

Purification and Characterization of an Extracellular Pectinolytic Enzymes Synthesized by *Xanthomonas sp.*

Sujata Chavan and M.M.V. Baig*

Department of Botany and Department of Biotechnology,
Yeshwant Mahavidyalaya, Nanded - 431 602, India.

(Received: 29 November 2013; accepted: 21 January 2014)

Thirty physiologically variant pathogenic strains of *Xanthomonas sp* from various orchards in and around Nanded district, Maharashtra were isolated. Of the thirty strains, one strain isolated from *Citrus lemon* from Nanded district, Maharashtra was found to be the most effective in the pectinolytic enzymes production. This strain was subjected for production of pectinolytic enzymes in surface culture and various factors affecting the enzyme production was studied. The optimum time for production of pectinolytic enzymes by this strain was 48 hrs. The optimum temperature and pH for production of pectinolytic enzymes by this strain was 30°C and 7 respectively. The effect of various carbon and nitrogen sources was studied and it was found that pectin was most suitable carbon source and yeast extract was most suitable nitrogen source for optimum production of pectinolytic enzymes.

Key words: *Xanthomonas sps*, pectinolytic enzymes.

Pectin is high molecular weight complex heteropolymers present in middle lamella and primary cell wall of higher plants. It is made up of D-galacturonic acid residues linked by α -1, 4 linkages¹. It is composed of α -D-galactopyranosyluronic acid (α -D-Galp A) and α -L-rhamnopyranose (α -L-Rhap), α -L-arabinofuranose (α -L-Araf) and B-D-galactopyranose(B-D-Galp)². Pectic substances are classified into protopectin, pectinic acid, pectin and polygalacturonic acid, depending upon degree of esterification³. Protopectin is parent pectin substance, yields pectin or pectinic acid by restricted hydrolysis. Pectic acid composed of galacturonans with negligible amounts of methoxyl groups. Pectinic acid is composed of galacturonans with various amounts of methoxyl groups. Pectin contains pectinic acids as the major components⁴. Pectinases maintain ecological balance by causing decomposition and recycling of waste plant materials⁵. The production of pectinolytic enzymes

and effect of different temperature, pH, carbon and nitrogen sources on their production has been discussed in this study. Pectinolytic enzymes include poly galacturonases (PG), pectin lyases (PL), pectate lyases (PgL), poly methyl galacturonases (PMG); this classification is based on their mode of action. Spoilage and decay of the food causes various problems which can be solved by treatment of efficient pectinases. In fruit and vegetable industries, pectinases are used to increase yield and clarification of juice⁶. It degrades homogalacturonan and rhamno-galacturonic acid of pectin to convert it into sugar and other useful compounds. They may be used in the pretreatment of waste water from vegetable food processing that contains pectin residues⁷. Very little attention had been paid for the study on the pectinolytic enzymes by *Xanthomonas* species that are involved in bacterial virulence.

MATERIALS AND METHODS

Isolation of *Xanthomonas* species

Thirty strains of *Xanthomonas* isolated from *Citrus lemon* from different locations in and

* To whom all correspondence should be addressed.
Tel.: +91- 09422170641; Fax No: +91- 0262-268560;
E-mail: mmvbaig@gmail.com

around Nanded district of Maharashtra were used in this study. All the strains were isolated from infected plant parts during mid-rainy season following standard method. All these strains were purified and maintained on YDC (Yeast extract - 1gm, D-glucose -2gm, Calcium carbonate -2gm) slants. Morphological characteristics were recorded for all these strains include colony characters like Gram staining, Cell morphology, Cell motility⁸. Bacteriological characteristics of the isolates were examined by using the biochemical test described by Goszczynska et al⁹, Aesculin test, Starch hydrolysis, Tween 80 lipolysis, H₂S production, Urease production, Milk proteolysis, Gelatin liquefaction, Oxidase test.

