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Production kinetics and characterization of pectinase enzyme from *Aspergillus niger*

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Abstract

Food grade pectinases from *Aspergillus niger* are predominantly used as processing aids in industries. In the present investigation, efforts towards standardization for pilot scale production of pectinase enzyme from *A. niger* were made. The crude enzyme was studied for its kinetics to optimize substrate concentration and processing parameters on the basis of polygalacturonic activity. Km (Michaelis-Mentenconstant) 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 2.43mg/mL and that the enzyme exhibited maximum activity at 40°C, beyond which the enzyme activity was affected negatively coupled with gradual reduction. The polygalacturonase recorded optimum activity of 5.78 µmol/ml/min at pH 4.5 with substrate concentration of 9 mg/ml respectively, of enzyme activity. Linear increase exhibited by pectinase with increased substrate concentration, remains in agreement with the previously recorded scientific literature.

Key words: Pectinase, *Aspergillusniger,* kinetics, polygalacturonase activity, enzyme.

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Profound increase in industrial applications of food grade enzymes laid down a milestone 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 fibers (linen and ramie fibers) and degumming of plant fibers (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 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).

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. The fundamental purpose of enzyme kinetic studies is to investigate enzyme substrates affinity for reaction characterization. The objective of this study was to assess the importance of biotechnological interventions in fruit processing and was designed to standardize the technology of preparation of pectinase enzyme and study its enzyme kinetics in terms of polygalacturonase activity.

Materials and Methods

The *A. niger* Van *Tiegh* as a protected organism was obtained from Bhabha Atomic Research Centre, Mumbai on agreement for commercial production of enzyme. The notified substrate standardized growth medium was used as provided by Hi-Media group from Latur (MS) India. Media and fermentation conditions specified in the production technology developed by Pawar (1994) were used for pilot scale production. Pectin and polygalacturonic acid sodium salt were obtained from Sigma Chemicals, and were 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 equipment and machines available in Department of Food Science and Technology, College of Agricultural Technology, VRNMKV, Parbhani, India 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 90mL inoculums of spore suspension (ca. $1x10^6$ spores / mL) of *A. niger* were inoculated in 4L 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 $28\pm2^{\circ}$C and incubation period of 100 hour. At the end of incubation period, the contents were filtered through Whatman filter paper No. 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 was 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 over 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). Temperature versus activity graph 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 over a pH range of 3.5 - 5.5 with an 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 of Km of pectinase: The Km (Michaelis-Menten constant) of the purified polygalacturonase was determined by measuring the reaction velocities (µ mole galacturonic acid / min) for various concentrations of pectin (mg/ml^{-1}) at 40^{o}C for 20 min. The data were plotted according to Lineweaver-Burk plot Lineweaver and Burk, (1934).

Results and Discussion

The standard process developed by Pawar, (1994) for 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 Agricultural 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 clear from the data that 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 was found to substantially increase i.e. (0.15 L) as compared to laboratory scale technology i.e. (0.1 L). Increase in 18% 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 increase in dry mycelial weight may be associated with provision of favorable 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 (PG): 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.

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.

Effect of pH on Polygalacturonase activity: The effect of pH on polygalacturonaseactivity was assessed 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 indicate that the pH optimum for its highest activity is 4.5. Fungal polygalacturonases are known to operate functionally in mild acidic

Table 1: Effect of methods on pectinase production.

