Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Scholars Research Library

Der Pharmacia Lettre, 2011, 3(2): 358-367
(http://scholarsresearchlibrary.com/archive.html)

Der Pharmacia Lettre
Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Extracellular Thermostable Polygalacturonase from *Bacillus* sp. AD 1

Arpita Dey, Moumita Karmakar and Rina Rani Ray*

Microbiology Laboratory, Department of Zoology, Molecular Biology & Genetics, Presidency University, Kolkata, India

---

ABSTRACT

The production of polygalacturonase (PG ase) by a locally isolated aerobic bacterium, Bacillus sp. in submerged fermentation (SmF) was optimized. The effects of the fermentation parameters namely initial pH, temperature, cultivation time and nitrogen source on enzyme production were studied using both pure pectin and citrus wastes as the sole carbon source. Polygalacturonase from the strain was maximally secreted at 37°C, initial pH 7.0 with 0.15% (w/v) of pure pectin as sole carbon source. Among citrus wastes, 1% (w/v) orange peel and lemon peel gave the most promising results at pH 7 and 5 respectively. Tryptone was found to be the best nitrogen source for enzyme synthesis. Under optimized conditions, highest PG ase production was achieved at 24th hour, at mid stationary phase of growth of the strain. The enzyme was extremely thermostable and 75% of the activity was restored even after the exposure of the enzyme protein at 80°Cfor 90 minutes. The enzyme was 86% stable in a broad range of pH of 4 to 9. The PG ase activity was found to increase in presence of Mn^{2+} and also by addition of exogenous thiols like DTT, Cysteine, GSH indicating the presence of thiol groups at the active site.

Keywords: Polygalacturonase, *Bacillus* sp, pectin, citrus wastes.

INTRODUCTION

Pectin is a family of complex polysaccharides that contain 1,4-linked α-D-galactosyluronic acid residues [1]. Although it is a natural part of human diet, it does not contribute significantly to nutrition and goes through the small intestine more or less intact. Enzymes that hydrolyze pectic polymers are broadly known as pectinases and microbial pectinaes have tremendous potential to offer mankind [2].Amongst the pectic enzymes, polygalacturonases (EC 3.2.1.15) catalyze the random hydrolysis of 1,4 α-D galacturonic acid linkages in smooth region of pectin [3] and have

attracted the attention of scientists from biotechnology or pharmaceutical industry because they are protein enzymes relevant to phytopathogens invasion, fruit ripening, and potential antimicrobial drug targets [4] . Commercially, pectinase has a share of 25% in the global sales of food enzymes [5] and is added to livestock feed to help the animals for better digestion of the food and are also sold as nutritional supplements for humans to aid digestion . The pectinase production from microorganisms has been reported under both submerged and solid state fermentations [6] . At present almost all the pectinolytic enzymes used for industrial applications are produced by fungi and there are a few reports of pectinase production by bacterial strains [7]. Hence an extensive research is warranted to isolate a bacterial strain for production of polygalacturonase within a short period of time and standardization of the conditions for maximum production of the enzyme in a cost effective way.

The aim of the present study was the optimization of production parameters for exo-polygalacturonase from potent soil isolated bacterial strain by submerged fermentation of citrus agro wastes and characterization of the enzyme produced.

MATERIALS AND METHODS

Chemicals

Pectin and D-galacturonic acid monohydrate were obtained from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals were of analytical grade. Various pectin wastes like peels of citrus fruits were collected from domestic and market effluents. Those were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure pectin.

Microorganism

A strain of *Bacillus* sp. was isolated from the decaying vegetation enriched soil of Presidency University, Kolkata. The strain was repeatedly sub cultured on pectin plates and maintained at 4 °C for further studies.

Cultivation of the Strain

The strain was cultivated in 100 mL Erlenmeyer flasks each containing 10 mL Basal Medium (BM) composed of (g L^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1; $MgSO_4.7H_2O$ 0.1 and pectin 0.15. (pH: 7) for 36 hours.

Measurement of Growth

The growth was measured by turbidometric method at 650 nm [8] .