Optimization of cultural parameters for pectinolytic enzyme production

Optimum time for production of Enzyme

The media used for production of pectinolytic enzymes contained Pectin - 5gm/200ml, KH₂PO₄ - 1.6gm, Na₂HPO₄ -1.6gm, MgSO₄.7H₂O - 0.2gm, CaCl₂.2H₂O -0.1gm, Yeast extract - 5 gm. total volume 800 ml (pH 7). Fifty ml of the medium was taken in each 100 ml Erlenmeyer flask and all flasks were autoclaved at 15 lbs. for 20min. The medium was inoculated with 1×10^7 cells/ml and mixed thoroughly. These flasks were subjected for different incubation period varied from 1-6 days at 30°C. Culture filtrate was collected every day and kept in refrigerator by adding 1-2 ml of toluene.

Optimum temperature for production of Enzyme

Five flasks (100 ml) containing medium (50 ml) which is previously used were autoclaved at 15 lbs. for 20min and inoculated with *Xanthomonas* culture (1×10^7 cells/ml). These flasks were incubated for 48 hrs. at temperature range from 25°C to 45°C in surface fermentation¹¹.

Optimum pH for production of Enzyme

By keeping other condition constant the pH of the medium was kept 6.5, 7, 7.5, 8 and 8.5 by using 1N HCl or 1N NaOH in the flasks (100ml) containing medium (50ml). Flasks were autoclaved at 15 lbs. for 20 min then 1×10^7 cells/ml of *Xanthomonas* cultures was inoculated in each flask and was kept for incubation at 30°C for 48 hrs. in static condition.

Effect of carbon on production of Enzyme

Time, temperature and pH of the medium were kept constant, five flasks (100ml) containing

50ml of production medium with different carbon source such as glucose, sucrose, starch and maltose were used in place of pectin at a concentration of 0.5% w/v. These five flasks were first autoclaved at 15 lbs. for 20 min and inoculated with same *Xanthomonas* culture. Flasks were kept for incubation at 30°C for 48 hrs.

Effect of nitrogen on production of Enzyme

Five flasks (100ml) containing medium (50ml) with different nitrogen source such as yeast extract, ammonium nitrate, ammonium sulphate, potassium nitrate and peptone at a concentration of 0.5% w/v by keeping other conditions constant. All these flasks were autoclaved at 15 lbs. for 20 min. Same *Xanthomonas* culture was inoculated in each flask then all flasks were kept for surface fermentation at 30°C for 48 hrs¹¹.

Extraction of enzymes

Firstly all incubated flasks were taken for filtration, the culture filtrate was collected, centrifuged at 6000 rpm for 15 min. Supernatant was collected and cell pellets were discarded. That supernatant was filtered through Whatman no. 1 filter paper. This filtrate was further precipitated by drop wise addition of cold acetone with stirring; the precipitate was collected by centrifugation and used as a crude pectinolytic enzyme solution during the course of study¹².

Assays for enzymes

Polygalacturonase (PG) activity was determined by measuring the increase in absorbance at 240 nm of substrate concentration of 2 ml of 1% polygalacturonic acid in 0.2 M phosphate buffer of pH 6.5 hydrolyzed by 1 ml of enzyme solution¹³. For Poly Methyl Galacturonase (PMG) assay 2 ml of 1% pectin in 0.2 M phosphate buffer of pH 6.5 is used as a substrate which is hydrolyzed by 1ml of enzyme solution is measured by absorbance at 240 nm¹⁴. Pectin Lyase (PnL) activity was measured at 240 nm by determining the increase in absorbance of substrate concentration as 2ml of 1% pectin in 0.2 M phosphate buffer of pH 4.5 is hydrolyzed by 1ml of enzyme solution¹⁵. Substrate concentration as 2 ml of 1% polygalacturonic acid in 0.2 M phosphate buffer of pH 4.5 is used which hydrolyzed with 1% of enzyme solution this reaction is determined by measuring increase in absorbance at 240 nm for the activity of Pectate Lyases (PgL)^{16,17}. One unit of enzymatic activity was defined as the amount of

enzyme which released 1 μ mol of reducing sugar per minute.

