Research Article **Microbiology**

International Journal of Pharma and Bio Sciences **ISSN 0975-6299**

PRODUCTION AND CHARACTERIZATION OF EXOPOLYGALACTURONASE FROM *FUSARIUM OXYSPORUM* F. SP. *LYCOPERSICI*.

D. NIRMALADEVI, M. ANILKUMAR AND C. SRINIVAS*.

Department of Microbiology & Biotechnology, Jnanabharathi Campus, Bangalore University, Bangalore-560 056, Karnataka, India

ABSTRACT

Polygalacturonases (PGs) are pectinolytic enzymes with wide industrial applications. The demand for the enzyme and its producing organism is rapidly increasing. Enzymes from filamentous fungi have been subject to vast number of studies due to their functional role in the organism as well as their advantage as models for enzyme production and their characteristics. The objective of the study was to screen a large population of *Fusarium oxysporum* isolated from tomato fields of Karnataka, for PG production. The ability of the cultures to produce extracellular PG was investigated. The culture conditions providing high level of enzyme production were determined. A typical exoPG producing strain was identified. The exoPG exhibited maximal activity at pH 4.0, 50°C, 40 min and thermal and pH stability. Various metal ions tested showed a high inhibitory effect on PG activity. The K_m and V_{max} values were 7.69 mg/ml and 0.20 µmol/ml/min. The molecular weight of the enzyme was found to be ~60 kDa.

KEYWORDS: ***F. oxysporum* f. sp. *lycopersici*, Exopolygalacturonase, Enzyme characterization.**

C. SRINIVAS
Department of Microbiology & Biotechnology, Jnanabharathi Campus, Bangalore University, Bangalore-560 056, Karnataka, India

INTRODUCTION

Enzymes are versatile biomolecules with ever-increasing biotechnological applications[1]. They demonstrate striking features such as specific biocatalysis, improved stability, high activity, and reduction of undesirable byproducts. They are biodegradable, thus being more environment-friendly and also a viable alternative in economic terms[2,3]. Pectin is a prominent structural constituent of primary cell walls and middle lamella providing firmness and organization to plant tissues[4]. Pectinases are group of enzymes that contribute to the degradation of pectin by various mechanisms. The family of pectinases include protopectinases, polygalacturonases, lyases and pectin esterases. Polygalacturonases (PGs) catalyze the hydrolytic cleavage of the polygalacturonic acid chain with the introduction of water across the oxygen bridge. They are the most studied among the pectinolytic enzymes[5]. Polygalacturonase is classified into glycosyl-hydrolases family 28[6]. Based on their mode of action, polygalacturonases are divided into two groups: (i) Endopolygalacturonases (E.C.3.2.1.15) break the polymer chain in a random pattern liberating saturated oligogalacturonides and galacturonic acid, (ii) Exopolygalacturonases (EC 3.2.1.67), cleave the penultimate polymer bonds releasing one saturated galacturonic acid residue from non-reducing end of homogalacturonan. In nature, pectinases are important for plants as they help in cell wall extension and fruit ripening. They have a significant role in maintaining ecological balance by causing decomposition and recycling of plant materials[7]. Plant-biomass-degrading fungi produce an extensive set of carbohydrate-active enzymes including pectinases specifically to degrade plant polysaccharides[8]. Pectin degradation plays an important role in plant diseases which generally begins with the production of polygalacturonases[9]. Besides plant cell wall degradation, PGs release oligogalacturonides that can act as elicitors of the plant defense response[10].