Enzyme Assay

The grown culture was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure the activity of polygalacturonase, the assay mixture (1 mL) containing an equal volume of enzyme and 1% (w/v) pectin dissolved in 0.1(M) phosphate buffer (pH-7) was incubated at 37°C for 15 min. The reducing sugar released was measured by the dinitrosalicylic acid method [9]. Blanks were prepared with inactivated enzymes .One unit of enzymatic activity (U) was defined as one µmol of galactunoric acid released per minute [10] .

Pectin waste Materials

Various pectin wastes were collected from domestic and market effluents, agricultural fields and temple wastes. Those were dried, pulverized and sieved as 40 mesh particle size before using in fermentation media in place of pure pectin.

Optimization of other Parameters

The concentrations of agro wastes used as sole carbon source were varied to optimize the substrate concentration of submerged culture of *Bacillus* sp. The optimum pH was determined by adjusting the initial pH of the fermentation media at a range from 4.0-9.0. Most favorable production temperature was studied by incubating the production medium at different temperatures (7°C, 17°C, 27°C, 37°C, and 47°C). To study the effect of concentration of optimized carbon source for maximal enzyme production, pure pectin as well as the citrus wastes were used at different concentrations (0.03% to 3% w/v) in the production media. Similarly, the effects of various nitrogen sources namely peptone, yeast extract, ammonium chloride, tryptone and urea 0.09% (w/v) were tested. Each experiment was carried out in triplicate and their values were averaged. The time course of growth and enzyme production by the strain under optimized culture conditions was studied by checking the enzyme production kinetics fro 0 to 36 hours at 37°C.

Enzyme characterization

The stability of the enzyme at different pH values was studied by incubating the enzyme (0.5ml) with buffers presenting pH from 4.0 to 9.0 was kept at 37°C for 120minutes followed by the estimation of the residual activity. Thermostability kinetics was determined by exposing the pectinase at 80°C for 0 – 90 minutes in water bath and then estimating the residual activity. The effect of metal ions and thiol compounds was measured by incubating the enzyme at 37°C for 30 minutes with the additives at a concentration of 10mM followed by the assay of enzyme activity in usual procedure.

RESULTS AND DISCUSSION

Out of the 14 bacterial isolates obtained, strain AD1 was selected for further studies. The strain was identified by their basic morphology and biochemical properties according to Bergey' s Manual of Systematic Bacteriology [11] as *Bacillus* sp.

Among the carbon sources used, pectin gave the best activity for production of PGase followed by other citrus pectins present in the peels of orange, lemon and sweet lime (Fig 1) suggesting that the organism utilized pure pectin more efficiently as compared to citrus pectin.

The maximum enzyme activity was obtained when the initial pH of the production media supplemented with pure pectin and orange peel were adjusted to 7.0 (Fig. 2), and there was a sharp decrease in enzyme activity at pH of 9.0. The preference for a neutral pH for PG ase production was similar to that of *Bacillus sphaericus* [7] and other *Bacillus* strains [12].On the other hand, lemon peel containing medium showed highest PG ase production at pH 5.0, similar to the optimum pH reported from the fungal strains [13] , [14].

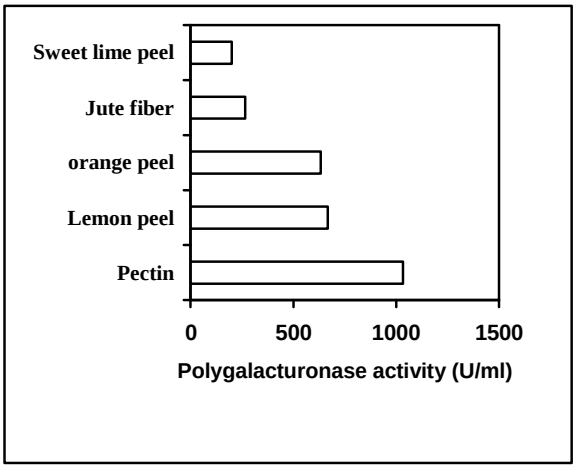

Fig 1.Effect of various substrates as inducer of polygalacturonase production by *Bacillus* sp AD 1