SDS-PAGE

SDS-PAGE was performed to check the purity and to determine molecular weight of pectinolytic enzymes by using standard protein marker. The protein bands were visualized on gel by staining it with coomassie brilliant blue.

RESULTS AND DISCUSSION

Effect of different incubation time on pectinolytic enzyme production

The flasks were incubated at different time period of 1st, 2nd, 3rd, 4th, 5th and 6th days for pectinolytic enzyme production by keeping all other

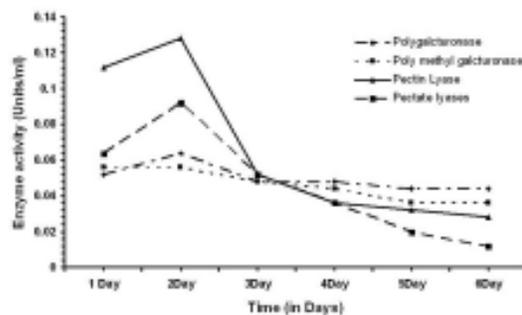


Fig. 1. Effect of incubation period on pectinolytic enzyme production

Effect of pH on pectinolytic enzyme production

The experiment was conducted for the study of effect of pH on pectinolytic enzyme production. Different pH namely 6.5, 7, 7.5, 8 and 8.5 were used by keeping all other conditions constant. As initial pH was increased from 6.5 to 7, the pectinolytic activity also increased. Further increase in initial pH beyond 7 reduced the pectinolytic activity. A maximum pectinolytic activity was seen by PG (0.088 U/ml), PMG (0.076 U/ml), PgL (0.096 U/ml) and PnL (0.100 U/ml) when pH of the medium was 7. Hence as seen in figure.3 optimum pH value was chosen as 7.

Effect of different C-Sources on pectinolytic enzyme production

Culture of *Xanthomonas* produced extra cellular enzymes which are influenced by different carbon and nitrogen sources and different environmental conditions like temperature, pH. Pectinolytic enzyme production is carried out using

process parameters same. Pectinolytic enzyme production PG (0.064 U/ml), PMG (0.056 U/ml), PgL (0.092 U/ml) and PnL (0.128 U/ml) is maximum on 2nd day of incubation as seen in figure 1.

Effect of temperature on pectinolytic enzyme production

The effect of temperature on pectinolytic enzyme production was studied by conducting experiment at different temperature namely 25°C, 30°C, 35°C, 40°C and 45°C. As temperature increases from 25°C-30°C, the pectinolytic activity was found to increase and maximum activity was found at 30°C. Further increase in temperature decrease the pectinolytic activity. Hence optimum temperature value was chosen as 30°C. The results are given in figure 2.

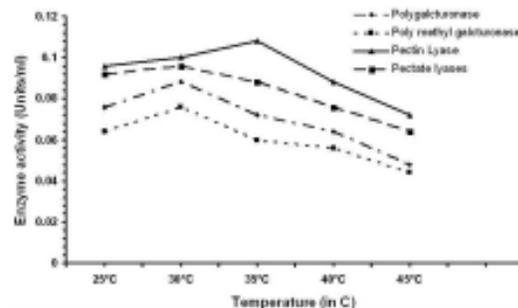


Fig. 2. Effect of temperature on pectinolytic enzyme production

different carbon-sources like glucose, sucrose, starch, maltose and pectin in the media and all other process parameters were same. Pectinolytic enzyme production is maximum when media contained pectin as a carbon-source. Maximum activity of PG (0.084 U/ml), PMG (0.076 U/ml), PgL (0.096 U/ml) and PnL (0.104 U/ml) was seen in medium containing pectin. PnL showed the highest activity among all these enzymes. The results are as shown in figure 4.

