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Purification and characterisation of an acidic pectin lyase produced by *Aspergillus ficuum* strain MTCC 7591 suitable for clarification of fruit juices

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Abstract - An acidic pectin lyase (E.C 4.2.2.10) produced by *Aspergillus ficuum* MTCC 7591 of molecular weight 31.6 kD was purified to apparent homogeneity by ion exchange and gel filtration chromatography. Eighty-six fold purification with 60% yield and a specific activity of 7.8 U/mg protein was obtained. The K_m and calculated turnover number (kcat) of the purified enzyme were found to be 0.60 mg/ml and 74 s^{-1} respectively using citrus pectin as the substrate. The pH and temperature optima were 5.0 and 50 ^{0}C respectively. Exposed to 24 hours at a particular pH the enzyme was found to be relatively stable in the pH range 2.0-9.0. Exposed to a particular temperature for 1 hour, the enzyme retains full activity up to 40 ^{0}C. Metal ions and protein inhibitors did not have significant effects on the activity of the enzyme. The enzyme has been found to be very effective in the clarification of sweet lime and orange juices.

Key words: *Aspergillus ficuum,* juice clarification, pectin lyase, pectinases, pectin, submerged fermentation.

INTRODUCTION

Pectic substances belong to a class of complex heterogeneous and multifunctional polysaccharides forming a hydrated cross-linked three dimensional network in the matrix of primary plant cell walls and play a diverse role in cell physiology, growth, adhesion and separation (Jarvis *et al*., 2003). The matrix polymer identified as an adhesion molecule is a pectic polysaccharide (Mollet *et al*., 2000). The pectin rich region of the plant cell wall the middle lamella, acts as a glue to hold adjacent cells together (O'Neill *et al*., 1990). The pectin degrading enzymes have the potential to degrade adhesion molecules and release the adjacent cells. According to the cleavage site, these enzymes are divided into three groups: (a) hydrolase consisting of polygalacturonase, PG (E.C. 3.2.1.15); (b) lyase/ transeliminase comprising of pectin lyase, PNL (E.C.4.2.2.10) and pectate lyase, PL (E.C. 4.2.2.2); (c) pectin esterase, PE (E.C.3.1.1.11). Among all pectinases, lyases are the ones which cleave α-1,4 bonds between galacturonic acid residues by transelimination mechanism to give oligosaccharides with terminal 4-deoxy-6-methyl-α-D-galact-4-enuronosyl groups (Linhardt *et al*., 1986). Acidic pectin lyases are specifically preferred in the fruit juice industries as they don't require the parallel action of any other enzyme. Instead they degrade highly esterified pectins (like those found in fruits) without disturbing the ester content of the polymer which is responsible for specific aroma of the fruit juices (Brawman, 1981; Taragano and Pelosof, 1999). Alkaline pectin lyases acquire an important place in textile industries as they are widely used in degumming of natural fibres. Oil extraction, coffee and tea leaf fermentation, pectic waste water treatment and purification of viruses are also some of the other uses of pectin lyase (Hoondal *et al*., 2002). Literature survey suggests that in spite of industrial potential, this particular enzyme has received a comparatively less attention than other pectinases (PGs, PEs and PLs). Since new applications for this enzyme are emerging (Satyanarayana and Kumar, 2005), there is a scientific need to identify new sources of this enzyme with properties suitable for different applications and to develop simple procedures for the purification of these enzymes.

Keeping in view the above points, authors have attempted enzymological studies on pectin lyases from indigenous fungal sources (Yadav and Shastri, 2004, 2005; Yadav *et al*., 2007). This communication reports purification; enzymatic characterisation and application of the pectin lyase in clarification of fruit juices from an indigenously isolated fungal strain identified as *Aspergillus ficuum* MTCC 7591.

MATERIALS AND METHODS

Chemicals. Citrus pectin, DEAE-cellulose and Sephadex G-100 were from Sigma Chemical Company (St. Louis, USA). The protein molecular weight markers used were: phosphorylase B (94.4kD), BSA (66 kD), ovalbumin (43 kD), carbonic anhydrase (29 kD), soyabean trypsin inhibitor (20.1 kD) and lysozyme (14.3 kD) from Genei (Bangalore,

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India). All other chemicals were either E-Merck (Navi Mumbai, India) or S.D fines (Mumbai, India) and were used without further purification.

