Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

Production and characterization of pectinase enzyme from *Penicillium chrysogenum*

A. Rasheedha Banu, M. Kalpana Devi, G. R. Gnanaprabhal, B. V. Pradeep and M. Palaniswamy
Department of Microbiology, Karpagam University, Coimbatore - 641 021, Tamil Nadu, India
m.palaniswamy@gmail.com

Abstract

Ten moulds isolated from municipal waste soil sample were screened for pectinolytic enzyme production when grown on pectin containing (YPSS) solid media. *Penicillium chrysogenum* was selected based on clearance zones and pectinase enzyme production was carried out in submerged fermentation. Enzyme production by *Penicillium chrysogenum* was higher at pH 6.5 and a temperature of 35°C using sucrose and ammonium per sulphate as carbon source and nitrogen source, respectively. The maximal activity of *P. chrysogenum* pectinase was at 50°C, pH 6.5 and was thermostable up to 40°C. $MgCl_2$ and $CaCl_2$ ions had little effect on pectinase activity. K_m and V_{max} values were 1.0 mg/mL and 85 U/mg protein, respectively and an apparent molecular weight of 31 kDa on SDS-PAGE.

Keywords: *Penicillium chrysogenum*; submerged fermentation, pectinase.

Introduction

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). The genus Penicillium is world wide known for production of secondary metabolites and extracellular enzymes of commercial value, including pectinases.

Pectinase are now an integral part of juice and textile industries (Kashyap *et al.,* 2001) such as maceration of tea leaves (Angayarkanni *et al.,* 2002); processing of cotton fabric (Solbak *et al.,* 2005) as well as in various biotechnological applications (Alkorta *et al.,* 1998, Jacob & Prema, 2006). The filamentous fungi are most often used in the commercial production of pectinases. Microbial production of pectinases has been extensively studied (Kashyap *et al.,* 2001, Torres *et al.,* 2006); actinomycetes (Bruhlmann *et al.,* 1994); *Aspergillus flavus* (Mellon & Cotty, 2004); *Aspergillus* sp. (Angayarkanni *et al.,* 2002); *Penicilluim italicum* (Alana *et al.,* 1990); *Penicillium viridicatum* RFC3 (Silva *et al.,* 2002); *Penicillium roqueforti* (Pericin *et al.,* 2007); *Penicillium expansum* (Cardoso *et al.,* 2007) and Pectolytic moulds (Fawole & Odunfa, 1992).

New enzymes for use in commercial applications with desirable biochemical and physico-chemical characteristics and a low cost production have been the focus of much research (Silva *et al.,* 2002; Malvessi & Silveira, 2004; Phutela *et al.,* 2005). Enzyme breakdown of the biomolecules depends up on the type of enzyme, application, temperature, incubation time, agitation, concentration, pH and use of different enzyme preparations (Dominguez *et al.,* 1994; Chadha *et al.,* 2003). In this context, the objective of the present study was to produce pectinolytic enzymes by a newly isolated strain of *Penicillium chrysogenum* by submerged fermentation and process evaluation. Furthermore, the physico-chemical characteristics of the purified enzymes are also presented.

Materials and methods

Isolation of fungal species

The soil fungi were isolated from municipal solid waste collected from Coimbatore, Tamil Nadu, India. Isolation medium of following composition, g/L (Pectin, 10; sucrose,10; tryptone, 3; yeast extract, 2; KCl, 0.5; $MgSO_4.7H_2O$, 0.5; $MnSO_{4\cdot5}H_2O$, 0.01; $(NH_4)_2SO_4$, 2) supplemented with mineral salt solution of composition g/100 mL ($CuSO_4.5H_2O$, 0.04; $FeSO_4$, 0.08; Na_2MoO_4, 0.08; $ZnSO_4$, 0.8; $Na_2B_4O_7$, 0.004, $MnSO_4$, 0.008), pH 5.5-6.0 was used. To the above medium, ampillicin (100 mg/l) was added to restrict bacterial growth. The inoculated plates were incubated at 50°C for 5-7 days. The cultures were further purified by sub-culturing on YPSS (Yeast soluble starch agar) medium having composition, g l^{-1} (starch, 15; yeast extract, 0.4; K_2HPO_4, 0.23; KH_2PO_4, 0.2; $MgSO_4.7H_2O$, 0.05; citric acid, 0.052; pH 5.5-6). Identification of the genus was based on morphological and biochemical characteristics (Phutela *et al.,* 2005).

