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Pilot scale production and characterization of pectinase enzyme from *Aspergillus niger*

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SUMMARY :

Food grade pectinases from *A. niger*, are predominantly used as processing aids in industries. In present investigation, efforts were made to standardize pilot scale production of pectinase enzyme by *Aspergillus niger*. The crude enzyme was studied for its kinetics to optimize substrate concentration and processing parameters on the basis of polygalacturonic activity. Km was determined by measuring reaction velocities while temperature and pH range was standardized on the basis of activity of polygalacturonase. The results revealed that km was observed to be 2.43 mg/ml. It was found that the enzyme exhibited maximum activity at 40°C. Increase in temperature above 40°C, the enzyme activity was affected negatively coupled with gradual reduction. The polygalacturonase recorded optimum activity (5.78 µmol/ml/min) at pH 4.5 and 9 mg/ml substrate concentration, respectively enzyme activity. Linear increase with in increased in substrate concentration, exhibited by pectinase is in agreement with the results recorded by earlier scientists.

KEY WORDS : Pectinase, *Aspergillus niger*, Kinetics, Polygalacturonase activity, Enzyme

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Profound increase in industrial applications of food grade enzymes laid down a mile stone to assess biotechnological potential in fruit and vegetable processing industries. The enzymes are used in processing agricultural and agro-industrial waste, clarification of fruit juices and wines, extraction of vegetable oils, reduction of viscosity of concentrates, fermentations of coffee and tea, production of paper, treatment of natural fibres (linen and ramie fibres) and degumming of plant fibres (Jin and Masako, 2001). Pectinolytic enzymes catalyzing the degradation of pectic substances are of great industrial importance (Meyrath and Volavsek, 1975). Pectinases have extensive applications in fruit juice industries in order to improve fruit juice yield and clarity (Sartoglu *et al.*, 2001). The use of liquefying enzymes for mash treatment results in improvement of free flowing juice flow, leading to a lower press time even in absence of suitable pressing aids. At the same time pectin is broken down into such

an extent that the viscosity of mash is considerably reduced (Mutlu *et al*., 1999). The importance of food grade enzymes for maceration induced liquefaction, pulping, homogenization, clarification, cloud stabilization and texturization become indispensable for monitoring quality parameters of fruit processed products (Bhat, 2000).

It significantly improves the process-ability of fruit mass and yield of the juice. The liquefaction technique is relatively simple and techno-economical to stimulate higher yield of clear or cloudy juices. This technology is applicable for pulpy fruits (mango, guava and banana) where tissue entrapped juice cannot be obtained by crushing and pressing. Liquefaction also helps in monitoring the losses of nutrients occurring during mechanical pressing, Production of carrot juice as multicomponent fleshy mass (Nilay, 2001). Enzyme kinetics is the study of the chemical reactions that are catalyzed by relative enzymes. The reaction rate is measured and also the overall effect of governance conditions of the reaction is critically investigated. The enzyme kinetics in this way can reveal the nature of catalytic mechanism role in metabolism, activity control and enzyme inhibition made characterization. The fundamental purpose of enzyme kinetic studies is to investigate enzyme substrates affinity for reaction characterization. The main objective of this study was to assess the importance of biotechnological applications in fruit processing present investigation is designed to standardize the technology of preparation of pectinase enzyme and to study its enzyme kinetics in terms of polygalacturonase activity.

EXPERIMENTAL METHODS

The *A. niger* Van *Tiegh* as a protected organism was obtained from BARC, Mumbai on agreement for commercial production of enzyme. The notified substrate standardized growth medium was used as provided by high-media group from Latur (M.S.) India. Media and fermentation conditions specified in the production technology developed by (Pawar, 1994) is used for pilot scale production. Pectin and polygalacturonic acid sodium salt were obtained from Sigma chemicals, used as the inducer as well as substrate for elaboration of pectinolytic enzymes. All the other chemicals used were of analytical grade. Predesigned experiments were undertaken with the help of equipments and machines available in Department of Food Science and Technology, College of Agriculture Technology, Marathwada Krishi Vidyapeeth, Parbhani were used as and when required.

Production of crude enzyme :

Crude enzyme extract (CE) was obtained by the process of submerged fermentation in Biostat B^+ fermenter. The 90 ml inoculums of spore suspension (ca. 1×10^6 spores/ml) of *A.niger* inoculated in 4 L synthetic media contained in a 5 L capacity vessel of fermentor. The fermentation process was carried out at controlled conditions of pH (4.5), temperature ($28 \pm 2°$ C) and incubation period of 100 h. At the end of incubation period, the contents were filtered through Whatman filter paper number 541. The culture filtrates (CF) were used as a source of crude pectinolytic enzyme. The mycelial mass from fermentor was dried at 70°C till a constant weight is attainted. The residual mycelia dry weights (biomass) were used as a measure of growth of the respective organisms.