Microbial production of polygalacturonases is mainly from filamentous fungi, yeasts and bacteria. Polygalacturonase producing fungi have been isolated from diverse habitats including soil, decomposed plant parts, infected host tissue of plants, mangroove environments and vegetable wastes. In laboratory conditions, the production of PGs generally depends on media composition such as pectin source, nitrogen source, pH of the medium, temperature and agitation[11, 12]. Endopolygalacturonase and exopolygalacturonase are of industrial interest as they act on pectin, hydrolyzing its internal and external glycosidic bonds, producing shorter pectin molecular structures, declining the viscosity and improving the yield of juices[13]. Pectinolytic enzymes are of significant importance in the current biotechnological era and have been described as one of the future enzymes of the commercial sector. They are used in the juice and food industries[14], paper and pulp[15], scouring of cotton, degumming of plant fibers, waste water treatment, tea & coffee fermentations, as poultry feed additives, alcoholic beverages[5], wood preservation[4] and during the maceration, liquefaction and extraction of vegetable tissues[16]. It has been estimated that microbial pectinases account for 25% of the global food enzymes sales[5]. The innumerable applications of PGs in industrial processes demand the isolation, selection of potential strains, investigating the production conditions and physico-chemical characteristics of the enzyme. Screening a large number of microorganisms for high active enzymes combined with molecular techniques can lead to more efficient strains and stable enzymes. Further, enzymes having a set of biochemical and physical properties can be generated for specific industrial process[17]. This study describes the screening, production and characterization of extracellular PG from *F. oxysporum* f. sp. *lycopersici.*

MATERIALS AND METHODS

(i) Isolation and Identification

F. oxysporum f. sp. *lycopersici* strains were isolated according to Norhito *et al*.[18] from wilted tomato plants and rhizosphere soil

samples, collected from major tomato growing areas in and around Karnataka. Colonies exhibiting the taxonomic features of *F. oxysporum* were identified according to Nelson *et al*. [19]. The identification was further confirmed from National fungal culture collection of India (NFCCI, Agharkar Research Institute). Pure cultures of the isolates were maintained on potato dextrose agar and stored as stock cultures at 4°C.

(ii) Species specific PCR assay

Species specific identification of *F. oxysporum* was performed by PCR using primers FOF1 5'- ACATACCACTTGTTGCCTCG -3', FOR1 5'- CGCCAATCAATTTGAGGAACG- 3' reported by Prashant *et al*. [20]. Standard PCR protocol was followed to perform the reaction. Amplicons were electrophoresed in a 1% agarose gel for observation. A 1kb ladder was used as a marker. Gels were documented using Gbox (GE health care, Mumbai).

(iii) Qualitative plate screening assay for pectinase production

Preliminary screening of 85 *F. oxysporum* f. sp. *lycopersici* isolates for pectinase production was performed on solid medium containing 0.5% Pectin, 0.1% Yeast extract and 1.5% Agar[21]. Five mm disc from a seven day old culture of the isolates was inoculated onto Petri dishes containing this medium and incubated for four days. The plates were flooded with 1% aqueous solution of hexadecyltrimethylammoniumbromide and checked for the formation of clear zones around the colonies[22].

(iv) Enzyme production in liquid medium

Fusarium oxysporum f. sp. *lycopersici* strain MB-39 which showed maximum zone of clearance was selected and grown in a synthetic medium (SM) containing 0.2 g $MgSO_4.7H_2O$, 0.4 g KH_2PO_4, 0.2g KCl, 1g NH_4NO_3, 0.01 g $FeSO_4$, 0.01 g $ZnSO_4$, 0.01 g $MnSO_4$ per liter. Cultures were supplemented with 1% Pectin. The flasks were maintained at 120 rpm on a shaker for five days[10].

(v) Preparation of crude enzyme

Fusarium oxysporum f. sp. *lycopersici* mycelia from the broth was removed by filtration through Whatman No. 1 filter paper and centrifuged. Cold Acetone was added to clarified supernatant up to 80% and centrifuged[23]. The precipitate was dissolved in 0.1M acetate buffer (pH 4.2) and stored at 4°C.

(vi) Enzyme Assay

The PG activity was determined by measuring the release of reducing groups by modified 3,5- dinitrosalycilic acid (DNS) method[24]. The reaction mixture contained equal volumes of the culture filtrate and substrate (0.1% in 0.1 M acetate buffer pH 4.2) incubated at 45°C for 30 minutes. The quantity of reducing sugar released was calculated from standards of D-galacturonic acid. The enzyme activity was expressed as the amount of reducing sugar released per mL per minute under the above conditions. The specific activity was calculated.

(vii) Protein determination:

The protein content of the culture filtrates and the crude enzyme was determined according to the method of Lowry using Bovine Serum Albumin (BSA) as standard[25].

(viii) Effect of culture conditions on enzyme production

The effect of pH, incubation temperature and incubation period on enzyme production in culture medium was studied between 2.0 and 8.0 for pH, 28°C and 50°C for temperature and 2-8 days for incubation period. Enzyme production was also studied at static and shaker conditions[26].