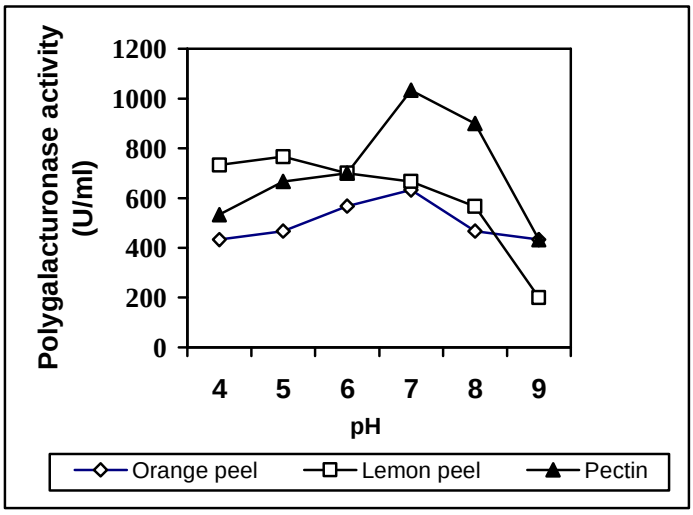

Fig 2. Effect of pH on Polygalacturonase production by *Bacillus* sp AD 1

Most favorable production temperature for PGase production by Bacillus sp AD 1 was found to be 37°C (Fig 3).Generally an optimal temperature of 30°C was found for PGase production by *Aspergillus* sp. [15] and *Peacilomyces clavisporus* [12] and a higher temp of 50°C was required for *Bacillus subtilis* CM5 [6].

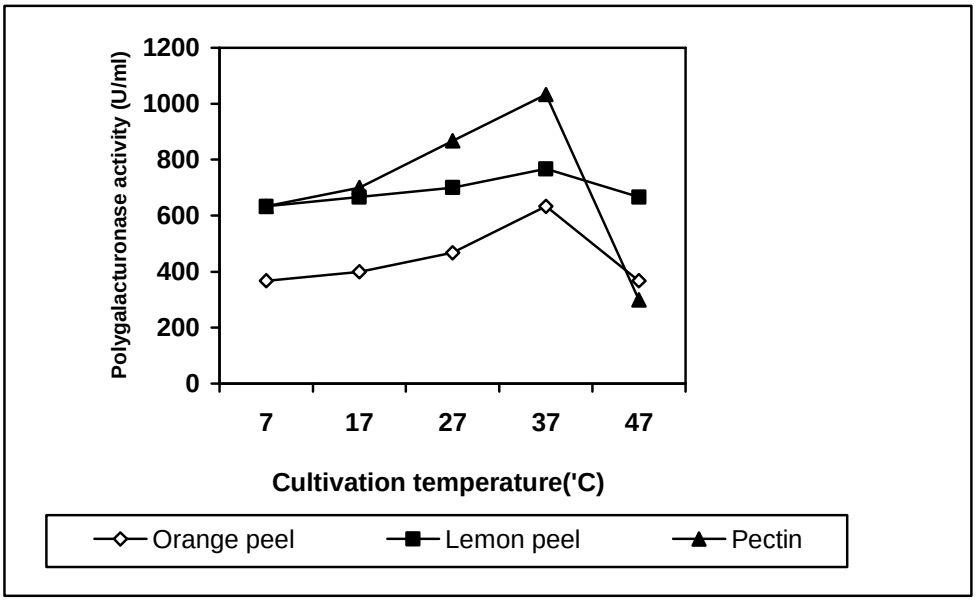

Fig 3. Effect of cultivation temperature on Polygalacturonase production by *Bacillus* sp AD 1