Screening of soil fungal isolates for pectinolytic activity

A total of ten isolates from soil were assayed for polygalacturonase (PG) activity using pectin containing agar medium. Culture plates with pectin-containing agarose were inoculated with each isolate and incubated for 3-5 days at 31°C. Isolates were replicated 2 to 3 times and tests were performed twice. After incubation, plates were stained with aqueous 0.05% ruthenium red solution for 1h and rinsed with deionised water. Cultures expressing pectinase activity exhibited a clear zone around the margins of the colony. Isolates without a clear zone usually exhibited a ring of intense staining around the colony.

Enzyme production by submerged fermentation

The fungal culture was used to produce pectinase enzyme using liquid medium (g/L) citrus pectin, 10; $(NH_4)_2SO_4$, 1.4; K_2HPO_4, 6; KH_2PO_4, 2; $MgSO_4.7H_2O$,

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

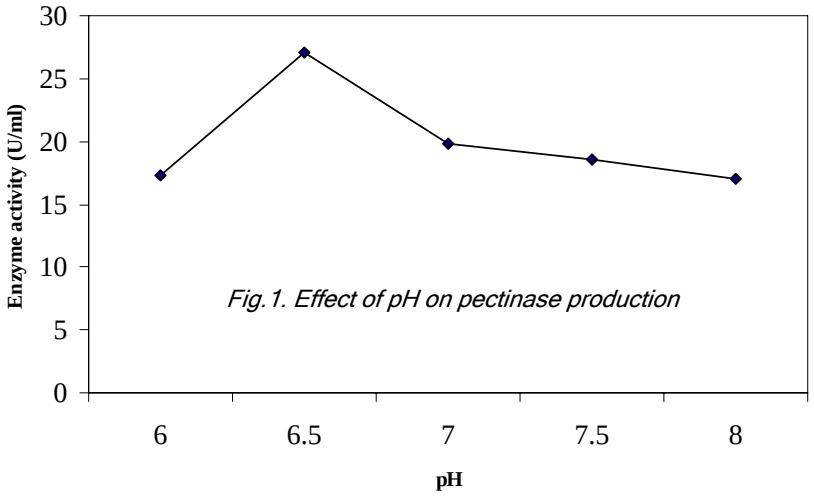


Fig.1. Effect of pH on pectinase production

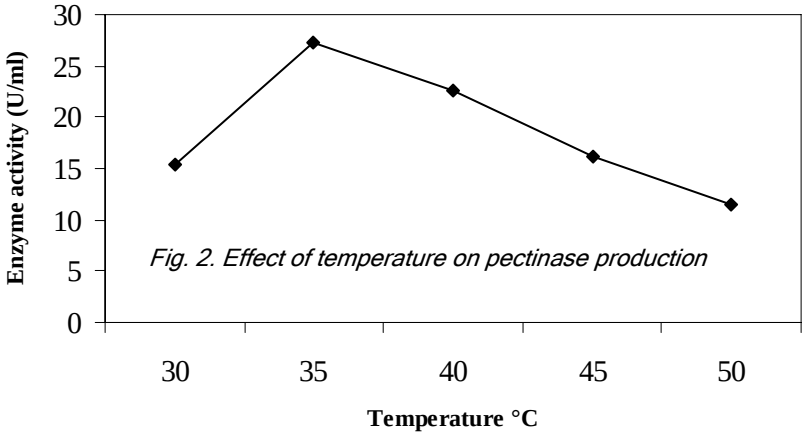


Fig. 2. Effect of temperature on pectinase production

0.1; pH, 6). Fermentation was carried out in 500 mL Erlenmeyer flask containing 250 mL of growth medium with 10% inoculum (10^6 spores/mL) and incubated at 30°C under shaking conditions (175 rpm) for 5 days (Angayarkanni *et al.,* 2002). The biomass was separated by centrifugation at 10,000 x g for 15 min at 4°C. The supernatant was used to evaluate the polygalacturonase enzyme activity.

Culture conditions

The cultural conditions [pH (6-8), temperature (30-50°C), different carbon (glucose, galactose, mannitol, sucrose & starch) and nitrogen sources (Ammonium persulfate, Ammonium nitrate, Ammonium chloride, Peptone & Ammonium oxalate)], on selected pectinase enzyme production was studied.

Polygalacturonase enzyme assay