(ix) Production of PG on different carbon sources:

The production of PG on different carbon sources was studied by growing the organism in synthetic media supplemented with 1% of Pectin, Galacturonic acid, Glucose, Maltose, Lactose and Pectin + Glucose (0.5% each)[10].

(x) Characterization of polygalacturonase

a) pH and temperature optima: The influence of pH on PG activity was measured by performing the enzyme-substrate reaction at various pH values between 3.0 and 9.0. To determine the optimal temperature for PG activity, the enzyme was incubated with the

substrate at various temperatures between 10°C -100°C.

b) The effect of metal ions and reagents: Effect of $CaCl_2$, $CuSO_4$, $HgCl_2$, $ZnSO_4$, $FeCl_3$, $MgSO_4$, $MnSO_4$, EDTA and SDS on enzyme activity was determined at a concentration of 2 mM[27].

c) The kinetic parameters K_m and V_{max} of PG towards Pectin at pH 4.5 and 50°C were obtained by double reciprocal Lineweaver-Burk plot utilizing substrate in the range of final concentration of 1.0-20 mg/ml[10].

d) Enzyme stability: The thermal stability of PG was assayed as residual activity after incubating the culture filtrate for 20, 40 and 60mins at 40-100°C followed by enzyme activity assay at 50°C. The pH stability was determined as the residual PG activity after the culture filtrate had been incubated (1:1) in 0.1 M buffer solutions pH 3.0-9.0, maintained at room temperature. An aliquot was drawn at 30, 60, 90 and 120mins and used to determine enzyme activity at 50°C[28].

(xi) Chromatographic separation of hydrolysis products and viscometric assay

Hydrolysis products from pectin by the culture filtrate and crude enzyme were analyzed by Thin Layer Chromatography (TLC) on silica gel using ethyl acetate/acetic acid/formic acid/water (9:3:1:4 v/v) as the mobile phase system and galacturonic acid as standard. Sugars were detected with 0.2% orcinol in sulphuric acid: methanol (10:90 ml) [29]. The endo or exo mode of action of the enzyme was determined by measuring the decrease in relative viscosity at regular time intervals using a basic viscometer. Reaction mixture contained 1% substrate in sodium acetate buffer, pH 4.5 and the culture filtrate in equal volumes. Simultaneously, the percentage of substrate hydrolysis was determined by the reducing sugar assay[30].

(xii) Analytical electrophoresis

The polyacrylamide gel electrophoresis was carried out in slab gels composed of 4% (w/v) stacking gel and 10% (w/v) resolving gel. Molecular mass markers were used to determine the molecular mass, of the enzyme[10].

(xiii)Analysis of Data

The mean values and the standard deviations from three independent experiments are represented.

RESULTS AND DISCUSSION

1. Isolation and identification

A total of Eighty five (85) isolates of *F. oxysporum* were isolated and identified. Morphological identifications were further confirmed by molecular method. A single band of 340 bp was obtained in all the isolates and that was on par with standard culture of *F. oxysporum* (Fig 1).

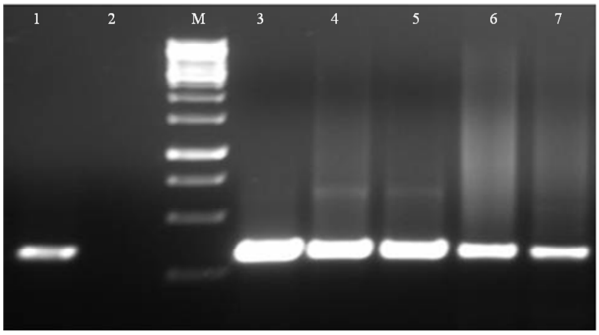

Figure 1

Species Specific PCR assay. Lane-1 and 2 positive and negative controls of F. oxysporum, Lane M- 1Kb DNA ladder, Lane-3-7 isolates of F. oxysporum.

2. Qualitative plate screening assay for pectinase production

All the strains were able to grow on the medium containing Pectin as the sole carbon source. Isolates varied widely in their reaction to the plate screening assay. Out of the Eighty five isolates screened for pectin hydrolysis, 95% of the isolates showed

positive for pectinase by the formation of clear zone around the colonies, the zone size ranging between 2 to 5mm. The strain MB-39 (weakly pathogenic to tomato) which showed maximum zone was used for enzyme production in liquid medium.