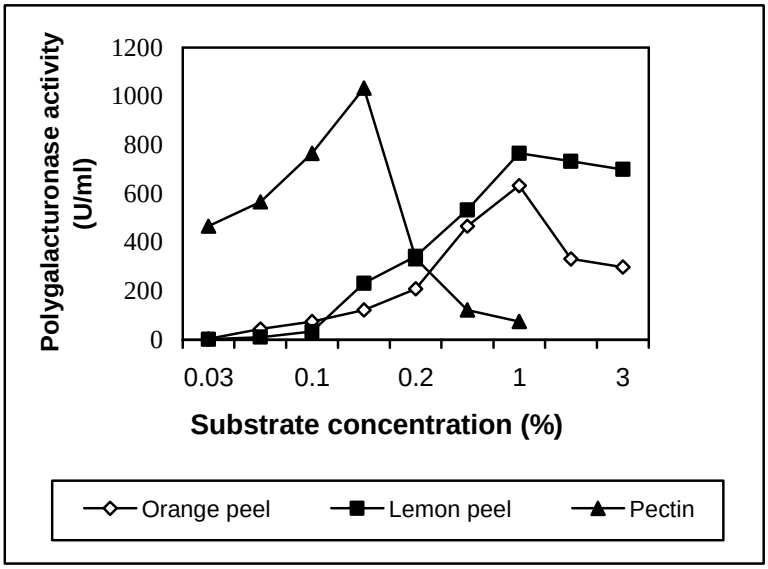

Fig 4. Effect of substrate concentration on Polygalacturonase production by *Bacillus* sp AD 1

Enzyme synthesis as found to increase gradually with increase in substrate concentration and highest production was achieved at 0.15% (w/v) of pure pectin was used. The enzyme synthesis decreased drastically with further increase in substrate concentration, which might be due to enzyme limitation. On the other hand, 6.7 times higher concentration of substrate was essential for PGase production from citrus wastes which indicated the presence of relatively lesser amount of degradable pectin molecules in these citrus wastes.

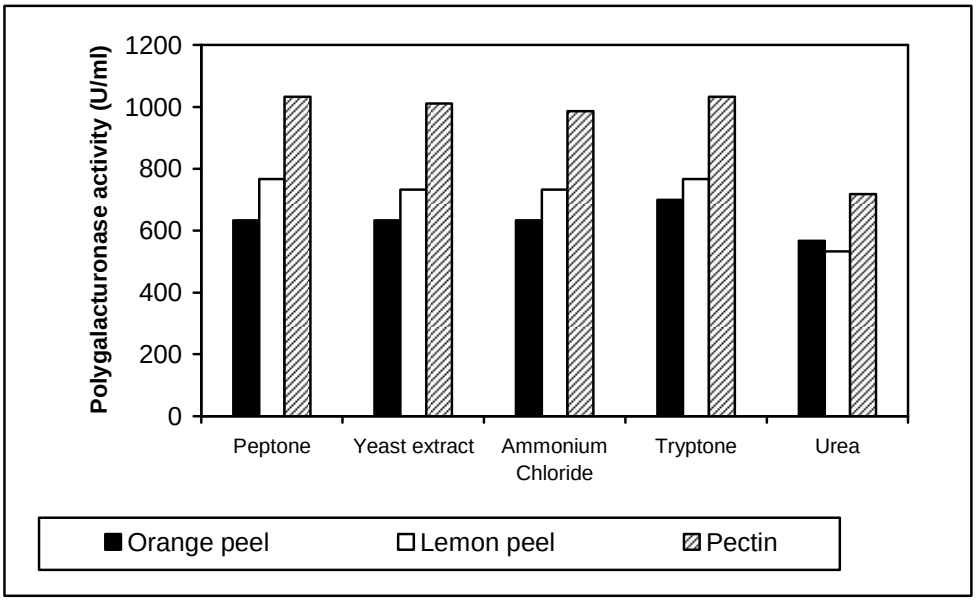

Fig 5. Effect of various nitrogen sources on Polygalacturonase production by *Bacillus* sp AD 1

Of the various nitrogen sources used, maximum PGase activity was observed when tryptone was used (Fig 5), although this increase was not so prominent in case of pure pectin supplemented medium. Ammonium chloride could not bring about any notable increase, a result contrary to [16], and urea reduced the PG ase production in all types of substrates.

