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Optimization, purification and characterization of pectinases from pectinolytic strain, *Aspergillus foetidus* MTCC 10559

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Abstract

A strain (MPUAT-2), isolated from coconut hull and identified as *Aspergillus foetidus* MTCC 10559, was used for pectinase production. Optimum pectinase production was obtained at pH 8.0 and temperature 35ºC under static conditions in submerged fermentation after 5 days of incubation. Orange peel, a by-product of fruit industry, was used as a sole carbon source (3% w/v) to produce high pectinase, thus making the process cost effective. The culture filtrate was analyzed for pectin methyl esterase (PME) and endo-polygalacturonase (endo-PG) enzymes. The enzymes, PME and endo-PG were purified using ammonium sulphate precipitation and molecular exclusion chromatography (Sephadex G-75) with corresponding recovery of 39.3 and 44.3%. The partially purified enzymes were also characterized for their kinetic properties.

Key words

Aspergillus foetidus MTCC 10559,Hanes plot, Molecular exclusion chromatography, Pectinases, Submerged fermentation

Introduction

Pectins are complex, high molecular weight, heterogeneous and acidic structural polysaccharides and are the major constituents of primary cell wall of cereals, vegetables, fruits and fibres. Pectins rank third as cell wall constituents after cellulose and hemicellulose, and form approximately 35% of the dry weight of dicot cell walls. The main component of pectin backbone is galacturonic acid residues, linked by -1-4 linkages, with neutral sugars such as arabinose, galactose and xylose present in side chains, whereas rhamnose constitutes a minor component of pectin (Kumar *et al.*, 2014). Pectins are present in considerable amount in fruits and vegetables thus, contributing to the strength of these tissues (Kumar *et al*., 2012; Mandhania *et al*., 2010). The carboxyl groups of galacturonic acid are partially esterified by methyl groups and partially or completely neutralized by sodium, potassium or ammonium ions. Pectinases breakdown complex polysaccharide/pectins into simpler molecules like galacturonic acid. The various enzymes of this group can be divided into depolymerizing enzymes (polygalactouronases [endo- and exo-type] and pectin lyases) and saponifying enzymes (pectin methyl esterases), each having discrete function (Padma *et al*., 2012). They have significant commercial value with a share of about 25% in global sale of food enzymes (Padma *et al*., 2012). Pectinases profoundly affect the fine structure of pectin and find applications in various industrial processes (Kumar *et al*., 2012; Mandhania *et al*., 2010). Acidic pectic enzymes are used in the extraction, clarification and liquefication of fruit juices (Kaur *et al*., 2004), while alkaline pectinases are employed in paper and textile industry (Ahlawat *et al*., 2009). In addition, these enzymes also have the potential to produce low degree methoxylated pectins (Kumar *et al*., 2012).

Biotechnological potential of micro organisms to produce pectinases in textile paper and fruit processing industries has drawn a great deal of attention worldwide (Reid and Ricard, 2000). Acidic pectinases used in fruit juice industries and wine making are often obtained from fungal sources, especially from *A. niger* (Bhat, 2000; Kashyap *et al.,* 2001), *A.awamori* (Blandino *et al*., 2000), *A. foetidus* (Sebastian *et al*., 1996), *A. japonicum (*Semenova *et al*., 2003*), A. carneus (*El-Sheekh *et al*., 2009) and *A. flavus* (Yadav *et al.,* 2008). However, there are various reports of pectinases from bacterial sources, especially *Bacillus* spp. (Ahlawat *et al*., 2009; Kashyap *et al.,* 2001). There are many advantages of using microorganisms as enzyme sources *viz.* easy culturing and propagation, less time and space requiring *etc.* Above all, majority of the microbial enzymes are extra cellular, heat stable and active over wide pH range. In the present study, a strain isolated from decaying coconut hull was optimized for pectinase production and the enzymes PME and endo-PG were partially purified and characterized for their kinetic properties.