3. Effect of culture conditions on PG production

Enzyme biosynthesis is influenced not only by the composition of the nutrient medium but also by the other conditions of cultivation including pH and temperature of the medium which limit the growth of the culture or exert influence upon the catalytic activity of the enzyme. The effect of the medium pH and incubation temperature on polygalacturonase biosynthesis was studied at seven different pH values in a range of 2.0-8.0 and at six different values for temperature between 28°C-50°C. PG activity was observed in the medium with acidic initial pH values within a range of 4.0-6.0 with the optimum pH of 5.0 (119.39U/mg). The optimum temperature for enzyme production was found to be 35°C (100.67U/mg). PG production increased after 48h of incubation (227.5U/mg) and was highest at the 4th day (255.57U/mg), while its production drastically declined thereafter. The enzyme production was increased by incubation on a shaker at 120rpm as compared to the static conditions.

4. Production of PG on different carbon sources

High PG activity was observed in cultures supplemented with Pectin + Glucose (0.5% each) followed by Pectin (1%). Glucose was found to be an active inducer of the enzyme when supplemented at low levels. However at high levels, glucose represses the enzyme production. Enzyme activity was observed at very low levels in the filtrates of FoI grown on carbon sources like glucose, galacturonic acid, maltose and lactose (Fig 2). Inhibition of polygalacturonase production by glucose and other simple sugars might be due to catobolite repression. Similar results showing a higher polygalacturonase yield with pectin polymer compared to simple sugars have been reported by Aminzadeh *et al.*[26] which is in accordance with the present study.

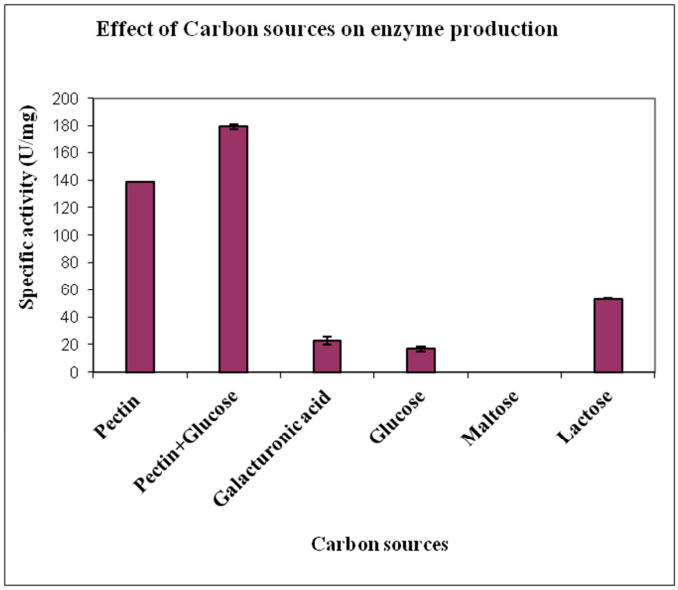

Figure 2
Production of PG in media supplemented with different carbon sources.

5. Characterization of polygalacturonase

The enzyme exhibited 65-100% activity at a broad range of temperature between 20-80°C. Maximum enzyme activity was observed at 50°C (Fig 3). Enzyme activity was observed between pH 4 to 6, the optimum at pH 4 with a sharp decrease in activity with increasing pH (Fig 4). Partial to significant inhibition of enzyme activity was observed with all the ions tested except Ca^{2+}. Maximum inhibition of

enzyme activity was by Cu^{2+} and Hg^{2+} which were around 94% and 92% respectively. SDS and EDTA had the strongest inhibitory effect on the enzyme (Fig 5). Inhibition of activity by thiol blocking agents such as $HgCl_2$ suggests the possible involvement of thiol group in the active site of the enzyme. Similar results were observed by Garcia *et al.* [10]. The enzyme exhibited 42, 33 and 8% stability at 40°C for 20, 40 and 60 mins respectively and was 12% stable at 80°C for 20 mins. At higher temperature there was complete loss of activity. Maximum stability of 87, 83, 70 and 58% was observed at pH 5.0 for 30, 60, 90 and 120mins respectively. At pH 4 the enzyme was 54% stable followed by 9% at pH 3 for 30 mins. At neutral and alkaline pH the enzyme exhibited 20-12 % stability and there was no activity above pH 9. The K_m and V_{max} as determined by Lineweaver-Burk plot, using Pectin as substrate, were 7.69 mg/ml and 0. 20 µmol/ml/min respectively (Fig 6).