Effect of different Nitrogen Sources on pectinolytic enzyme production

Different nitrogen sources like yeast extract, ammonium nitrate, ammonium sulphate, potassium nitrate, peptone were used for the production of pectinolytic enzymes by keeping other conditions constant. When media contained yeast extract as nitrogen-source pectinolytic enzyme production is maximum. All enzymes PG (0.088 U/ml), PMG (0.072 U/ml), PgL (0.092 U/ml)

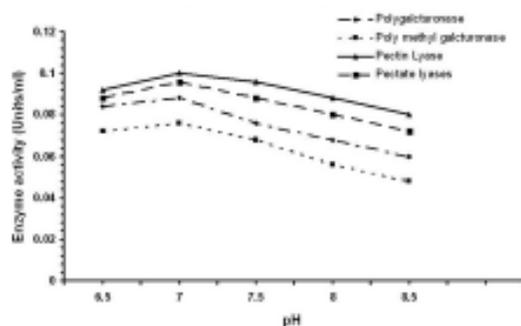


Fig. 3. Effect of pH on pectinolytic enzyme production

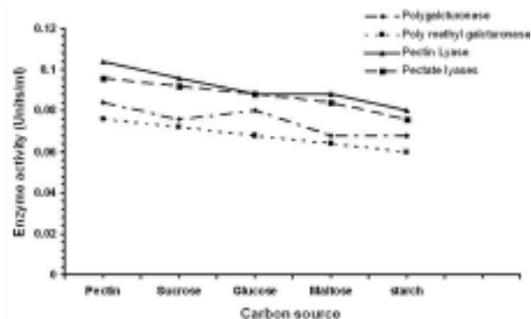


Fig. 4. Effect of C source on pectinolytic enzyme production

and PnL (0.100 U/ml) showed maximum activity when media contained yeast extract as nitrogen-source. The results are given in figure 5.

SDS-PAGE

The purified pectin lyase exhibited a single band on SDS-PAGE. When compare it with standard molecular weight markers, it showed molecular weight of 35 kDa. The *pghAxc* gene encodes an exo-PG of 47 kDa, and *pghBxc* encodes an endo-PG of 60kDa¹⁸. A major endopolygalacturonase excreted by *Pseudomonas solanacearum* was purified to >95% homogeneity and shown to have an isoelectric point of 9.0 and a subunit molecular mass of 52 kilodaltons (kDa)¹⁹. The molecular weight of alkaline PLs produced by

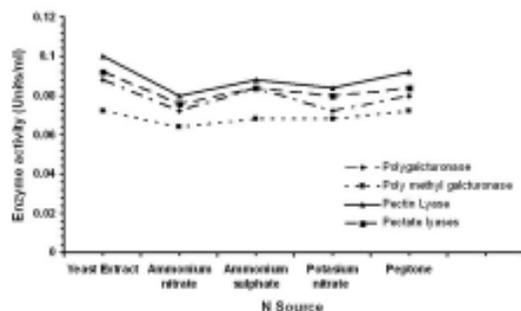


Fig. 5. Effect of N source on pectinolytic enzyme production

P. viridiflava, *P. fluorescens*, *X. campestris*, and *C. johnsonae* were estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis to be 42,000, 41,000, 41,500, and 35,000, respectively²⁰. PNL from *E. chrysanthemi* has a molecular weight of 34,500, a pH optimum of 8.3²¹.

The identification and characterization of pectinase produced by bacteria with different biochemical activities can be potentially very interesting from their application point of view. The characterization of pectinases from *Xanthomonas* will impart an insight in the bacterial pathogenesis and these enzymes can be industrially important also.