The polygalacturonase activity was determined by measuring the amount of reducing substances liberated from citrus pectin. The reaction mixture consisted of substrate buffer (0.125 g citrus pectin dissolved in 25 mL of 0.2 M sodium citrate buffer, pH 6.5) and enzyme solution (0.5 mL). This mixture was incubated at 37°C for 30 min., heated in a boiling water bath for 5 min and the reaction was stopped by using 3 mL of DNS reagent. The absorbance was read at 570 nm. One unit of enzyme activity (U) was defined as 1 µmol of galacturonic acid released per min (Silva *et al.,* 2002).

Protein content

Protein content was determined by the method of Lowry *et al.,* (1951), using Bovine Serum Albumin (BSA) as standard.

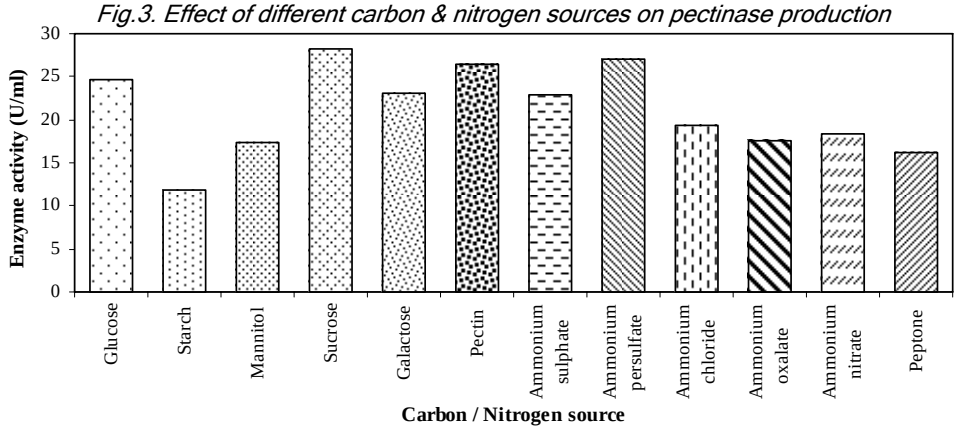
Enzyme purification

The crude enzyme filtrate was dialyzed and clear supernatant (200 mL) was mixed with three volumes of cold ethanol and kept undisrupted for overnight at 4°C. The resulting precipitate was collected by centrifugation at 9,000×g for 30 min at 4°C. The precipitate was dissolved in 10 mL of sodium citrate buffer (pH 6.5) and dialyzed against distilled water. For further purification, the dialyzed sample was lyophilized to 5 mL and passed through Sephadex G-100 column (1.5 x 45 cm) equilibrated with sodium citrate buffer (pH 6.5) and eluted with the same buffer. About 20 fractions were collected at a rate of 10mL/h. The fraction of the enzyme that showed highest enzyme activity was then pooled and used for characterization (Angayarkanni *et al.,* 2002)

Enzyme characterization

The enzyme PG activity was determined at 35°C in different pH using acetate (pH 4.0-5.0), citrate (pH 5.0-6.0), sodium citrate (pH 6.0-7.0) and Tris-HCl (pH 7.0-8.0) as buffers. The optimum temperature was assayed by incubating each reaction mixtures at 20-80°C and its thermo stability. The reaction speed V_{max} and K_m were determined for the enzyme by varying the substrate concentration from 2-20 mg/mL and plotting substrate/velocity as function of substrate concentration (Line-Weaver & Burk, 1934).