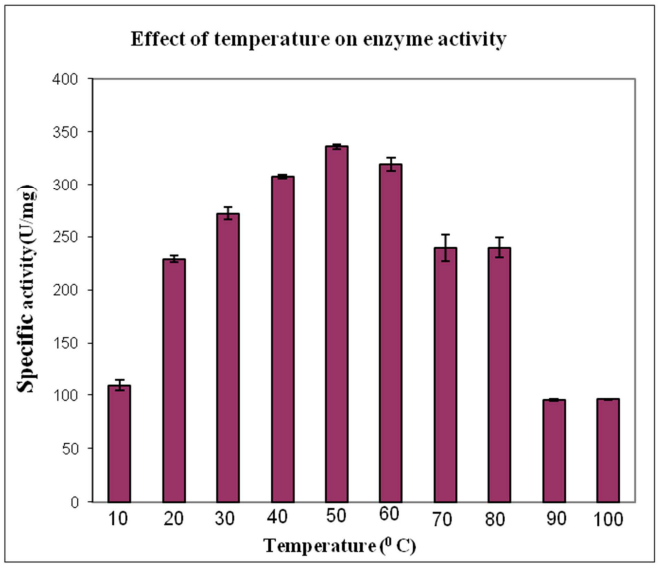

Figure 3
Effect of temperature on enzyme activity

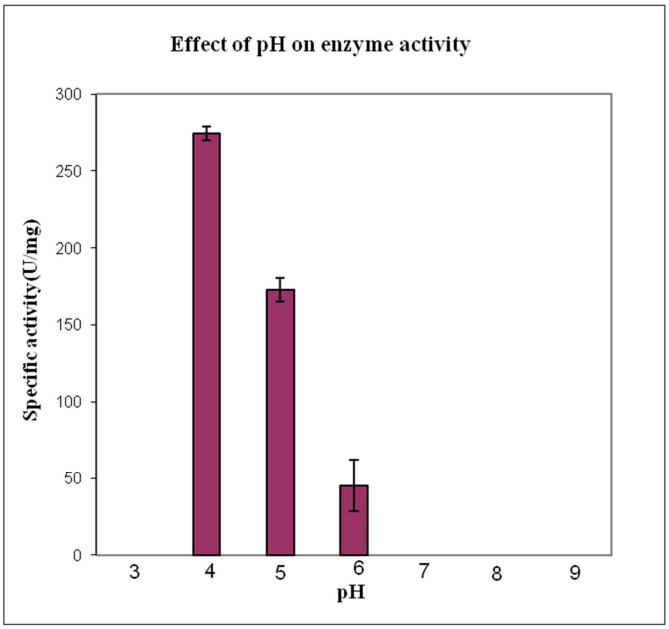

Figure 4
Effect of pH on enzyme activity.

