinases for it influences the sort and content of those enzymes produced by fungus. The fermentation was carried out in medium containing 1.0% pectin at 35 °C and incubated at 72 hrs under pH 5.0, 5.5, 6.0, 6.5 and 7.0.

In our study the maximum activity of 6.09 U.mL⁻¹ was found at pH 6.0. Either increase or decrease of pH beyond the optimum value showed decline in enzyme production (Figure 4). However, the mechanism by which the pH acts

on the production of pectic enzyme is not known. The increasing levels of pH significantly affected the enzyme production. The enzyme activity was increased from 3.62 U.mL⁻¹ to 6.09 U.mL⁻¹ at pH 5.0 to 6.0 respectively then it decreased progressively to 4.58 U.mL⁻¹ at pH 7.0.

The results were more or less similar to those reported by Banu et al. (2010). They reported that the enzyme production by *Penicillium chrysogenum* was higher at pH 6.5 and a temperature of 35 °C. Piccoli-Valle et al. (2001) observed that a high polygalacturonase and pectin esterase activity was showed by *P. griseoroseum* in more acid pH of 4.5 and 5 and of pectinlyase, pH was close to the

Table 5 Effect of pH on the pectinase activity.

pH	Absorbance at 550nm	Volumetric activity (U.mL ⁻¹)
5.0	1.408	2.24
5.5	1.596	2.54
6.0	1.811	2.89
6.5	1.985	3.16
7.0	1.816	2.89
7.5	1.581	2.52
8.0	1.404	2.23

Table 6 Effect of heat treatment on the pectinase activity.

Temperature (°C)	Volumetric activity (U.mL ⁻¹)	Relative activity (%)	% loss of activity
40 °C	3.03	100	0
50 °C	2.76	91	9
60 °C	2.71	89	11
70 °C	2.23	74	26
80 °C	0.87	28	72

Table 7 Effect of incubation time on the pectinase activity.

Time (min.)	Relative activity (%)
10	95
20	95
30	100
40	93
50	85
60	72
70	68
80	60
90	52

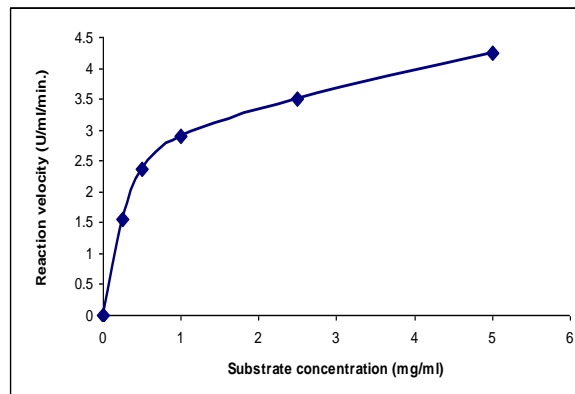


Figure 8 Effect of substrate concentrations on pectinase activity of *R. oryzae*.

neutral, 5 – 7. *P. viridicatum* showed maximum production of polygalacturonase and pectinase at a pH of 4.5 and 5 respectively (Silva et al., 2002).

Effect of temperature of cultivated media on pectinase production

The fermentation was carried out in medium containing 1.0% pectin at pH 6.0 and incubated at 72 hrs under different temperatures such as 30 °C, 32.5 °C, 35 °C and 37.5 °C. In our study the maximum activity of 6.09 U.mL⁻¹ was found at 35 °C. The pectinase production increased with rise in temperature 30 °C to 35 °C and exhibited maximum activity at 35 °C. With a rise in temperature over 35 °C the pectinase production was decreased (Figure 5).

Similar result was found by Banu et al. (2010). They reported that the enzyme production by *Penicillium chrysogenum* was higher at pH 6.5 and a temperature of 35 °C using sucrose and ammonium sulphate as carbon and nitrogen source, respectively. However, Chellegatti (2002) reported that the temperature optima of 50 °C were obtained from a purified culture fluid of *P. frequentans*.

Characterization of pectinase

After separation of cells from fermentation media, pectinase solution was subjected to characterization to find the optimum conditions for its best activity. Data regarding characterization of pectinase would be a guide line for its application in food products. For this purpose, pectinase produced in the study was characterized for temperature, pH, heat stability, incubation time and K_m values.

Effect of temperature on the pectinase activity

The effects of temperature on enzymatic activities were investigated at pH 6.5 under different temperature and the

results showed in Table 4. The maximum activity (3.03 U.mL⁻¹) was found at temperature 40 °C and minimum activity (2.72 U.mL⁻¹) was found at temperature 55 °C.

The Figure 6 depicts the effect of different temperatures on relative activity of pectinase. It is obvious that when enzyme assay was performed at various temperatures, the pectinase activity increased with rise in temperature up to 40 °C and exhibited maximum activity. However, further increase in temperature caused a decrease in activity. Minimum activity was observed at 55 °C; the highest temperature studied in the present study.