Fig.3. Effect of different carbon & nitrogen sources on pectinase production



Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

For determination of the influence of $CaCl_2$, $CuSO_4$, $CoCl_2$, $HgCl_2$, $ZnCl_2$, $MgCl_2$ EDTA and $BaCl_2$ on PG activity, the salts were added to the reaction mixture (5 mM), and the enzyme activity was determined as described above. SDS-PAGE of protein samples was performed using 12% gel. After migration protein bands were stained with Coomassive Brilliant Blue (CBB) (Laemmli, 1970).

Table 1. Purification of pectinase from P. chrysogenum

Fraction	Total protein (mg)	Total activity (U)	Specific activity (U/mg)	Purification (fold)	Recovery (%)
Culture filtrate	523.0	3818	7.30	1.0	100
Ethanol precipitation	67.8	2205	32.53	4.45	57.77
Sephadex G-100	6.3	918	145.84	17.24	48.12

Table 2. Properties of pectinase

Properties	*P. chrysogenum*
Optimum pH	6.5
Optimum temp. (◦C)	50
Thermostability (min)	60
V_{max} (U/mg protein)	85
K_m (mg/mL)	1.0
Molecular wt (kDa)	31

Results and discussion

Screening of isolates

Ten fungal species isolated from municipal solid waste material and morphological characteristics were examined. Fungal cultures were further screened by yeast soluble starch agar plate method and the zone of clearance was calculated. *Penicillium chrysogenum* and *Aspergillus niger* cultures had a zone of clearance above 3 mm, *A. alacatus*, *A. flavus*, *A. fumigatus*, *A. oryzae* and *P. italicum* cultures had a zone of clearance between 1 and 2 mm and *Rhizopus oryzae*, *Rhizopus sp.* and *Mucor sp.* isolates had a zone of clearance below 1 mm or no clearance zone around the colonies (data not shown). On the basis of screening method, the isolate *Penicillium chrysogenum* found to be a potential source of pectinolytic enzyme. This culture was used for optimization of pectinase production using submerged fermentation.

Table 3. Effect of metal ions & inhibitors(5 mM) on pectinase activity

Metal ions	Residual activity (%)
None	100
$CaCl_2$	103.56
$MgCl_2$	73.44
$ZnCl_2$	83.79
$HgCl_2$	41.23
$CoCl_2$	41.50
$CuSO_4$	40.50
$BaCl_2$	60.53
EDTA	61.35

Optimization of cultural conditions

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 strong effect of pH on the production endo PG was clearly observed in flask cultures, were pH value 6-8 was tested (Fig. 1). The maximum PG activity occurred with an initial pH of 6.5, activity reaching 27.07 U/mL on 5th day. Either increase or decrease beyond the optimum value show decline in enzyme production. However, the mechanism by which the pH acts on the production pectic enzyme is not known. Piccoli-valle *et al.,* (2001) observed that a high PG 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 neutral, 5-7. *P. viridicatum* showed maximum production of polygalacturonase and pectinlyase at a pH of 4.5 and 5 respectively (Silva *et al.,* 2002). In the present study *P. chrysogenum* exhibited maximum PG production at 35°C (27.21 U/mL) (Fig. 2). The temperature optima of 50°C, was obtained from a purified culture fluid of *P. frequentans* (Chellegatti, 2002)

Supplementation of sucrose to the production medium increased the pectinolytic activity of *P. chrysogenum*. However the production rate was highly repressed in the presence of starch (Fig. 3). Piccoli-valle *et al.* (2001) obtained significant Pectin lyase (PL) production by growing *P. griseoroseum* in medium containing 60 to 74 mM sucrose. PL produced by *P. griseoroseum* does not appear to be influenced by concentrations of sucrose used or by the free glucose in the medium.