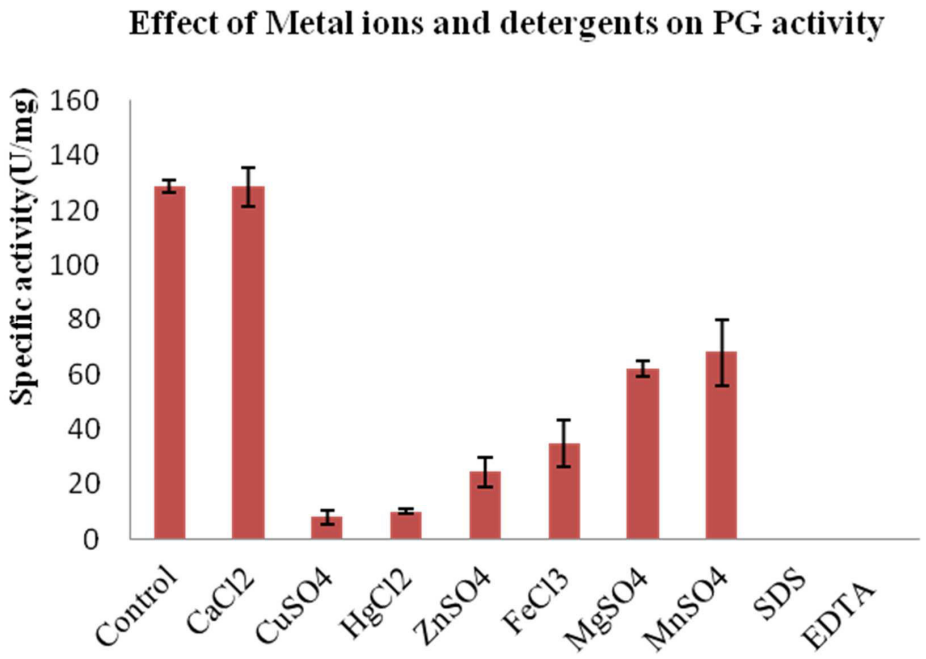

Figure 5
Effect of metal ions and reagents on enzyme activity.

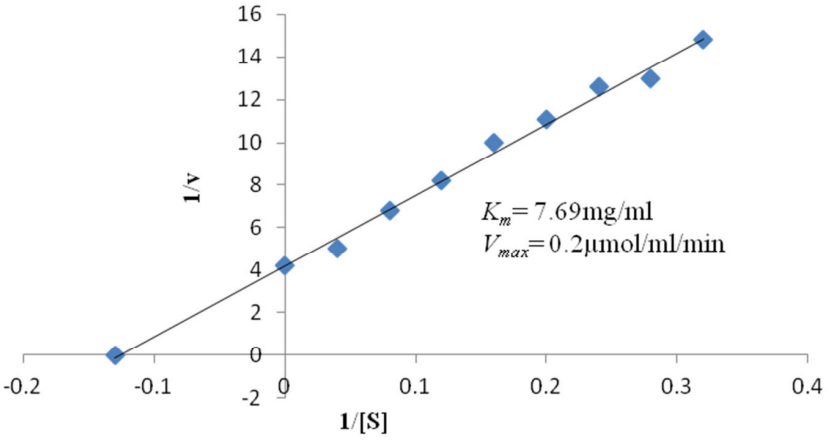

Figure 6
LB plot. PG reaction velocity (1/v) versus substrate concentration (1/[S])

6. Mode of action of the enzyme

The viscometric assay and the identification of hydrolysis products were used to determine the mechanism of action of the enzyme. Measurement of the decrease in relative viscosity of pectin solution showed that, at 50% viscosity reduction, more than 30% of the glycosidic bonds were hydrolyzed. These results are typical to an exopolygalacturonase. This is in accordance to the study of Renta *et al.*[31]. The exo mode of action of the enzyme was further confirmed when the reaction products were analyzed by TLC. Galacturonic acid was the only degradation product released during enzyme activity corresponding to the standard and no high molecular weight products were detected (Fig 7). This finding is similar to the results of Garcia *et al.*[10] and Tohru *et al.*[32].

Figure 7
TLC of Reaction products. Lane 1- Galacturonic acid standard, Lane 2- Culture filtrate + Pectin, Lane 3- Crude enzyme + Pectin.

7. SDS-PAGE of the culture filtrate and the crude enzyme showed a strong protein band corresponding approximately to 60kDa. The results of SDS PAGE, the pH and temperature optima for enzyme activity and the TLC obtained in the present study are typical to an Exopolygalacturonase [10, 27].

CONCLUSION

In our present study, we have screened a large population of *F. oxysporum* f. sp. *lycopersici* for PG production and characterized an exopolygalacturonase. The enzyme was active at acidic pH and at a broad temperature range. It exhibited properties such as high activity and pH and temperature stability. Since *F. oxysporum* are saprophytes and widespread phytopathogens, the industrial applications of their enzymes are limited. Endo-PGs are widely distributed among micro organisms and plants. In contrast, the occurrence of exo-PGs is less frequent in fungi, being reported mainly in bacteria. The PG from *F. oxysporum* f. sp. *lycopersici* is a typical fungal exo-PG. The functional role of this enzyme in phytopathogens and saprobes could be explored for industrial applications. Non pathogenic strains could serve as potential sources of polygalacturonases. In order to use *F. oxysporum* as a suitable industrial biocatalyst and to apply these in various research fields, the purification and improved knowledge of enzyme properties is essential. Engineering approaches can allow the development of systems for the over expression of functional heterologous proteins in yeasts and other hosts making them more suitable for application in food industries. Recombinant genetic techniques may help to breed *F. oxysporum* and *Saccharomyces* strains exhibiting high yield of PG.