Materials and Methods

Microorganism and process variables : The present investigations were carried out at AICRP on Post Harvest Technology, Department of Processing and Food Engineering, MPUAT, Udaipur from January, 2009 to September 2010. A strain (MPUAT-2), isolated from coconut hull dipped in distilled water at 35±2°C was optimized for optimum pectinase production. The strain was identified from the Institute of Microbial Technology (IMTECH) Chandigarh, India, as *A. foetidus* and included as collection at IMTECH with identification number MTCC 10559.

Submerged fermentation was carried out initially in 250 ml flasks, by taking 50 ml of pectin containing medium (pectin 1%, yeast extract 0.1%, pH 7.0). The flasks were inoculated with fungal spores and incubated at 35°C for 8 days under static conditions, in a BOD incubator (Shivam, India). For maximum production of pectinases, various culture conditions *viz.* pH, carbon source and concentration, nitrogen source and concentration, incubation period and temperature were optimized, as per the conventional method of one variable at a time approach, which involved varying a single independent variable and maintaining others at constant level.

The strain was optimized for higher yield of enzyme for different media parameters such as pH, carbon and nitrogen source and incubation time. The pH employed in this investigation ranged from 3.5 to 8.5 with 0.5 incremental while the carbon sources (lime peel, orange peel, guava fruit powder and commercial pectin) were used in the range of 1-4 % with 1 % incremental. In case of nitrogen sources, two organic sources (yeast extract and peptone) and two inorganic sources ($(NH_4)_2SO_4$ and $NaNO_3$) were used in the range of 0.1 to 1.0 % with 0.15 % incremental. The incubation time varied from 1 to 11 days, with one day incremental and incubation temperature varied from10 to 50°C, with 5°C increment.

Production of culture filtrate and analysis of various parameters : After incubation, the broth was filtered through Grade-4 filter paper (Axiva), centrifuged at 12,000 rpm for 20 min and the resulting culture filtrate was analyzed for PME, endo-PG and soluble protein. The filtrate was stored at 4°C until use, for estimating pectinolytic enzyme activities. The procedures adopted for enzymatic and non-enzymatic analysis were as depicted below:

The PME (3.1.1.11) activity was estimated by the method of Kertesz (1955), based on the principle of measuring released carboxylic groups by titration against NaOH. Ten ml of 1% pectin (M_r 27,000) in 0.15 M NaCl (pH 7.0) was incubated with ample amount of enzyme extract in 100 ml flask at 35°C for one hr. After 1 hr of incubation, the reaction was stopped by keeping the reaction flask in boiling water bath for 10 min. The contents of the flask were cooled and titrated against 0.02 N NaOH, using phenolphthalein as indicator. A blank without enzyme under similar conditions was also run as control. Enzyme unit was expressed as the amount of enzyme required to produce carboxylic groups to be titrated by one micro-equivalent of NaOH and the activity was expressed as µmoles of carboxylic acid produced $ml^{-1} min^{-1}$.

The activity of endo-PG (3.2.1.15) was measured as described by Martins *et al.* (2007). Suitable aliquot of enzyme was added to 12.0 ml of 1% pectin in 0.2 M sodium acetate buffer (pH 5.5) and incubated for 3 hr at 50 °C. The blank contained thermally inactivated crude enzyme. The enzyme activity was calculated using a viscometer (JSGW) and expressed as percent loss in viscosity of the substrate $ml^{-1} min^{-1}$.