The results of current work are in close conformity to the findings of Martin et al. (2004). They reported that the purified polygalacturonase from the fungus *Penicillium sp EGC5* exhibited highest activity at 40 °C. Likewise, Riou et al. (1992) calculated optimum activity of exopolymethylgalacturonase from the fungus *Sclerotinia sclerotiorum* at 45 °C. However, Banu et al. (2010) reported 50 °C as optimum temperature for the activity of pectinase enzyme from the fungus *Penicillium chrysogenum*.

Effect of pH on the pectinase activity

The effects of pH on enzymatic activities were investigated at temperature 40 °C and the results showed in Table 5. For estimation of optimum pH, the enzyme assay was carried out at optimum temperature (40 °C) in different pH levels ranging from 5 to 8.0. The increasing level of pH influenced the activity of pectinase enzyme. At pH 5.0 to 6.5, the activity of pectinase increased from 2.24 U.mL⁻¹ to 3.16 U.mL⁻¹ then it decreased progressively to 2.23 U.mL⁻¹ at pH 8.0.

The graphical representation of the relative activity of produced enzymes under the influence of different pH

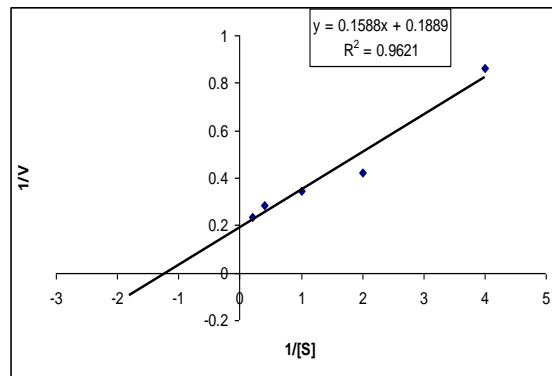


Figure 9 Lineweaver-burk plot for the hydrolysis of pectin by the pectinase activity of *R. oryzae*.

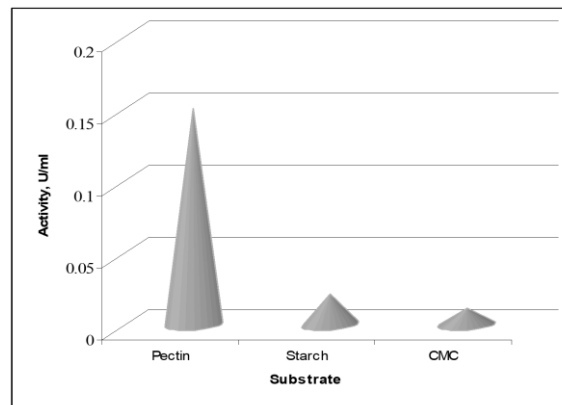


Figure 10 Substrate specificity of extracted enzyme at optimized pH and temperature.

values indicated that the enzyme exhibited minimum relative activity at pH 5.0 (2.24 U.mL⁻¹) (Figure 7). When the pH was raised above 5.0, a gradual increase in enzyme activity was observed and at pH 6.5 the enzyme exhibited maximum activity (3.16 U.mL⁻¹). However, further increase in pH showed a gradual decrease in pectinase activity.

The results of current work are similar with the findings of **Banu et al. (2010)**. They found the maximum pectinase activity from *Penicillium chrysogenum* in the pH 6.5. Likewise, **Arotupin et al. (2008)** reported that the optimum temperature for pectinmethylesterase (PME) activity was 30 °C and most active at pH 6.5. **Martin et al. (2004)** reported that polygalacturonase (PG) from *Penicillium sp.* was stable at pH range of 3 – 8 and maintained 70% of initial activity at 70 °C. Pectin lyase (PL) produced by this microorganism was stable in acidic to neutral pH (4.0 – 8.0) and was stable in temperature lower than 40 °C.

Heat stability of pectinase enzyme

Heat stability of the enzyme under different temperatures is important regarding its application in different processes. The responses of produced enzymes against heat are shown in Table 6.

When the activity of the pectinase was detected at different temperatures, it showed maximum activity at 40 °C (Table 6). Considering this activity as 100%, the enzyme was subjected to the elevated temperature in order to find out the stability of pectinase. The increase in temperature showed a negative effect on the enzyme performance and activity loss was higher as the incubation temperature was kept higher than 60 °C. When the

temperature was 70 °C, the enzyme exhibited 74% enzyme activity infers that it lost 26% of the total activity. However, when the enzyme activity was calculated at 80 °C, it exhibited only 28% enzyme activity infers that it lost 72% of the total activity. The decrease in the heat stability is certainly being due to enzyme denaturation at higher temperatures.

The findings of the present study are supported by the results found by **Banu et al. (2010)**; they produced pectinase from the fungus *Penicillium chrysogenum* that was stable at a temperature 40 °C up to 60 min.

Effect of incubation time on the pectinase activity

The effects of incubation time on enzymatic activities were investigated at pH 6.5 and 40 °C under 10 to 90 min (Table 7).