ACKNOWLEDGEMENT

The authors are grateful to UGC (University Grants Commission), Ref No: F. No. 34-244/2008 (SR), New Delhi, India for providing the financial support under UGC-Major Research Project.

REFERENCES

1. Demarche P, Junghanns C, Nair RR and Agathos SN, Harnessing the power of enzymes for environmental stewardship. Biotech. Adv, 05: 013, (2011).

2. Hoondal G, Tiwari RP, Tewari R, Dahiya N and Beg Q, Microbial alkaline pectinases and their industrial

applications: a review. Appl. Microbiol. Biotechnol, 59: 409-418, (2002).
3. Lara-Marquez A, Zavala-Paramo MG, Lopez-Romero E and Camacho HC, Biotechnological potential of pectinolytic complexes of fungi. Biotechnol. Lett, 33: 859-868, (2011).
4. Gummadi SN and Panda T, Purification and biochemical properties of microbial pectinases-/a review. Process Biochem, 38: 987-996, (2003).
5. Jayani RS, Saxena S and Gupta R, Microbial pectinolytic enzymes: A review. Process Biochem, 40: 2931-2944, (2005).
6. Coutinho PM and Henrissat B, Carbohydrate-active enzymes: an integrated database approach. In "Recent Advances in Carbohydrate Bioengineering", Gilbert, H. J., Davies, G., Henrissat, B. and Svensson, B., Eds. Cambridge: The Royal Society of Chemistry 1999, 3-12.
7. Jacob N, Pectinolytic enzymes. In Nigam, P. S. and Pandey, A., (Eds.), Biotechnology for Agro-Industrial Residues Utilisation., 2009, Springer Science+Business Media B.V. pp 383-396.
8. Joost van den Brink and Ronald P de Vries, Fungal enzyme sets for plant polysaccharide degradation. Appl Microbiol Biotechnol, 91:1477-1492, (2011).
9. Baayen RP, Schoffelmeer EAM, Toet S and Elgersma DM, Fungal Polygalacturonase activity reflects susceptibility of carnation cultivars to *Fusarium* wilt. European Journal of Plant Pathology, 103: 15-23, (1997).
10. Garcia Maceira I, Antonio Di Pietro and Roncero MIG, Purification and characterization of a novel exopolygalacturonase from *Fusarium oxysporum* f.sp*. lycopersici.* FEMS Microbiology Letters, 154: 37-43, (1997).
11. Niture SK, Comparative biochemical and structural characterizations of fungal polygalacturonases. Biologia, 63: 1-19, (2008).
12. Patil RC, Tushar PM and Shamim AS, Extraction of Pectinase from Pectinolytic bacteria isolated from carrot waste. Int. J. Pharma Bio Sci, 3: 261-266, (2012).
13. Souza JVB, Silva TM, Maia MLS and Teixeira MFS, Screening of fungal strains for pectinolytic activity: endopolygalacturonase production by *Peacilomyces clavisporus* 2A.UMIDA.1. Process Biochem, 4: 455-458, (2003).
14. Kashyap DR, Vohra PK, Chopra S and Tewari R, Applications of pectinases in commercial sector: a review. Biores. Technol, 77: 215-227, (2001).
15. Viikari L, Tenkanen M and Suurnakki A, Biotechnology in the pulp and paper industry. In: Rehm HJ (ed) Biotechnology, 2nd edn, vol 10. VCH-Wiley, 2001, pp 523–546.
16. Bohdziewiez J and Bodzek M, Ultrafiltration preparation of pectinolytic enzymes from citric acid fermentation broth. Proc. Biochem, 29: 99-107, (1994).
17. Pedrolli DB, Monteiro AC, Gomes E and Carmona EC, Pectin and Pectinases: Production, Characterization and Industrial Application of Microbial Pectinolytic Enzymes. The Open Biotechnology Journal, 3: 9-18, (2009).
18. Norhito Y, Jyuichi S, Mamoru S, Seizo S and Takashi S, Physiological races and vegetative compatibility groups of butterhead lettuce isolates of *Fusarium oxysporum* f. sp. *lactucae* in Japan. J Gen Plant Pathol, 70: 308-313, (2004).
19. Nelson PE, Toussoun TA and Marasas WFO, *Fusarium* species., An Illustrated manual for identification., University Park, PA, USA: Pennsylvania State University. 1983.
20. Prashant KM, Roland TVF and Alastair C, Development of a PCR-based assay for rapid and reliable identification of pathogenic Fusaria. FEMS Microbiology Letters, 218: 329- 332, (2003).
21. Hyuk Woo Kwon, Ji Hwan Yoon, Seong Hwan Kim, Seung Beom Hong, Youngah Cheon and Seung Ju Ko, Detection of extracellular enzyme activities in various *Fusarium* spp. The Korean Society of Mycology, 162-165, (2007).
22. Abdel-Raheem A and Shearer CA, Extracellular enzyme production by fresh