Microorganism and growth conditions. Twenty fungal strains were isolated from decaying wood using serial dilution technique (Cappuccino and Sherman, 1998). The strains were maintained on Czapek dox agar slants and sub cultured after every 15 days. The isolated fungal strains were screened for the secretion of pectinases by the method reported in literature (Molina *et al*., 2001). The fungal strain was got identified by the Microbial Type Culture Collection (MTCC) centre and Gene Bank Institute of Microbial Technology, Chandigarh, India as *Aspergillus ficuum* and deposited there with fungal strain no. MTCC 7591.

The fungal strains showing prominent halo zone formation were further screened for the secretion of pectin lyase (PNL) in a liquid culture medium containing 10 g of citrus pectin, 2 g of L-asparagine, 0.5 g of $MgSO_4 \cdot 7H_2O$ and 0.2 g of KH_2PO_4 per litre of distilled water. The initial pH of the medium was adjusted to 4.5 with dilute NaOH solution. Spore suspension in distilled water (1 ml, spore density: 10^6 spores/ml) was inoculated aseptically in 25 ml of the sterilised liquid culture medium contained in 100 ml culture flasks. The flasks were incubated in a BOD incubator at 25 ^{0}C and were allowed to grow under static condition. Aliquots (1 ml) of the growth medium were withdrawn at regular intervals of 24 h, filtered through Whatmann filter paper no. 41 and assayed for the activity of pectin lyase by the reported method (Albersheim, 1966).

Enzyme assay and protein determination. The activity of pectin lyase was spectrophotometrically evaluated on citrus pectin as substrate, by monitoring the increase in optical density at λ = 235nm due to liberation of 4,5 unsaturated galacturonide product from citrus pectin. The assay solution consisted of 2 ml 100 mM citrate phosphate buffer pH 5.0 containing 0.8 ml of 1% (w/v) citrus pectin maintained at 37 ^{0}C. The reaction was initiated by addition of 200 µl of the purified enzyme and initial absorbance and after 20 min were recorded on UV/Vis spectrophotometer (U-2000, Hitachi, Japan) which was fitted with an electronic temperature control unit having a least count of 0.001 absorbance unit. One unit of pectin lyase was defined as the amount of the enzyme that releases 1 µmole of 4,5 unsaturated galacturonide formed per minute. Protein concentration was determined by Lowry method (Lowry *et al*., 1951) using bovine serum albumin as standard.

Enzyme purification. For purification of the enzyme, the fungal strain was grown in six 100 ml culture flasks each containing 25 ml of the liquid culture medium. On 6th day all the growths were pooled and mycelia were removed by filtration. The culture filtrate, 100 ml was concentrated to 2 ml (8200Amicon Cell, PM 10 ultrafiltration membrane 10 kD cut off). The concentrated crude enzyme was dialysed against 2 l of 50 mM sodium phosphate buffer pH 7.0 with three changes at the intervals of 8 h. The sample was then centrifuged for 10 min at 10000 *x g* to remove the particles and the supernatant was loaded on DEAE cellulose column (6.5 x 2.0 cm) equilibrated with 50 mM sodium phosphate buffer pH 7.0. The adsorbed protein was washed with 2 times of bed volume of the same buffer. The protein was eluted using 100 ml of 1 M linear gradient (Gradient mixer GM-1, Pharmacia) of NaCl in the same buffer at the flow rate of 10 ml/h. Fractions of 5 ml were collected and analysed for activity of pectin lyase and protein. The most active fractions were combined and concentrated to 1 ml, by putting the enzyme sample in a dialysis bag and cover it with powdered sucrose. The concentrated enzyme solution was dialysed against 20 mM sodium phosphate buffer pH 7.0 and dialysate was loaded on Sephadex G-100 column (67 x 1.6 cm) equilibrated with 20 mM sodium phosphate buffer pH 7.0. The flow rate was 7 ml/h, fractions of 3.5 ml were collected and analysed. The most active fractions were pooled, concentrated by solid sucrose as mentioned above, stored at -20 ºC in deep fridge (Vestfrost, Denmark).