Fig. 6. SDS-PAGE of purified pectinolytic enzymes

REFERENCES

- Patil, N.P. and Choudhari, B.L. Production and Purification of pectinases by soil isolate *Penicillium* species and search for better Agro
- residue for its SSF, *Recent Research in Science and Technology*, 2010; **2**(7):36-42.
- Capek, P., A. Kardosová, and D. Lath, A Neutral Heteropolysaccharide from the Flowers of *Malva mauritiana* L. *Chem. Papers*, 1999; **53**(2):

- 131-136.
3. Gummadi, S.N. Manoj, N. and Kumar D.S. Structural and Biochemical properties of pectinases J. Palaina and A.P. MacCabe (eds.), *Industrial Enzymes*, 2007; 99-115.
 4. Kiro Mojsov. The effects of different carbon sources on biosynthesis of pectinolytic enzymes by *Aspergillus niger* *Applied Technologies and Innovations*, 2010; 3(3): 23-29.
 5. Jayani, R.S., Saxena, S., Gupta, R., Microbial pectinolytic enzymes: A review, *Process Biochem.*, 2005; 40: 2931-44.
 6. Sharma, N.R., Sasankan, A., Singh, A. and Soni G., Production of polygalacturonase and pectin methyl esterases from Agrowaste by using various isolates of *A. niger*, *Insight Microbiol.*, 2011; 1(1).1-7.
 7. Pedrolli, D.S., Moteriro, A.C., Eleni Gomes and Carmona E.C., Pectin and Pectinases: Production, Characterization and Industrial Application of microbial pectinolytic enzymes, *The Open Biotechnol J.*, 2009; 3: 9-18.
 8. Bradbury, J.F. Genus I. *Xanthomonas* Dowson. In *Bergey's Manual of Systematic Bacteriology*, 1984; 1: 63-90. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
 9. Goszczynska, T., Serfontein, J.J. & Serfontein, S. Introduction to practical phytobacteriology. 2000; SAFRINET SDC Switzerland.
 10. Starr, M.P. and Nasuno, S., Pectolytic activity of phytopathogenic Xanthomonads, *J. Gen. Microbiology* 1966; 46: 425-433.
 11. Jayani, R.S. Surendra Kumar Shukla and Reena Gupta, Screening of Bacterial Strains for Polygalacturonase Activity: Its Production by *Bacillus sphaericus* (MTCC 7542) *Enzyme Research* Volume 2010; 1-5. (Article ID 306785)
 12. Weber and Parola, Pectinolytic Enzymes of Oral Spirochetes from Humans. *Appl. and Environmental Microbiol.*, 1984; 48 (1) 61-67.
 13. Gewali, M.B., Maharjan, J. Sambhu, T and Jaya Krishna Studies on Polygalacturonase from *A. flavus* *Scientific World*, 2007; 5:19-22
 14. Mahadevan, A. and Sridhar R. Methods in Physiological Plant Pathology. 1986; Sivakami publishers, Madras, pp.103-104.
 15. Ramanujam, P. K. Saritha, N., Palani Subramanian, Production of pectin lyase by solid state fermentation of sugarcane bagasse using *Aspergillus niger* *Advanced Biotech.* 2008; 30-33
 16. Viviani F. Roberto da Silva, D'enis Silva, and Eleni Gomes Production of Pectate Lyase by *Penicillium viridicatum* RFC3 in Solid-State and Submerged Fermentation *Int. J. of Microbiol.*, 2010; 1-8
 17. Visser, J. and Voragen, A.G.J. Pectines and pectinases *Progress in Biotechnology* 1996; 14: 3-990
 18. Wang, L., Rong, W and He, C Two *Xanthomonas* Extracellular Polygalacturonases, PghAxc and PghBxc, are regulated by Type III secretion regulators HrpX and HrpG and are required for virulence. *Mol Plant Microbe Interact.* 2008; 21(5): 555-63.
 19. Schell, M.A., Roberts, D.P. and Denny, T.P. Analysis of the *Pseudomonas solanacearum* polygalacturonase encoded by pglA and its involvement in phytopathogenicity. *J. Bacteriol.* 1988; 170(10):4501.
 20. Ching-Hsing Liao Analysis of Pectate Lyases Produced by Soft Rot Bacteria Associated with Spoilage of Vegetables *Appl And Environmental Microbiol*, 1989; 1677-1683.
 21. Kotoujansky, A. Molecular Genetics of Pathogenesis by Soft-Rot Erwinias. *Annual Rev. of Phytopathol.* 1987; 25: 405-430.