Purification and characterization of PME and endo-PG : Unless stated otherwise, all steps of enzyme purification were carried out at 0-4°C. Extracellular enzymes were separated by filtering through grade-4 filter paper (Axiva) and then centrifuging at 10,000 rpm for 20 min and clarified supernatant was precipitated with ammonium sulphate. The enzymes endo-PG and PME were partially purified by $(NH_4)_2SO_4$ fractionation and then through molecular exclusion chromatography (MEC). Based on the preliminary standardizations, the crude extract was subjected to 25-70% $(NH_4)_2SO_4$ saturation. The precipitate collected after 5 hr by centrifugation (10,000xg, 20 min) had very high activity (72.1%) of endo-PG, so dissolved in 0.05 M acetate buffer (pH 5.5) and dialyzed for 24 hr against the same buffer, with repeated changes of buffer while the supernatant left after 70% $(NH_4)_2SO_4$ saturation was subjected to 100% $(NH_4)_2SO_4$ saturation. The resulting precipitate collected after 5 hr by centrifugation (10,000xg, 20 min) had 54.2% of total PME activity, so dissolved in 0.05 M phosphate buffer (pH 7.0) and dialyzed for 24 hr against the same buffer, with repeated changes of buffer. The dialyzed fraction (70% saturation) was concentrated by

osmosis against solid sucrose and loaded onto a Sephadex G75 (2.5 x 44 cm) column pre-equilibrated with 0.05 M acetate buffer (pH 5.5) for 10 hr. The enzyme was eluted with same buffer at a flow rate of 18 ml hr^{-1}. The fractions of 3.0 ml each were collected, analyzed for protein (280 nm) and for endo-PG and PME activity. This portion showed maximum endo-PG activity and the active fractions were concentrated using sucrose and used as partially purified endo-PG. The column was washed with 0.05 M phosphate buffer (pH 7.0) for 24 hr for reuse. The fraction (100% saturation) was concentrated against sucrose and loaded onto the same Sephadex G75 column. The enzyme was eluted with same buffer at a flow rate of 18 ml h^{-1}. The fractions of 3.0 ml each were collected, analyzed for protein (280 nm) and for endo-PG and PME activity. This portion showed maximum PME activity and the active fractions were concentrated using sucrose and used as partially purified PME. The concentration of protein at every step was determined by the method of Bradford (2002), using bovine serum albumin (BSA) as standard.

Molecular weight of the two partially purified enzymes was determined by MEC on a Sephadex G-75 column. The column was calibrated for molecular weight with standard molecular weight markers; cytochrome-*c* (12.4 kDa), β-lactoglobulin (18.0 kDa), carbonic anhydrase (29.0 kDa), pepsin (34.7 kDa), ovalbumin (45.0 kDa), albumin bovine (66.0 kDa) and alcohol dehydrogenase (150.0 kDa). The molecular weights were determined using a plot of elution volume *vs.* log molecular weights of standard proteins, according to the method of Whitaker (1963). A common factor y = - 0.0073x + 2.6208 was computed statistically and used to know the log M_r of purified proteins. The effect of pH on activity of endo-PG and PME was determined in 50 mM each of sodium acetate (pH 3.5-5.5), sodium phosphate (pH 6.0-7.5) and Tris buffer (pH 8.0-8.5) and the optimum temperature was determined between 25ºC and 70ºC. The K_m and V_{max} were calculated for both the enzymes using Hanes plots.

Statistical analysis : Statistical analysis of the data was done by in a complete randomized design (CRD). All the experiments were performed in triplicate and the data were statistically analyzed at $p \leq 0.05$, using 'ANOVA' table.

Results and Discussion

Microbial pectinases are produced from high yielding strains by fermentation under controlled conditions in surface or submerged culture. At present, majority of the commercial preparations of pectinases are obtained from fungi, especially species of genus *Aspergillus*, such as *A. niger, A. oryzae* and *A. wentii,* since the fungi produce different extracellular enzymes with pectinolytic activity (Bhat, 2000; Kashyap *et al.,* 2001). Various strains of *Aspergillus* have long been used in the fruit processing industry where they play crucial role in the extraction and clarification of fruit juices. Although, the pectinases produced by *A. niger*, *A. oryzae*, *Rhizopus oryzae* etc. have long been used in fruit juice clarification, yet the search for specific enzyme activities is still increasing/ under the way due to their demand for clarification of new types of fruit juices (Mandhania *et al*., 2010). During the present investigations, out of five isolates (data not shown) isolated from decaying coconut and jamun, a strain, MPUAT-2 was found to be a better pectinase producer and selected for further studies. Microbial cultures, regardless of the nature of their end products and the type of bio-processing, have certain specific requirements for their growth which have to be optimized for their maximum production (Mandhania *et al*., 2010).