SDS-PAGE. The purity of the enzyme preparation was checked by SDS-PAGE using standard method (Laemmli, 1970). The separating gel was 10% acrylamide in 37 mM Tris-HCl buffer pH 8.8 and stacking gel was 5% acrylamide in 12 mM Tris-HCl buffer pH 6.8. The SDS-PAGE was performed under constant current of 20 mA. Silver staining was employed to identify protein bands. A calibration curve was drawn showing log of molecular weights of markers against their relative mobilities and the molecular weight of the purified enzyme was calculated from the calibration curve.

Characterisation of purified PNL. The K_m and calculated turnover number (kcat) values of the purified enzyme were determined by measuring steady state velocities of the enzyme catalysed reaction at different concentrations of citrus pectin and drawing double reciprocal plots (Engel, 1977). The pH optimum was determined by measuring steady state velocities in the buffered reaction solution in the pH range 1.0-10.0 and plotting steady state velocity against pH of the reaction solution. The pH stability of the enzyme was evaluated by exposing the enzyme to buffers of different pHs for 24 h at 20 ºC and assaying the residual activities and plotting the results in the form of percent residual activity versus pH. Similarly, the temperature optimum was determined by measuring steady state velocities of the enzyme catalysed reaction at different temperatures and plotting steady state velocity against temperature. Thermal stability of the enzyme was tested by incubating an enzyme aliquot at a particular temperature for 1 h and assaying its residual activity and plotting the percent residual activity against temperature.

Effect of metal ions and protein inhibitors on the activity of the enzyme. The enzyme activity was determined in the presence of various metal ions namely Ca^{++}, Mg^{++}, Mn^{++}, Co^{++}, Zn^{++}, Hg^{++}, Cu^{++}, K^+, Na^+, Ag^+ and protein inhibitors like sodium arsenate, sodium azide, potassium permanganate, potassium ferrocyanide and EDTA. The assay solutions were containing 0.2, 0.5 and 1.0 mM of these metal ions or protein inhibitors.

Studies on clarification of fruit juices using purified enzyme. Four types of fruits were purchased from local fruit market and their juices were extracted. The pHs of juices recorded were: apple (4.6), sweet lime (4.1), orange (4.3) and grape (4.0). The clarification of fruit juices by the purified pectin lyase was studied by using the method

reported by Ishii and Yokotsuka (1972). Aliquots (50, 100, 150 200 µl) of purified enzyme stock (0.34 IU/ml) were added to each 1 ml of the fruit juice, which was further incubated at 37 ºC for 1 h. The reaction was stopped by keeping the reaction mixture in boiling water bath for 5 min and centrifuged at 2000 *x g* for 5 min. Percentage transmittance of supernatants was measured at 660 nm with respect to controls that contained the same volume of water in place of added enzyme aliquot.

RESULTS AND DISCUSSION

The time course of pectin lyase production in medium containing 1% citrus pectin by *A. ficuum* is reported in Fig. 1. The maximum activity appeared after 6th day of incubation and the maximum activity was 0.047 IU/ml. This activity value is comparable to those reported in the literature (Friedrich *et al.*, 1990; Acuna-Arguelles *et al*., 1995). Attempts were made to concentrate the enzyme using ammonium sulphate precipitation from the culture filtrate but the activity of the enzyme was distributed in all fractions from 10 to 90% saturation. Even 90% saturation with ammonium sulphate could not bring the precipitation of most of the activity. The enzyme did not loose any activity under ultrafiltration process therefore ultrafiltration method was used for enzyme concentration.

The elution profile of the enzyme from DEAE cellulose column is reported in Fig. 2. There is a single activity peak in anion exchange chromatography. The most active fractions obtained after DEAE cellulose were combined, concentrated, dialysed and loaded on Sephadex G-100 column. The elution profile from gel filtration is shown in Fig. 3. Most of the activity comes in a single protein peak indicating that the enzyme after gel filtration is almost pure. All the purification steps are summarised in Table 1, which shows 86.7 fold purification with 60.9% yield and the specific activity of 7.8 U/mg. The homogeneity of the enzyme preparation was checked by SDS-PAGE and is reported in Fig. 4. Lane 1 shows a single protein band of the purified enzyme and lane 2 shows the markers. The purified enzyme is homogeneous in molecular weight. The molecular weight of the purified enzyme, determined by plotting the log of molecular weights of the markers versus their relative mobilities (Werber and Osborn, 1969) is approximately 31.6 kD.