Of the different nitrogen sources used, ammonium persulphate has enhanced the production of *P. chrysogenum* pectinase (Fig. 3). Phutela *et al.,* (2005) reported that $(NH_4)_2SO_4$ stimulated pectinase production, as in its absence fungus displayed a slight proteolytic activity and did not produce extracellular pectinases.

Enzyme purification

Pectinase enzyme was partially purified by Sepahdex G-100 column chromatography. It showed an increased specific activity of *P. chrysogenum* pectinase to 145.84 IU/mg protein and enzyme recovery of 48.12% (Table1.). Preparations of the enzyme obtained from culture filtrates of *P. italicum* were subjected to ion exchanger DEAE-cellulose and carboxymethyl cellulose chromatography at pH 8 and 6, respectively. The preparations yielded the elution pH value of 8.6 and the elution volume of 15.1mL and about three-fold higher specific activity (Alana *et al.,* 1990).

Enzyme characterization

The pectinase activity of *P. chrysogenum* was found to be highest at pH 6.5 using sodium citrate buffer (Fig. 4). Marcia *et al.,* (1999) studied the stability of PG against pH. Their results indicated that the enzyme was stable in a pH range of 6-8 and showed highest activity at pH 6. Martin *et al.,* (2004) reported that PG from *Penicillium* sp. was stable at pH range of 3-8 and maintained 70% of initial activity at 70°C. PL produced by this microorganism was stable in acidic to neutral pH (4-8) and was stable in temperature lower than 40°C.

The optimum temperature that showed highest enzyme activity for *P. chrysogenum* was 50°C (Fig. 5). Alana *et al.,* (1990) reported that the temperature effect on *P. italicum* PL activity consisted of an increase up to 50°C. PL from *P. italicum* was more thermostable than

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

other pectin lyases described for *Penicillium* culture filtrates. The pectinase enzyme produced by *P. chrysogenum* was stable up to 60 min and showed maximum activity (Fig. 6). Denis *et al.,* (2002) reported a similar result with polygalacturonase produced by *Penicillium viridicatum* exhibited maximal activity and was stable at pH 5-8 and maintained 80% of its activity at pH 9. Pectin lyase was more sensitive to pH variation, presenting maximum stability at pH 3.5-4.5 which declined to 80% at pH 5 and to 60% at pH 6.