The endo-PG activity increased from 0.047 (pH 3.5) to 0.247 U ml^{-1} min^{-1} (pH 8.0) and thereafter declined. The PME activity was also reported highest (13.19 U ml^{-1} min^{-1}) at pH 8.0. So, pH 8.0 was incorporated as optimized pH for further optimization steps (Table 1). This variation in pectinase production due to change in pH may be due to maximum availability of nutrients at that particular pH or the strain has the ability to grow at a particular pH (Joshi *et al*., 2006). However, the maximum production of pectinase in acidic medium was reported for *A. japonicum* (Semenova *et al*., 2003) and *A. heteromorphus* (Mandhania *et al*., 2010). The influence of wide range of pH from 2.3 to 7.2 (Sebastian *et al*., 1996) on the production of microbial pectinase from different substrates has been reported. Wide range of pH of the medium during upstream bioprocesses makes the end product either acidic or alkaline, which tends to have varied applications (Hoondol *et al*., 2002).

Orange peel as carbon source, with 3% concentration, was found optimum for pectinases. Data revealed that the activity of endo-PG and PME enhanced from their optimum values of 0.247 and 13.19 U ml^{-1} min^{-1} at 1% pectin concentration to respective values of 0.916 and 38.69 U ml^{-1} min^{-1}, at 3% concentration of orange peel for the isolated strain. This dramatic

Table 1 : Effect of pH on pectinase production for the strain *A. foetidus* MTCC 10559

pH	Endo PG activity (U ml^{-1} min^{-1})	Pectin methyl esterase (U ml^{-1} min^{-1})
3.5	0.047	1.37
4.0	0.058	3.28
4.5	0.126	6.68
5.0	0.169	6.55
5.5	0.189	6.40
6.0	0.205	6.98
6.5	0.238	7.45
7.0	0.229	6.93
7.5	0.232	10.98
8.0	0.247	13.19
8.5	0.225	8.05
SEM±	**0.02**	**1.32**
CD (5%)	**0.07**	**3.90**

(n = 3)

Table 2 : Summary of purification of endo-PG and PME from the strain *A. foetidus* MTCC 10559

Purification step	Total volume (ml)	Total protein (mg)	Total activity (units)	Specific activity (units mg^{-1} protein)	Fold purification	Yield
		Endo-PG				
Crude extract	5450.0	608.4	224703.5	369.3	1.0	100.0
Ammonium sulfate saturation (25-70%)	37.0	129.1	161949.0	1254.4	3.4	72.1
Sephadex G75	42.0	28.8	99523.0	3455.7	9.4	44.3
		PME				
Crude extract	5450.0	608.4	1482400.0	2436.5	1.0	100.0
Ammonium sulfate saturation (70-100%)	23.0	75.7	804000.0	10620.9	4.4	54.2
Sephadex G75	38.0	21.3	583379.0	27350.2	11.2	39.3

increase in pectinase activity might be due to the probable presence of metal ions and growth promoting factors in orange peel, which were lacking in pure pectin. The selection of orange peel as carbon source greatly reduced the cost of the enzyme production as orange peel is a fruit waste and underutilized, though it has bioactive compounds. Orange peel and pulp scrap, excluded as waste in orange juice industry and waste citrus peel have successfully been used in the production of pectinase by *A. carneus* NRC1 (El-Sheekh *et al*., 2009) and *A. heteromorphus* (Mandhania *et al*., 2010). However, the highest pectinase activity was reported in *A. niger* and *P. chrysogenum,* when wheat bran was used as the sole carbon source (Okafor *et al*., 2010).