The K_m and kcat, determined by double reciprocal plots using citrus pectin as the substrate, are 0.60 mg/ml and 74 s^{-1} respectively. The literature reported K_m values of pectin lyase from *Penicillium italicum*, *Aspergillus japonicus* and *Rhizopus oryzae* ranged from 0.16 and 3.2 mg/ml (Alana *et al*., 1990; Dinnella *et al*., 1994; Hamdy, 2005). Thus the K_m value determined for pectin lyase of *A. ficuum* is in the range reported in the literature and taking in account that

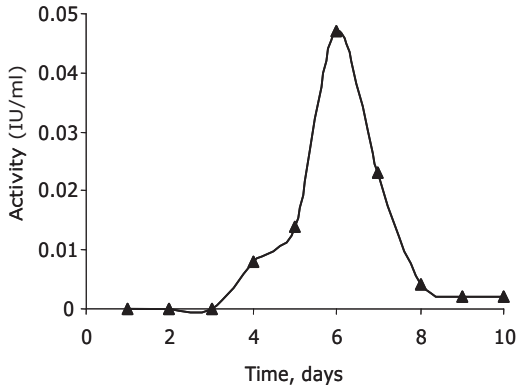

FIG. 1 - Production of pectin lyase by *Aspergillus ficuum* MTCC 7591.

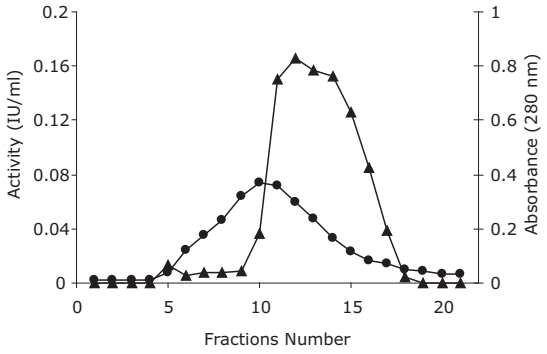

FIG. 2 - DEAE-cellulose column chromatography of PNL produced by *Aspergillus ficuum* MTCC 7591. Proteins (●) and PNL activity (▲).

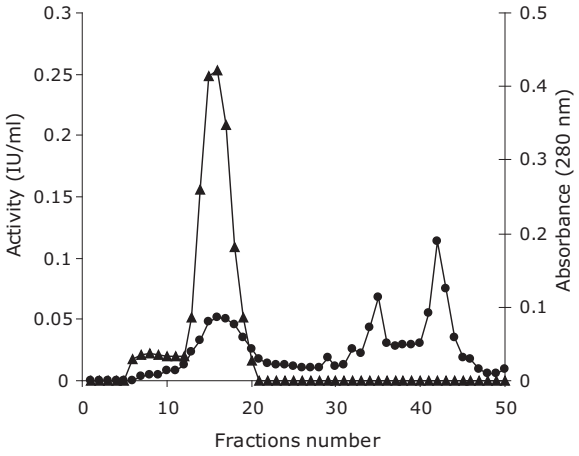

FIG. 3 - Sephadex G-100 column chromatography of PNL produced by *Aspergillus ficuum* MTCC 7591. Proteins (●) and PNL activity (▲).