Effect of temperature on activity of polygalacturonase :

The optimum temperature of the enzyme was determined by incubating the enzyme with citrus pectin at 30-60°C at interval of 5°C for 20 min and pH 5.0. The activity was then assayed as per the method of (Natalla *et al*., 2004). A plot of temperature versus activity was plotted to obtain the optimum temperature of the enzyme by the method of Stauffer and Etson (1969).

Effect of pH on activity of polygalacturonase :

The optimum pH of the enzyme was determined using acetate buffer of pH 3.5 - 5.5 at interval of 0.5 by dispersing the enzyme in 0.05 M acetate buffer in the various pH and then taking 0.1 ml of the dispersed enzyme for assay as per the method of Miller (1959). A plot of enzyme activity versus pH was plotted to determine the optimum pH of the enzyme.

Determination km of pectinase :

The Michaelis constant (km) of the purified PG was determined by measuring the reaction velocities (μ mole galacturonic acid / min) at various

concentrations of pectin (mg/ml^{-1}) at 40°C for 20 min. The data were plotted according to Lineweaver-Burk plot Lineweaver and Burk (1934).

EXPERIMENTAL FINDINGS AND ANALYSIS

The standard process developed (Pawar, 1994) for production of technological pectinase preparation at laboratory level was used as a bench mark base to scale up the production by using Biostat B$^+$ Fermentor (5 L capacity) from college of Food Technology and standard culture *Aspergillus niger* borrowed from BARC, Mumbai. The standardized fermentation conditions were used to scale-up the pectinase production.

Pectinase production :

It is cleared from the data that there were on pilot scale production of pectinase (Table 1) scaling-up of production technology recorded satisfactory results in comparison with laboratory scale production. The production of pectinase per unit weight of dry mycelia in case of pilot scale process found substantially increased *i.e.* (0.15 L) as compared to laboratory scale technology *i.e.* (0.1 L). 18 per cent increase in yield of crude enzyme extract under similar conditions in case of Biostat B$^+$ fermentation technology is encouraging to underline the strategic guidelines to proceed for large scale production. Seven fold increases in dry mycelial weight may be associated with provision of favourable growth conditions explored by Biostat $^+$ fermentor. Similar trend of pectinase production was also recorded by (Panda, 2004); Busto (2006) and Lee (2006).

Effect of temperature on activity of polygalacturonase :

The temperature as a variable for monitoring PG activity was assessed by reaction of Polygalacturonase in 20 mm phosphate buffers pH 5.0 at the temperature ranging from 30 to 60°C for 20 min. the relative activity of CE Pectinase presented in Fig. 1 indicated that the enzyme exhibited maximum activity at 40°C. Increased temperature above 40°C recorded negative effect leading to gradual reduction in activity, whereas, similar trend was also recorded at a temperature below 40°C. Denaturation of the enzyme protein occurs at elevated temperatures therefore, rapid decrease in the activity was also recorded along with increased temperature. From the tabulated data it was appeared that enzyme is most efficient at 40°C as compared with other tested temperatures. The present results are in harmony with the recent findings of Rashmi *et al*. (2008), Buga *et al*. (2010). Similar trends of result as temperature variable exhibited at 35° C, 40° C and 45° C as optimum was also recorded by earlier scientists.

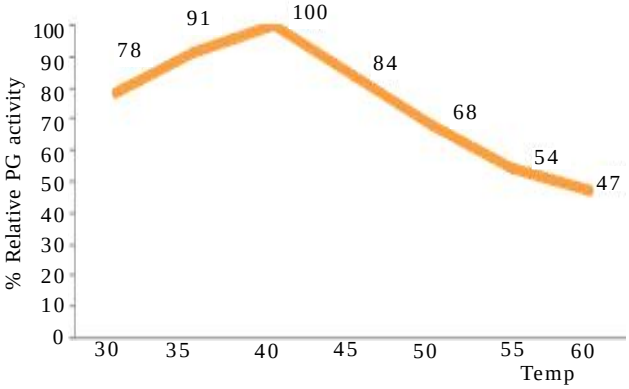

Fig. 1 : Effect of temperature on activity of polygalacturonase

Effect of pH on polygalacturonase activity :

The effect of pH of polygalacturonase activity was assessed by using pH range of 3.5 to 5.5 at the standard reaction time and temperature, respectively (20 min and 40° C). The data on effect pH on PG activity presented in (Fig. 2) indicated that the pH optimum for its highest activity is 4.5. Fungal polygalacturonases are known to operate functionally in mild acidic environment. Sebastian *et al*. (1996) and De Vries and Visser (2001) reported a very wide range of optimum for *A. niger* polygalacturonases pH (3.8-9.0). Lucie (2000) reported pH optima for