Yeast extract proved to be the best producer of pectinase at 0.1% concentration having endo-PG and PME activity of 0.916 and 38.69 U ml^{-1} min^{-1}, respectively and further increase in yeast extract concentration decreased the enzymatic titre. Peptone at 0.1% concentration also produced good enzyme titre for endo-

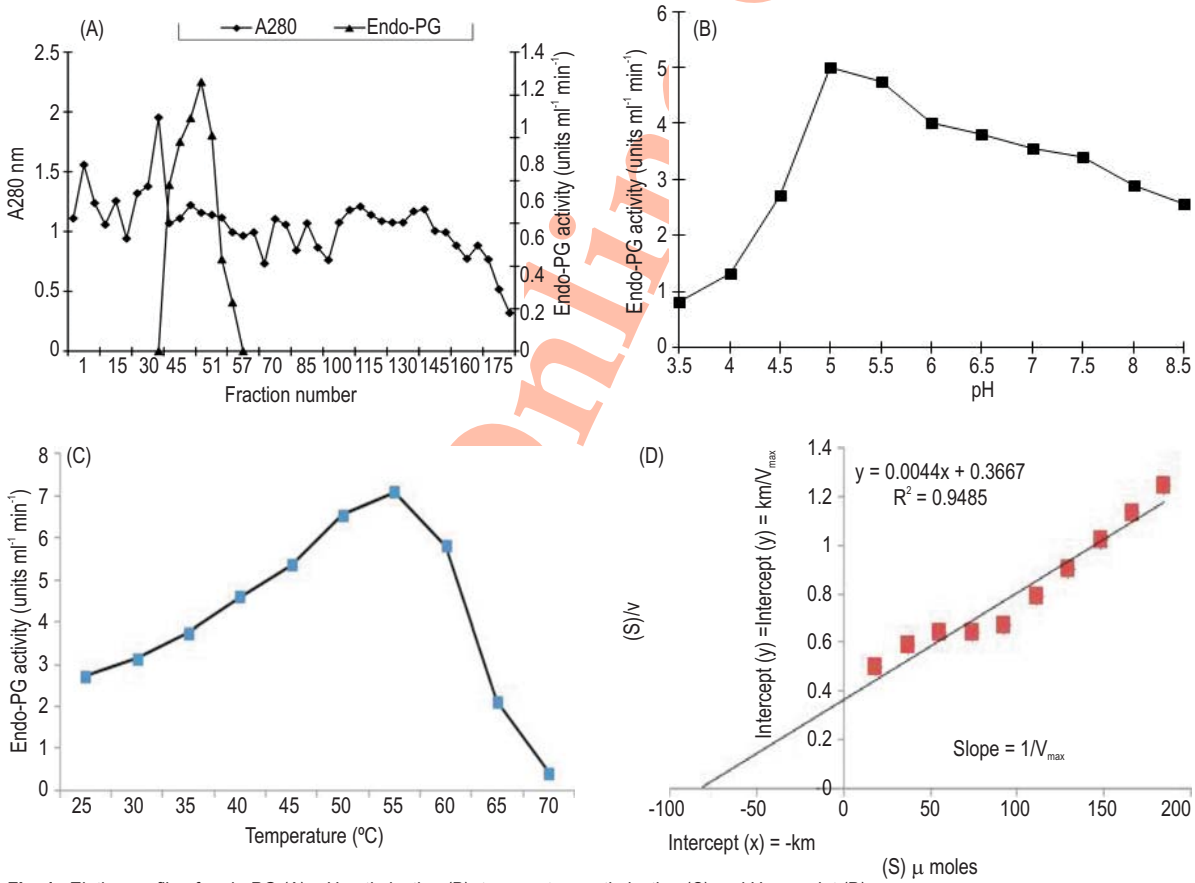

Fig. 1 : Elution profile of endo-PG (A), pH optimization (B), temperature optimization (C) and Hanes plot (D)

PG but activity of PME drastically decreased. Inorganic sources, $NaNO_3$ and $(NH_4)_2SO_4$, also yielded some results at lower concentration, but further increase decreased enzymatic titre. The results are contradictory to those *A. hetromorphus* (Mandhania *et al*., 2010) where activity of PME got inhibited due to yeast extract inclusion. The inhibitory effect of ammonium salts for pectinase production by *A. hetromorphus* (Mandhania *et al*., 2010) and *Bacillus sp.* (Kashyap and Soni, 2003) is in concurrence with the present results.