TABLE 1 - Purification table of PNL produced by *Aspergillus ficuum* MTCC-7591

Fraction	Volume (ml)	Total activity (U)	Total protein (mg)	Specific activity (U/mg)	Purification fold	Yield (%)
Crude	2.0	4.6	51.0	0.1	1	100
DEAE- Cellulose	25.0	3.5	2.3	1.5	16.7	77.2
Sephadex G-100	15.0	2.8	0.4	7.8	86.7	60.9

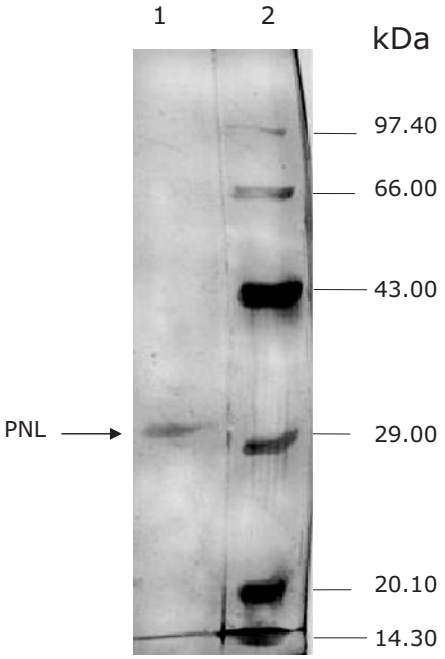

FIG. 4 - SDS- PAGE of PNL produced by *Aspergillus ficuum* MTCC 7591. Lane -1: purified PNL, lane-2: markers.

the substrate of this enzyme does not have well defined molecular weight therefore the K_m values cannot be compared accurately. The kcat of this enzyme on the basis of V_{max} is 74 s^{-1}.

The optimum pH of the purified pectin lyase is 5.0 (Fig. 5). It is also clear from the percentage residual activity versus the pH to which the enzyme has been exposed for 24 h at 20 ^{0}C that the enzyme shows good stability in the pH range 2.0-9.0 after which it looses the activity with increasing of pH. Above 83% residual activity of the enzyme, is maintained in the pH range 2.0-7.0 (Fig. 5).

The variation of the activity of the purified enzyme with temperature of the reaction solution and also the percentage residual activity of the enzyme, which has been kept at a particular temperature for 1 h versus temperature, are

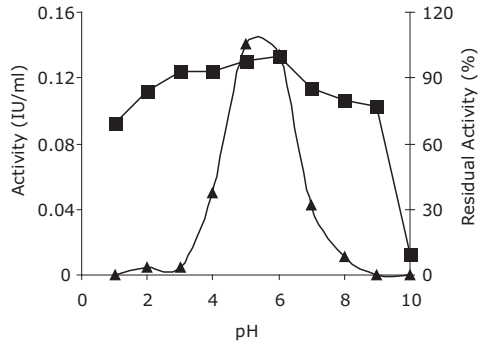

FIG. 5 - Optimum pH (▲) and pH stability (■) of pectin lyase produced by *Aspergillus ficuum* MTCC 7591.

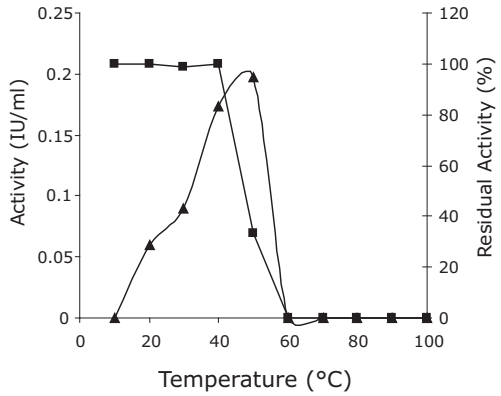

FIG. 6 - Optimum temperature (▲) and thermostability (■) of pectin lyase produced by *Aspergillus ficuum* MTCC 7591.

shown in Fig. 6. The temperature optimum of the enzyme is 50 ºC (Fig. 6). The percentage residual activity versus temperature at which enzyme has been kept for 1 h shows that the enzyme retains its full activity up to 40 ºC and above which activity decreases with increase in temperature. The residual activity was 33.3% at 50 ºC and the enzyme looses all its activity if it is exposed to 60 ºC for 1 h (Fig. 6).