As shown in Table 7, the influence of different incubation time on the enzyme activity indicated that the activity of pectinase enzyme produced by *Rhizopus oryzae* was increased up to the first 30 mins. After then enzyme activity was decreased gradually with increasing reaction time. It indicated that the reaction was seized within this time due to completion of hydrolysis of all most all substrate within this time. The further increase in incubation time resulted in a gradually decrease in relative activity. This might due to the change of its structure or conformity during prolong exposure in solution.

The findings of the present study are supported by the results found by **Banu et al. (2010)**; they produced pectinase from the fungus *Penicillium chrysogenum* that was stable at a temperature 40 °C up to 60 min.

Determination of K_m and V_{max} of pectinase enzyme from *R. oryzae*

The activity of the enzyme increased from 1.16 to 4.25 U/ml/min with increasing in the substrate concentration from 0.25 to 5.0 mg.mL⁻¹ and was found maximum activity at 5 mg.mL⁻¹ and further increase in substrate concentration above the optimum level will not produce any increase in the enzyme activity (Figure 8). This is happened when residual substrate is reached too low to continue further reaction.

The kinetic parameter of pectinase enzyme from *Rhizopus oryzae* was determined. The Lineweaver-Burk plot for the hydrolysis of pectin showed a K_m of 0.84 mg.mL⁻¹ and V_{max} of 5.294 U.mL⁻¹ of enzyme (Figure 9).

The results of current work are similar with the findings of Arotupin et al. (2008). They reported that the activity of the enzyme produce by *Aspergillus repens* increased with increase in substrate concentration reaching maximum at 4 mg/mL and the Lineweaver-Burk plot for the hydrolysis of pectin indicated approximately 1.3 mg.mL⁻¹. Likewise Banu et al. (2010) reported that the pectinase produced from *Penicillium chrysogenum* have the K_m and V_{max} values of 1.0 mg.mL⁻¹ and 85 U.mg⁻¹ of protein respectively.

Substrate specificity

Pectin is the major substrate of pectinase enzyme. To assess the presence of different enzymes other than pectinase enzymes, extracted enzyme solution was incubated with starch and CMC, other than pectin. The activity of produced enzymes in different substrates is shown in Figure 10. As shown in Figure 10, there is hardly any activities observed in starch and CMC.

CONCLUSION

The current exploration was an effort to produce pectinase enzyme by *R. oryzae*. In the production medium dextrose at three concentrations (1%, 1.5% and 2%) were used to optimize the growth rate of *R. oryzae*. Dextrose replaced with pectin was used as a carbon source by the organism through submerged fermentation technique. Pectin replaced at three concentrations (0.5, 0.75 and 1.0%), five pH levels of the culture medium (5.0, 5.5, 6.0, 6.5 and 7.0), four incubation temperatures (30 °C, 32.5 °C, 35 °C and 37.5 °C) and five fermentation periods (24, 48, 72, 96 and 120 hrs), were used to optimize the conditions for maximum pectinase activity. The outcomes of the fermentation process showed that at 2% sugar solution *R. oryzae* showed rapid growth and higher cell mass and dextrose replaced with 1% pectin was the best carbon sources for pectinase production as compared to that of 0.5% and 0.75% pectin. It showed maximum pectinase activity 6.09 U.mL⁻¹ at 35 °C, pH 6.0 and 72 hrs fermentation period. After optimization of culture conditions, the laboratory scale production of pectinase was carried out using dextrose replaced with 1% pectin as carbon source at 35 °C, pH 6.0 and 72 hrs of incubation for its further use. The produced pectinase was then stored at refrigerated temperature in sterilized test tubes and subjected to characterize for its optimum pH (5.0 – 8.0), temperature (30 – 70 °C), heat stability, and substrate

(pectin) concentration (0.25 to 5.0 mg.mL⁻¹). The kinetic parameter K_m and V_{max} of the produced pectinase enzyme was also determined. Later on, to assess the presence of different enzymes other than pectinase enzymes, extracted enzyme solution was incubated with starch and CMC, other than pectin. It was found that the maximal activity of *R. oryzae* secreting pectinase was found at pH 6.5 and 40 °C whereas showed 91% heat stability at 60 °C. The activity of the enzyme increased with increasing the substrate (pectin) concentration from 0.25 mg.mL⁻¹ to 5.0 mg.mL⁻¹ and was found maximum activity at substrate concentration of 5.0 mg.mL⁻¹. K_m and V_{max} values were found 0.84 mg.mL⁻¹ and 5.294 U.mL⁻¹, respectively at optimized conditions. The activity of produced enzymes in different substrates was shown that there is hardly any activity observed in starch and CMC other than pectin. In conclusion, pectinase enzyme is an important tool for many processing industries especially for fruit juice, wine and textile industry. However, a limited supply of this enzyme, high cost and complicated produces for production locally restricts wider use. The results obtained here will be useful for further research in low cost production of pectinases from *R. oryzae* for domestic consumption in many industries. Research revealed that solid state fermentation using low cost renewable substrate is excellent alternative in reducing cost of enzyme production. By solid state fermentation it is possible to produce enzymes with higher activity and higher concentration of protein. Therefore, further research of producing pectinase enzyme by employing solid state fermentation might result in a low cost and economically viable process.

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