The enzymatic activities increased from 2nd day to 5th day after inoculation and thereafter started decreasing. The optimized enzyme activities reported during this experiment were 2.049 and 56.51 U ml^{-1} min^{-1}, respectively, for endo-PG and PME. So, for further studies, incubation time of 5 days was followed. However, Sunnotel and Nigam (2002) and Mandhania *et al*. (2010) reported maximum enzyme production after 6 days of incubation. In case of *A. foetidus*, prolonged fermentation up to 12 day resulted in comparatively small decline in enzyme activities, while the activity of PME decreased significantly after 6 d in case of *A. heteromorphus*. The pectinase behavior, in the present case, is somewhat contradictory to *A. foetidus* (Sunnotel and Nigam, 2002) while in conformity to that of *A. heteromorphus* (Mandhania *et al*., 2010).

Finally, the strain (MPUAT-2) was optimized for incubation temperature and the activities were found optimum at 35°C temperature. At 10, 15, 45 and 50°C temperature, however, the strain was unable to grow. Also, optimal PME production by *A. terreus* (Okafor *et al*., 2010) and by *Fusarium oxysporum* f. sp. m*elonis* (Joshi *et al*., 2006) was reported to occur at 25°C and 26°C, respectively. Though, the aim was to find certain thermotolerant strain which could make pectinases to withstand higher temperatures during industrial uses, but the strain failed to grow above 40°C.

The enzyme endo-PG was purified sequentially by ammonium sulphate precipitation and MEC. The enzyme had a high yield (72.1%) with fold purification of 3.4 in 25-70% $(NH_4)_2SO_4$ saturation. Finally, it was purified to 9.4 fold with an overall yield of 44.3% (Table 2). An endo-PG was isolated and purified from *A.foetidus* EGEK145 using freeze drying, CM-sephadex C-50 and concanavalin-A-sepharose column to a fold purification of 0.28, with 10% recovery (Taskin and Stratilova,