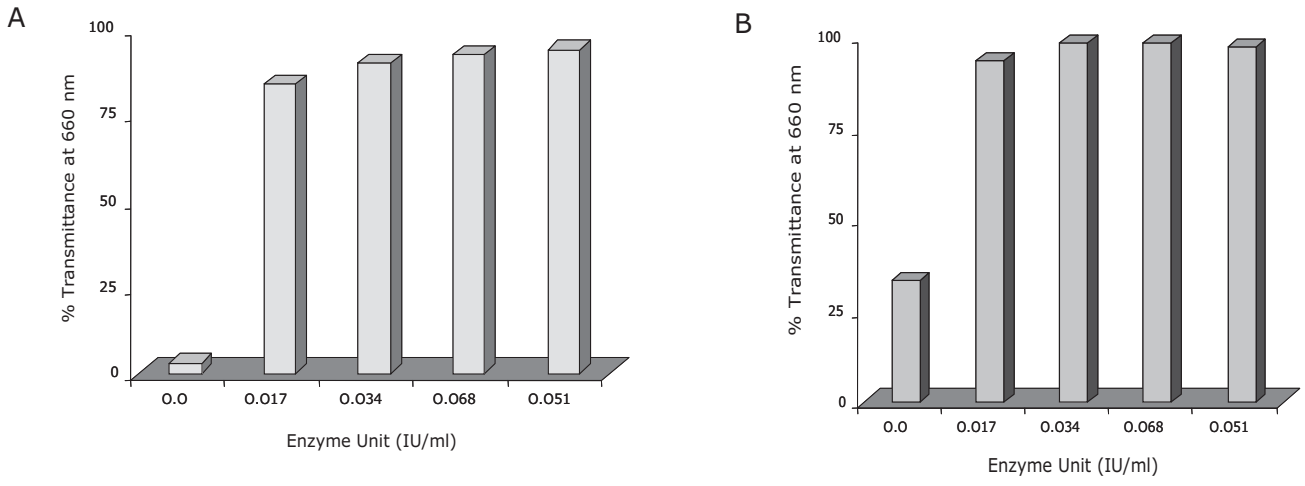

FIG. 7 - Fruit juice clarification using purified pectin lyase. A: sweet lime, B: orange.

Since pectate lyases and pectin lyases belong to the same group of enzymes i.e. transeliminases and pectate lyases are generally activated by Ca^{++} ions, the effect of metal ions like Ag^{+}, Ca^{++}, Co^{++}, Cu^{++}, Hg^{++}, K^{+}, Mg^{++}, Mn^{++}, Na^{+} and Zn^{++} on the activity of the purified pectin lyase were studied to see if any of the metal ions enhance the activity of pectin lyase. None of the ions studied have activating effect. This observation is in contrast to the report of activation of the activity of pectin lyase produced by *Rhizopus oryzae* by Mg^{++}, Na^{+} and K^{+} ions (Hamdy, 2005). The reported pectin lyase is completely inhibited by Ag^{+}, Cu^{++} and Hg^{++} even at 0.2 mm concentration. The effects of protein inhibitors like EDTA, sodium arsenate, sodium azide, potassium permanganate, potassium ferrocyanides on the activity of purified pectin lyase were also determined. Potassium permanganate and potassium ferrocyanide inhibits the activity of the enzyme completely at 0.2 mM concentration whereas the other inhibitors have no effect on the enzyme activity even at 1mM concentration.

The results of studies of fruit juice clarification by the purified pectin lyase are summarised in Fig. 7. Juice clarification is most noticeable in case of sweet lime (Fig. 7A) where percentage transmittance at λ = 660nm has increased from 3 to 94% after 1 h of incubation. The clarification is also appreciable in case of orange juice (Fig. 7B) where percentage transmittance has increased from 33.2 to 98.5% though it is not very effective for grape and apple juice clarification.

In conclusion, the reported enzyme has reasonable activity in the pH range 4.0 to 7.0 indicating that it will be useful in fruit industries for clarification fruit of juices especially sweet lime and orange.

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REFERENCES

Acuna-Arguelles M.E., Gutierrez-Rojas M., Viniegra-Gonzalez G., Favela-Torres E. (1995). Production and properties of three pectinolytic activities produced by *Aspergillus niger* in submerged and solid state fermentation. Appl. Microbiol. Biotechnol., 43: 808-814.

Alana A., Alkorta I., Dominguez J.B., Liama M.J., Serra J.L. (1990). Pectin lyase activity in a *Penicillium italicum* strain. Appl. Enivron. Microbiol., 50: 3755-3759.