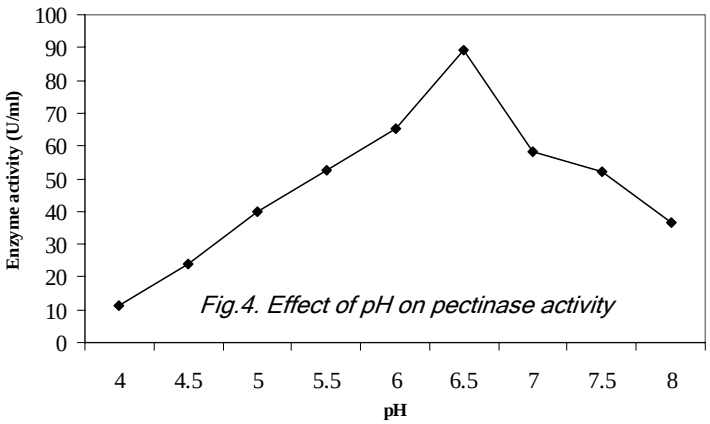


Fig.4. Effect of pH on pectinase activity

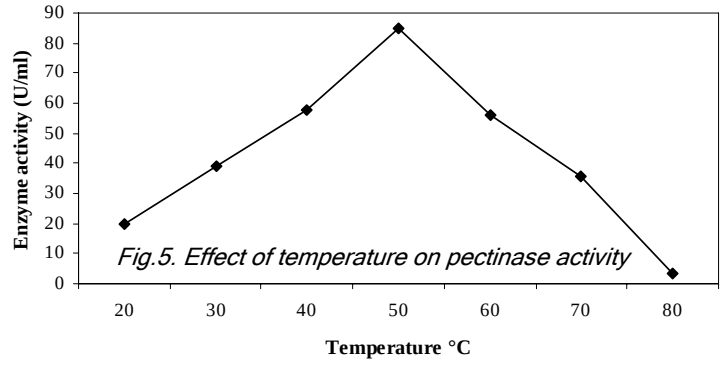


Fig.5. Effect of temperature on pectinase activity

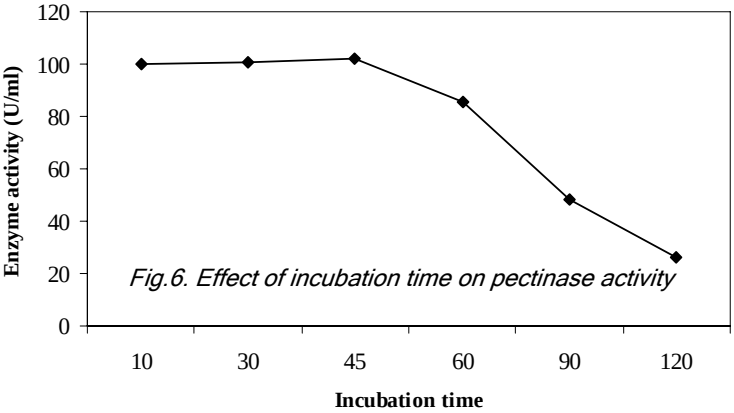


Fig.6. Effect of incubation time on pectinase activity

The kinetic parameter of purified pectinase enzyme from *P. chrysogenum* was determined. The pectinase enzyme from *P. chrysogenum* showed a V_{max} of 85 U/mg protein and K_m of 1 mg/mL (Table 2.). Sohel *et al.,* (2007) reported that the V_{max}/K_m values of pectinase enzyme were increased from 11 to 14. Among the metal ions tested, addition of 5 mM $CaCl_2$ enhanced the activity of pectinase enzyme produced by *P. chrysogenum* by 3.56%. $MgCl_2$ and $ZnCl_2$ inhibited pectinase enzyme activity to the level of 21.2 and 14.8%. Similarly $HgCl_2$, $CoCl_2$ and $CuSo_4$ have been reported to inhibit activity of pectinase of *P. chrysogenum* up to 60%. $BaCl_2$ and EDTA exhibited maximum inhibition of 40% on pectinase enzyme activity (Table 3.). Alana *et al.,* (1990) reported that Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} did not affect pectin lyase activity of *P. italicum* at 5 mM, while Co^{2+} reduced it by 14%. However Cu^{2+} and Fe^{2+} at the same concentration produced complete inhibition. When a chelating agent (ethylene dinitrilotetra aceticacid) was added at 0.2 mM to the incubation mixture, pectin lyase activity was not affected.

Optimal activity was observed at 0.05 mM of $CaCl_2$, although the enzyme produced by *Clostridium stercorarium* was more or less activated throughout a range of $CaCl_2$ concentrations from 0.05 to 0.2 mM. The addition of 0.2 mM EDTA inhibited activity to less than 5% of maximum (Hla *et al.,* 2005).

The pectinase enzyme produced by *P. chrysogenum* showed a molecular weight of 31 kDa (Fig. 7). The molecular mass of *P. italicum* (22 kDa) pectin lyase was lower than those reported for the enzyme isolated from a fungal source (30-35 kDa). A pectin lyase with a molecular mass of 18.2 kDa has been found in culture filtrates of *Botrytis cinerea* (Alana *et al.,* 1990).