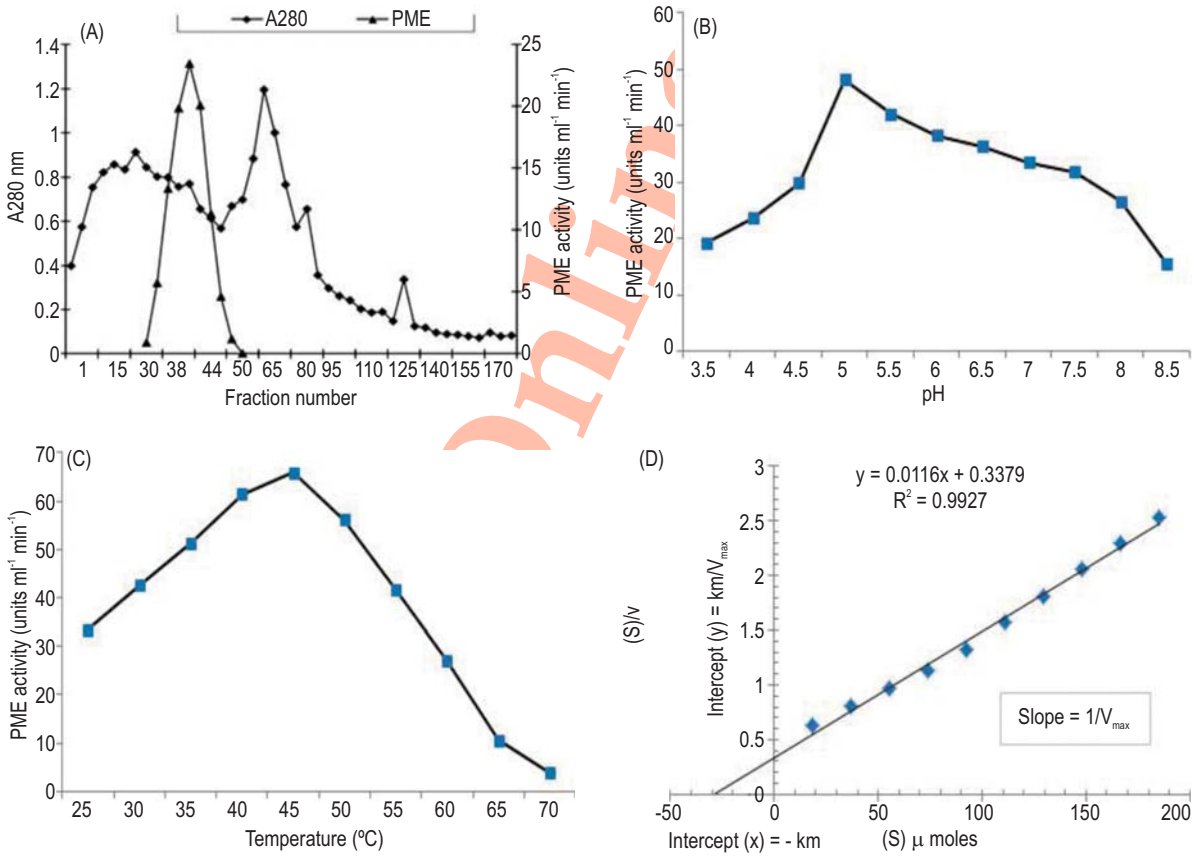

Fig. 2 : Elution profile of PME (A), pH optimization (B), temperature optimization (C) and Hanes plot (D)

2008). Contreras-Esquivel and Voget (2004) purified an endo-PG from the culture filtrate of *A. kawachii* IFO 4033, using acetone precipitation and Sepharose-Q chromatography to 470-fold, with a recovery of 40%. Molecular weight of endo-PG, as judged by MEC, was found to be 31 kDa. The endo-PG got eluted at 154 ml (from fraction number 45 to 56 with maximum at fraction number 51) (Fig.1A). The results obtained in the present investigation are in conformity with that of endo-PG from *A. foetidus* EGEK145 (Taskin and Stratilova, 2008) (M_r 31 kDa) but different from that of *A. awamori* IFO4033 (Nagai *et al*., 2000)(M_r 41 kDa). Optimum pH for endo-PG was found to be 5.0 (Fig.1B). Various workers have reported pH optima of endo-PG in acidic range of 4.5 to 5.0 *viz.* 4.5 for *Moniliella* SB9 (Martin *et al*., 2004), 4.6 for *A. niger* (Dinu *et al*., 2007), 4.8 for *A. foetidus* EGEK 145 (Taskin and Stratilova, 2008) and 5.0 for *A. awamori* IFO4033 (Nagai *et al*., 2000). The optimum temperature for endo-PG was 55°C (Fig.1C). Similar observations were observed for temperature optima for 3 variants of PGs (PG-I, II and III), respectively, 55, 50 and 50°C (Anjana and Appu-Rao, 1996). *Moniliella* SB9 also had 55°C as temperature optimum for PG (Martin *et al*., 2004). These observations are in conformity to that of *A. carbonarius* (Anjana and Appu-Rao, 1996) and to that of *Moniliella* SB9 (Martin *et al*., 2004). However, optimum temperature for endo-PG from *A. foetidus* EGEK 145 (Anjana and Appu-Rao, 1996) was observed to be 30°C. Temperature optima of 40°C was observed for PGs from *A. niger* MIUG16 (Dinu *et al*., 2007) and *A. awamori* IFO4033 (Nagai *et al*., 2000). The V_{max} and K_m of purified endo-PG, as judged by Hanes plot, was found to be 216 µmoles min^{-1} and 82 µmoles min^{-1}, respectively (Fig.1D). TheV_{max} of 22.62 and 153.84 µmols min^{-1} and K_m of 0.452 and 0.462 µmols min^{-1} were reported, for *A. foetidus* strains EGEK145 and EGEK635 (Taskin and Stratilova, 2008), whereas *A. niger* MIUG16 had V_{max} and K_m values of 3114.3 U mg^{-1} and 0.94 g l^{-1}, respectively (Dinu *et al*., 2007).