Albersheim P. (1966). Pectin lyase from fungi. Methods Enzymol., 8: 628-631.

Brawman J.W. (1981). Application of enzymes in fruit juice technology. In: Brich G.G., Blakcorough N., Barker J.K., Eds, Enzymes and Food Processing, London, Applied Science, pp. 247-261.

Cappuccino J.G., Sherman N. (1998). Microbiology: A Laboratory Manual, 5th edn., Benjamin/Cumming, California, pp. 14-19.

Dinnella C., Lanzarini G., Stagni A. (1994). Immobilization of an endopectinlyase on γ-alumina: study of factors influencing biocatalytic matrix stability. J. Chem. Technol. Biotechnol., 59: 237-241.

Engel P.C. (1977). Enzyme Kinetics: A Steady State Approach, Chapman and Hall London, pp. 14-25.

Friedrich J., Cimermenm A., Steiner W. (1990). Production of pectinolytic enzymes by *Aspergillus niger*: effect of inoculum size and potassium hexacyanoferrate II-trihydrate. Appl. Microbiol. Biotechnol., 33: 377-381.

Hamdy H.S. (2005). Purification and characterization of the pectin lyase produced by *Rhizopus oryzae* grown on orange peels. Ann. Microbiol., 55 (3): 205-211.

Hoondal G.S., Tewari R.P., Tewari R., Dahiya N., Beg Q.K. (2002). Microbial alkaline pectinases and their industrial applications: a review. Appl. Microbiol. Biotechnol., 59: 409-418.

Ishii S., Yokotsuka T. (1972). Clarification of fruit juice by pectin transeliminase. J. Agric. Food. Chem., 20: 787-791.

Jarvis M.C., Briggs S.P.H., Knox J.P. (2003). Intercellular adhesion and cell separation in plants. Plant. Cell .Environ., 26: 977-989.

Laemmli U.K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London), 227: 680-685.

Linhardt R.J., Galliher P.M., Cooney C.L. (1986). Polysaccharide lyases. Appl. Biochem. Biotechnol., 12: 135-176.

Lowry O.H., Rose Brough N.J., Farr A.L., Randall R.J. (1951). Protein measurement with Folin phenol reagent. J.Biol .Chem., 193: 265-275.

Molina S.M.G., Pelissari F.A., Vitorello C.B.M. (2001). Screening and genetic improvement of pectinolytic fungi for degumming of textile fibres. Braz. J. Microbiol., 32: 320-326.

Mollet J.C., Park S.Y., Nothnagel E.A., Lord E.M. (2000). A lily stylar pectin is necessary for pollen tube adhesion to an *in vitro* stylar matrix. Plant Cell, 12: 1737-1749.

O'Neill M.A., Albersheim P., Dravill A.G. (1990). The pectic polysaccharides of primary cell walls. In: Dey P.M., Ed., Methods in Plant Biochemistry, Vol. 2, Academic Press, London, pp. 415-441.

Satyanarayana N.G., Kumar S. (2005). Microbial pectic transeliminases. Biotechnol. Lett., 27: 451-458.

Taragano V.M., Pelosof A.M.R. (1999). Application of Doehlert design for water activity, pH and fermentation time, optimization for *Aspergillus niger* pectinolytic activity, production in solid state and submerged fermentation. Enz. Microbiol .Technol. 25: 411-419.

Werber K., Osborn M. (1969). The relative mobility or molecular weight determination by dodecyl sulfate-polyacrylamide gel electrophoresis. J. Biol. Chem., 244: 4406-4412.

Yadav S., Shastri N.V. (2004). Partial purification of an extracellular pectin lyase from a strain of *Aspergillus niger*. Indian J. Microbiol., 44: 201-204.

Yadav S., Shastri N.V. (2005). Partial purification and characterization of a pectin lyase produced by *Penicillium oxalicum* in solid-state fermentation (SSF). Indian J. Biotechnol., 4: 501-505.

Yadav S., Yadav P.K., Yadav K.D.S. (2007). Pectin lyases of a few indigenous fungal strains. J. Sci. Ind. Res., 66: 601-604.