Table 1 : Effect of method on pectinase production

Sr. No	Methodology	Substrate Vol. (L)	Inocul. Vol. (ml)	Dry mycelia wt.(g)	Crude pectinase extract (L)	Pectinase extract per unit wt. of dry mycelia	% yield
1.	Pilot scale method (5 L capacity)	4	90	21	3.2	0.15	80
2.	Laboratory method (1L flask)	0.5	10	3.1	0.31	0.10	62

polygalacturonases isolated from various species within the range of pH 3.8-6.5. The pH optima for polygalacturonases (30 fungal species) reported by Suryakant *et al*. (2001) ranged from 2.5 - 6.0. The graphic illustration elaborates on enzyme activity at different pH values; the highest enzyme activity was recorded at 4.5 pH. Increase in pH up to 5.5, reported a declined trend in the enzyme activity. Similarly enzyme activity at lower pH (4.5) also exhibited decreased trends. Moreover, the enzyme reported maximum activity at pH 4.5. The results are in close conformity with those of Buga *et al*. (2010) who reported optimum pH for Pectinase in the range 4.5-5.0. Rashmi *et al*. (2008), also reported that the characterization of Pectinase enzyme obtained from *Aspergillus* sp. submerged fermentation as 1 per cent pectin concentration as stimulator and pH 4.5 speciality fermentation character.

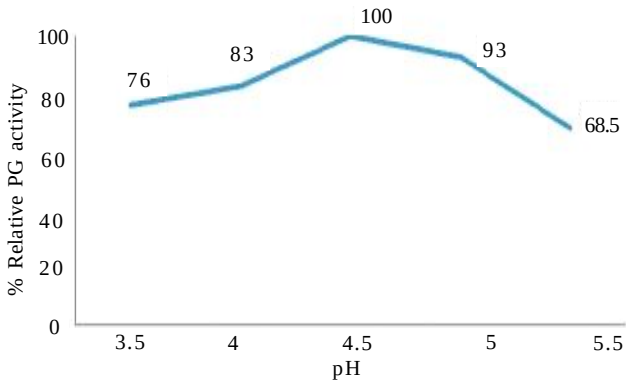

Fig. 2 : Effect of pH on activity of polygalacturonase (PG)

Effect of substrate concentration on PG activity :

Effect of substrate concentration on the activity of PG was assessed by incubating the enzyme with 1, 3, 5, 7, 9 and 11 mg/ ml of pectin and the results are summarized in (Fig. 3) indicated that PG activity increased with the increase in substrate concentration, and reached maximum (5.78 µmol/ml/min) at 0.9 mg/ml concentration of pectin. Thibault and Mercier (1978) also reported maximum activity of PG from *A. spergillus niger* at 0.5 per cent of pectin. Linear increase in PG activity with increase in substrate concentration may be attributed to the successive binding of the substrate to the active sites of enzyme. Further increase in substrate concentration above optimal level will not exhibit further increase in the enzyme activity because no enzyme molecule will be available to react with the substrate Segel (1976).

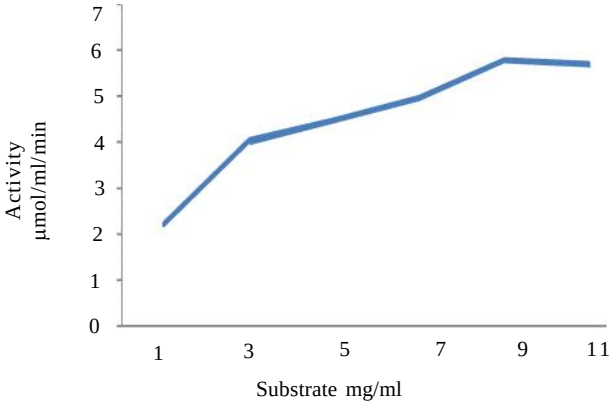

Fig. 3 : Effect of substrate concentration on PG activity

Km of pectinase :

The km value as an indicator of the affinity of pectinase to substrate was determined by using double reciprocal Lineweaver and Burk plot. Lineweaver and Burk plot based on reaction parameters of PG and hence the km value is characterized by 2.43 mg/ml at 4.5 pH and 9 mg/ml pectin as an accessible substrate concentration. The graphical data presentation (Fig. 4) revealed that apparent km value was calculated from the line weaver burk plot. The km was found to be 2.43 mg.ml^{-1} (Fig. 4). The km of PG acting on citrus pectin seemed to be relatively high, indicating comparative low affinity of the enzyme to its substrate associated with its crude nature, against km value for PG from *Aspergillus niger* using polygalacturonic acid as substrate.

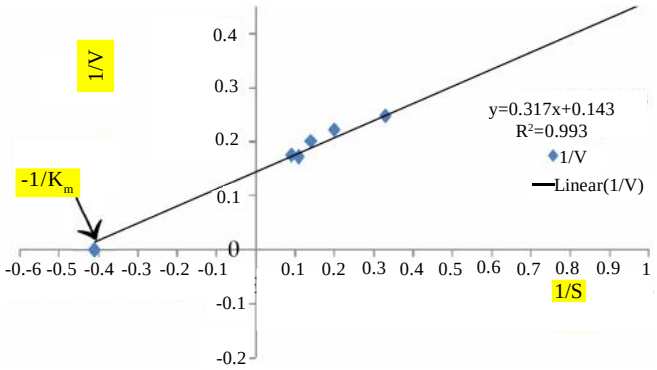

Fig. 4 : Lineweaver - Burk plot

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