The PME from *A. foetidus* MTCC 10559 was purified having overall 11.2 fold purification with 39.3% yield, using ammonium sulphate precipitation (70-100% saturation) and MEC (Sephadex G75) (Table 2). PME from *A. repens* (Arotupin *et al*., 2008) was purified using ammonium sulphate, MEC (sephadex G-100) and ion exchange chromatography to 7.93 fold with an overall yield of 21.47%. The purified enzyme was better in terms of yield than that of *A. japonicas* (Semenova *et al*., 2003).The PME got eluted at 126 ml (between fraction number 36 to 48 with maximal elution at fraction number 42) (Fig. 2A) and the M_r was found to be 50 kDa using standard curve of MEC. The molecular weight of purified pectinase generally ranges from 25 to 50 kDa (Semenova *et al*., 2003). Two forms of pectinase *i.e.*PME I and PME II, with molecular weights 46 and 47 kDa, with same pI value of 3.4, were purified from *A. japonicas* (Semenova *et al*., 2003). PME purified from *A. oryzae* KBN-616 had molecular weight of 38.5 kDa (Kitamoto *et al*., 1993). PME from *Arthrobotrys oligospora* had 50 kDa M_r as determined by Sephadex G-100 column and SDS-PAGE (Jaffar and Oommen, 1993). The optimum pH for partially purified PME was found to be 5.0 (Fig. 2B). The pH optima for most of the fungal pectinases were in range of 4.0 to 5.0. Pectin methyl esterases, with pH optima of 5.0 from *A. niger* (Maldonado *et al*., 1994) and *A. oryzae* KBN-616 (Kitamoto *et al*., 1993) have been reported. PME purified from *Curuvlariaina equalis* NRRL-13884 had pH optima of 4.4 (Afifi *et al*., 2002). Similarly, recombinant PME with pH optima of 4.5 was obtained from *A. aculeatus* (Christgau *et al*., 1996). A fungal PME could de-esterify citrus pectin at pH 4.5 and 8.0 but had an acidic isoelectric pH (pI) and acidic pH optima (Duvetter *et al*., 2006). The partially purified PME had optimum temperature of 45°C (Fig. 2C). The optimum temperature of PME lies in the range of 40-50°C (Shevchik *et al*., 1996). The recombinant PMEI from *A. aculeatus* which had maximum activity at 45°C (Christgau *et al*., 1996) and similar temperature optima (45°C) of PME was observed from *A. niger* (Maldonado *et al*., 1994). PME from *A. oryzae* KBN-616 (Kitamoto *et al*., 1993) showed maximum activity at 55°C. The optimum temperature of 37°C was reported for PME from *Arthrobotrys oligospora* (Jaffar and Oommen, 1993). The V_{max} and K_m of purified PME, as judged by Hanes plot, were found to be 84.8 µmoles min^{-1} and 28 µmoles min^{-1}, respectively (Fig. 2D). The affinity constant K_m, for PME from *Clostridium thermosaccharolyticum* was found to be 0.12% pectin with V_{max} value of 440 units mg^{-1} (Rijssel *et al*., 1993).

The strain MPUAT-2, isolated from coconut hull, dipped in distilled water at 35±2°C, was characterized as *A. foetidus* MTCC 10559. The strain produced highest pectinase activities using orange peel as sole carbon source, which may greatly reduce the cost of enzyme production.

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