The properties of pectinase enzyme produced by *P. chrysogenum* were determined. The results revealed that the pectinase enzyme of *P. chrysogenum* can tolerate high temperature and neutral pH (Table 2.).

Conclusion

Pectinase enzyme was isolated from the native strain, *P. chrysogenum* that can be used for various industrial applications including extraction and clarification of fruit juices, processing of cotton fabric in textile industries, bleaching of paper, removal of pectic waste waters and maceration of tea leaves.

Acknowledgements

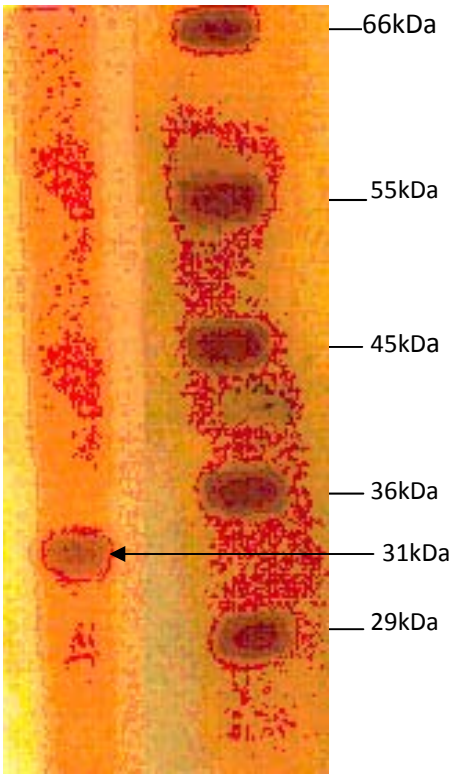
The authors are sincerely grateful to the management, Karpagam University, Coimbatore, Tamil Nadu, India for encouragement and support.

References

1. Alana A, Alkorta I, Dominguez JB, Llama MJ and Serra JL (1990) Pectin lyase activity in a *Penicillium Italicum* strain. *Appl. Envt. Microbiol.* 56, 3755-3759.
2. Alkorta I, Garbisu C, Llama MJ and Serra JL (1998) Industrial applications of pectic enzymes: a review. *Proc. Biochem.* 33, 21-28.

Indian Journal of Science and Technology Vol. 3 No. 4 (Apr. 2010) ISSN: 0974- 6846

Fig. 7. SDS - PAGE showing the molecular weight of pectinase enzyme produced by Penicillium chrysogenum (31 kDa)