S. 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

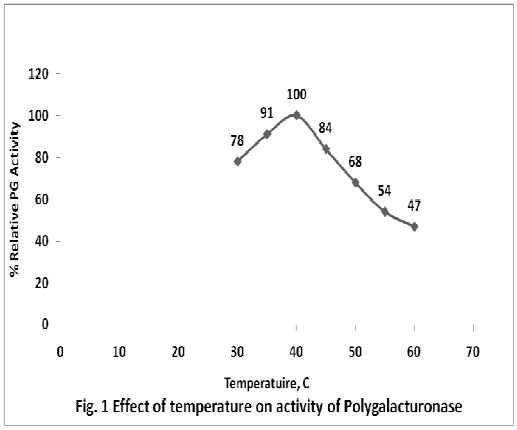

Fig. 1 Effect of temperature on activity of Polygalacturonase

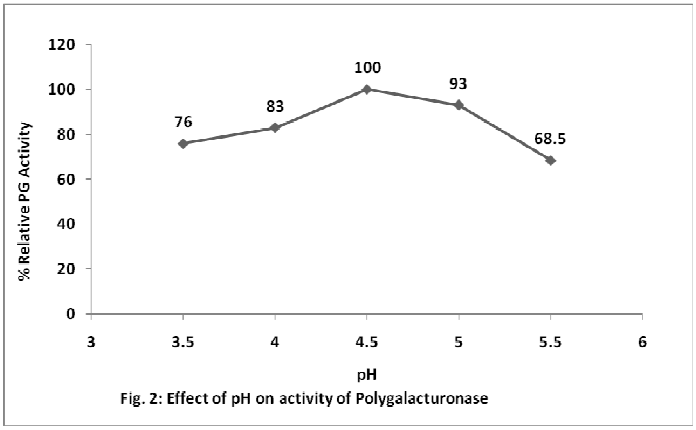

Fig. 2: Effect of pH on activity of Polygalacturonase

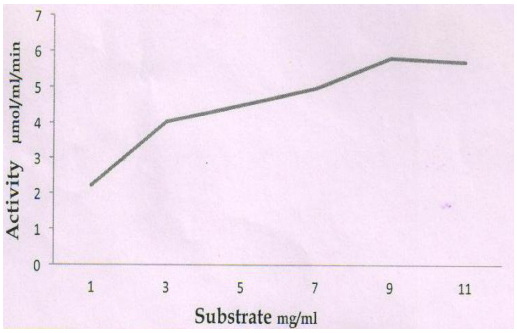

Fig. 3: Effect of substrate concentration on PG activity

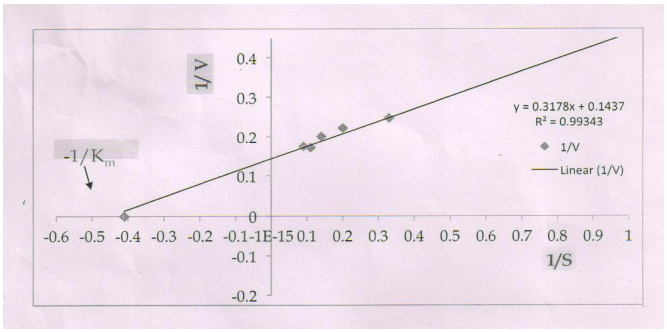

Fig. 4: Lineweaver-Burk Plot

environment. Sebastian *et al*., (1996) and De Vries and Visser, (2001) reported a very wide range of optimum pH (3.8-9.0) of polygalacturonases for *A. niger*. Lucie, (2000) reported pH optima for 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 pH 4.5. Increase in pH up to 5.5, recorded a declined trend in the enzyme activity. Similarly enzyme activity at lower pH (4.5) also exhibited decreased trends. However, 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.

Effect of substrate concentration on Polygalacturonase 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 summarized results at Fig. 3 indicate that PG activity increased with the substrate concentration and reached maximum (5.78µmol/ml/min) at 0.9 mg/ml concentration of pectin. Thibault and Mercier, (1978) also reported maximum activity of PG from *Aspergillus niger* at 0.5% 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 increase in the enzyme activity because no enzyme molecule will be available to react with the substrate (Segel, 1976).

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 is based on reaction parameters of PG and hence the Km value is characterized by 2.43 mg/ml at 4.5 pH and 9mg/ml pectin as an accessible substrate concentration.

The graphical data presentation (Fig. 4) reveals 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.

Conclusion

In order to increase the productivity of processed products and to compensate with innovative demands of overseas and domestic consumers, it is the need of the hour to upgrade the conventional status of Indian food industry. This aspiratory achievement could be positively restructured by introducing modern processing technologies like enzymatic processing of pulpy fruits, juice and pulp concentration with aroma recovery, diffusion extraction of fruit juice, ultra filtration, reverse osmosis and nano-processing etc. In view of emerging importance of bio-technological applications in fruit processing endowed by the use of enzymes in liquefaction to impart easiness in extraction of finished products, provides an option to upgrade the in-line-processing scenario to compensate with their qualitative and quantitative future needs. Hence present investigation is designed and formulated with specific intension to achieve techno-economical feasibility of fruit processing by involvement of biotechnology. The standard laboratory process developed for production of technological pectinase preparation by *A. niger van Tigh* was used as a bench 54 mark base to scale up the enzyme production by using Biostat B+ Fermentor (5 L capacity) under previously standardized culture conditions.The efforts were also made to study effect of method on pectinase production. The resulting 18% increase in yield of crude enzyme extract under controlled conditions in case of Biostat B+ fermentation technology is encouraging to underline the strategic guidelines to proceed for commercial production by co-relating it with seven-fold increase in dry mycelial weight. It may be associated with provision of favorable growth conditions explored by Biostat B+ fermentor. Protein content of crude enzyme extract obtained from pilot scale process using Biostat B+ fermentor was found to increase significantly as compared to conventional method of enzyme production (i.e. from 0.30 to 0.45mg/ml). Coincidently the activities of constituent enzyme of heterogeneous preparation were also found at par with that of laboratory scale production. The data emerging from Km investigation helped in characterization of pectinase activity profile. The 55 pilot scaling up method of pectinase production was found to be significantly encouraging over laboratory method.

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