water Ascomycetes. Fungal Diversity, 11: 1-19, (2002).
23. Antonio Di Pietro, Garcia Maceira, Dolores Huertas-Gonzales, Carmen Ruiz Roldan, Zaira Caracuel, Andrea Barbieri and Isabel G. Roncero, Endopolygalacturonase PG1 in different Formae speciales of *Fusarium oxysporum.* Applied and Environmental Microbiology, 1967-1971, (1998).
24. Saleh A. Mohamed, Abdulrahman L. Al Malik and Taha A. Kumosani, Characterization of a Polygalacturonase from *Trichoderma harizianum* grown on citrus peel with application for Apple Juice. Australian Journal of Basic and Applied Sciences, 3(3): 2770-2777, (2009).
25. Lowry OH, Roseburgh NJ, Farr AL and Randall RJ, Protein measurement with the folin phenol reagent. Journal of Biological Chemistry, 241: 5298-5306, (1951).
26. Aminzadeh Saeed. Naderi-Manesh Hossein and Khajeh Khosro. Isolation and characterization of polygalacturonase produced by *Tetracoccosporium* sp. Iran. J. Chem. Chem. Eng, 26(1) 47-54, (2007).
27. Christine Riou, Georges Freyssinet and Michel Fevre, Purification and characterization of extracellular Pectinolytic enzymes produced by *Sclerotina sclerotium*. Applied and Environmental Microbiology, 578-583, (1992).
28. Paula Mendes de Freitas, Natalia Martin, Denis Silva, Roberto da Silva and Eleni Gomes, Production and partial characterization of Polygalacturonases produced by Thermophilic *Monascus* sp N8 and by Thermotolerant *Aspergillus* sp N12 on solid substrate fermentation. Brazilian Journal of Microbiology, 37: 302-306, (2006).
29. Maria De Lourdes, Polizeli TM, Joao A. Jorge and Hector F. Terenzi, Pectinase production by *Neurospora crassa*: Purification and biochemical characterization of extracellular polygalacturonase activity. Journal of General Microbiology, 137: 1815-1823, (1991).
30. Antonio Di Pietro and M. Isabel G. Roncero, Endopolygalacturanase from *Fusarium oxysporum* f.sp*. lycopersici*: Purfication, Characterization and Production during infection of tomato plants. Phytopathology, 86: 1324-1330, (1996).
31. Renata I. Barense, Maria Angelica dos, S. C. Chellegatti, Maria Jose V. Fonseca and Suraia Said, Partial Purification and characterization of ExoPG II and III of *Penicillium frequentans*. Brazilian Journal of Microbiology, 32: 327-330, (2001).
32. Tohru Kobayashi, Norihiko Higaki, Noriyuki Yajima, Atsushi Suzumatsu, Hiroshi Hagihara, Shuji Kawai and Susumo Ito, Purification and properties of galacturonic acid- releasing Exopolygalacturonase from a strain of *Bacillus*. Biosci. Biotechnol. Biochem, 65(4): 842-847, (2001).