3. Angayarkanni J, Palaniswamy M, Murugesan S and Swaminathan K (2002) Improvement of tea leaves fermentation with *Aspergillus* spp. pectinase. *J. Biosci. Bioengg*. 94, 299-303.
4. Bruhlmann F, Kim KS, Zimmerman W and Fiechter A (1994) Pectinolytic enzymes from actinomycetes for the degumming of ramie bast fibers. *Appl. Envt. Microbiol*. 60, 2107-2112.
5. Cardoso PG, Queiroz MV, Pereira OL and Araújo EF (2007) Morphological and molecular differentiation of the pectinase producing fungi *Penicillium expansum* and *Penicillium griseoroseum*. *Braz. J. Microbiol*. 38, 71-77.
6. Chadha R, Kumbhar BK and Sarkar BC (2003) Enzymatic hydrolysis of carrot for increased juice recovery. *J. Food Sci. Technol*. 40, 35-39.
7. Chellegatti MASC, Fonseca MJV and Said S (2002) Purification and partial characterization of exopolygalacturonase I from *Penicillium frequentans*. *Microbiol. Res*.157, 19-24.
8. Dalal S, Sharma A and Gupta MN (2007) A multipurpose immobilized biocatalyst with pectinase, xylanase and cellulase activities. *Chem. Central J.*16, 1-5.
9. Dominguez H, Nunez MJ and Lema JM (1994) Enzymatic pretreatment to enhance oil extraction from fruits and oil seeds: A review. *Food Chem*. 49, 271-286.
10. Fawole OB and Odunfa SA (1992) Pectolytic moulds in Nigeria. *Lett. Appl. Microbiol*. 15, 266-268.
11. Hla SS, Kurokawa J, Suryani Kimura T, Ohmiya K and Sakka K (2005) A novel thermophilic pectate lyase containing two catalytic modules of *Clostridium stercorarium*. *Biosci. Biotechnol. Biochem*. 69, 2138-2145.
12. Jacob N and Prema P (2006) Influence of mode of fermentation on production of polygalacturonase by a novel strain of *Streptomyces lydicus*. *Food Technol. Biotechnol*. 44, 263-267.
13. Kashyap DR, Vohra PK, Chopra S and Tewari R (2001) Applications of pectinases in the commercial sector: A review. *Bioresour. Technol*. 77, 215-227.
14. Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature*. 227, 680-685.
15. Lineweaver H and Burk D (1934) The determination of enzyme dissociation constants. *J. Am. Chem. Soc*. 56, 658-666.
16. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ (1951) Protein measurement with folin phenol reagent. *J. Biol. Chem*. 193, 265–275.
17. Malvessi E and Silveira MM (2004) Influence of medium composition and pH on the production of polygalacturonases by *Aspergillus oryzae*. *Braz. Arch. Biol. Technol*. 47, 693-702.
18. Martin N, Souza SR, Silva R and Gomes E (2004) Pectinase production by fungal strains in solid state fermentation using agro-industrial bioproduct. *Braz. Arch. Biol. Technol*. 47, 813-819.
19. Mellon JE and Cotty PJ (2004) Expression of pectinase activity among *Aspergillus flavus* isolates from southwestern and southeastern United States. *Mycopathologia*.157, 333-338.
20. Miller GL (1959) Use of Dinitrosalicylic acid for determination of reducing sugar. *Anal. Chem.* 31, 426-428.
21. Pericin DM, Madarev SZ, Radulovic LM and Skrinjar M (2007) Production of exo-pectinase by *Penicillium roqueorti* using pumpkin oil cake. *Nat. Sci*. 113, 313-320.
22. Phutela U, Dhuna V, Sandhu S and Chadha BS (2005) Pectinase and polygalacturonase production by a thermophilic *Aspergillus fumigatus* isolated from decomposting orange peels. *Braz. J. Microbiol*. 36, 63-69.
23. Piccoli-valle RH, Passos FML, Passos FJV and Silva DO (2001) Production of pectin lyase by *Penicillium griseoroseum* in bioreactors in the absence of inducer. *Braz. J. Microbiol*. 32,135-140.
24. Silva D, Martins ES, Silva R and Gomes E (2002) Pectinase production by *Penicillium viridicatum* RFC3 by solid state fermentation using agro-industrial by-products. *Braz. J. Microbiol*. 33, 318-324.
25. Soares MMCN, Silva R and Gomes E (1999) Screening of bacterial strains for pectinolytic activity: characterization of the polygalacturonase produced by *Bacillus* species. *Rev. Micobiol*. 30, 229-303.
26. Solbak AI, Richardson TH, McCann RT, Kline KA, Bartnek F, Tomlinson G, Tan X, Parra-Gessert L, Frey GJ, Podar M, Luginbuhl P, Gray KA, Mathur EJ, Robertson DE, Burk MJ, Hazlewood GP, Short JM and Kerovuo J (2005) Discovery of pectin degrading enzymes and directed evolution of a novel pectate lyase for processing cotton fabric. *J. Biol. Chem*. 280, 9431-9438.
27. Torres EF, Sepulveda TV and Gonzalez GV (2006) Production of hydrolytic depolymerising pectinases. *Food. Technol. Biotechnol*. 44, 221-227.