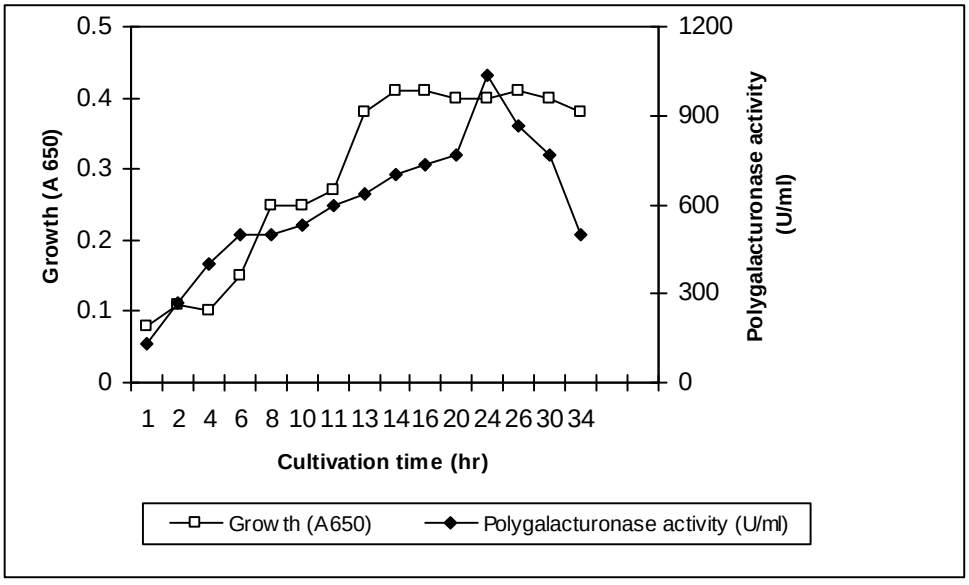

Fig 6. Kinetics of growth and polygalacturonase production by *Bacillus* sp AD 1
(Substrate: 0.15% pure pectin, pH: 7.0, temperature 37°C)

When the growth and enzyme production profile of *Bacillus sp* AD 1 was studied, a rapid increase in biomass during the first 8 hours of fermentation and then after 14 hour the growth became almost stationary probably due to exhaustion of nutrients in the culture medium. The enzyme production started increasing after 8 hours and reached its maximum after a period of 24

hours (Fig 6) after which it gradually declined. This was relatively a very rapid rate of production in comparison to that already reported strains of bacteria and fungi which were 72-73 hours [17, 18, and 19].

The optimum pH of the extracted enzyme was found to be 8.0 (Fig 7), which was higher than those reported from other PG ase secreting bacterial [12] and fungal strains [13, 20, 21, 22, 23]. The enzyme was found to be stable at a broad pH range of 4 to 9, which would be advantageous during industrial applications of the enzyme.

The optimum temperature of the enzyme was found to be at 60°C (Fig. 8), slightly higher than that reported from different microbial strains [12,20,21,23].

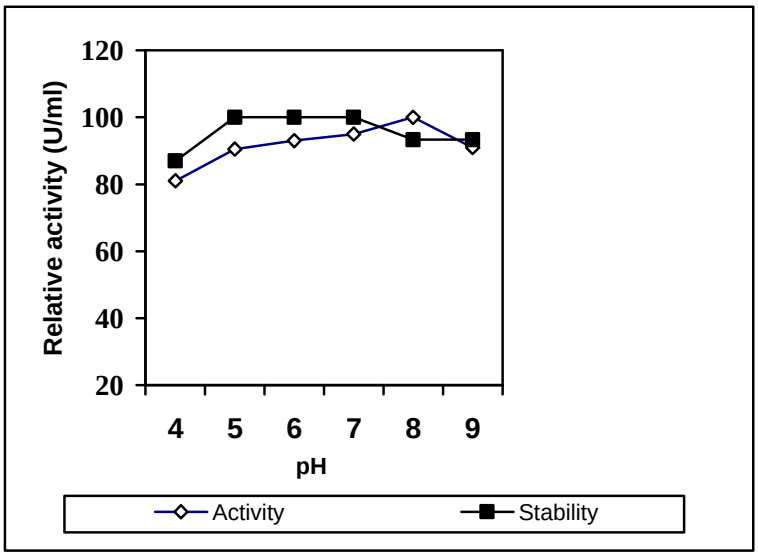

Fig 7. Effect of pH on activity and stability of polygalacturonase from *Bacillus* sp AD 1

The thermo inactivation kinetics of PG ase (Fig 9) showed that 75% the residual enzyme activity was retained even after 90 minutes of exposure at 80°C indicating the thermostable nature of the enzyme. This thermostability would make the enzyme applicable for commercial use.

Addition of various mono and divalent ions, surfactants and thiol compounds (Table 1) indicated that amongst metallic ions tested, only Mn^{2+} could increase the enzyme activity. The ability of the exogenous thiol compounds like dithiothritol (DTT), reduced glutathione (GSH) and cysteine to enhance the activity of the enzyme revealed the presence of thiol at the active site of the enzyme.

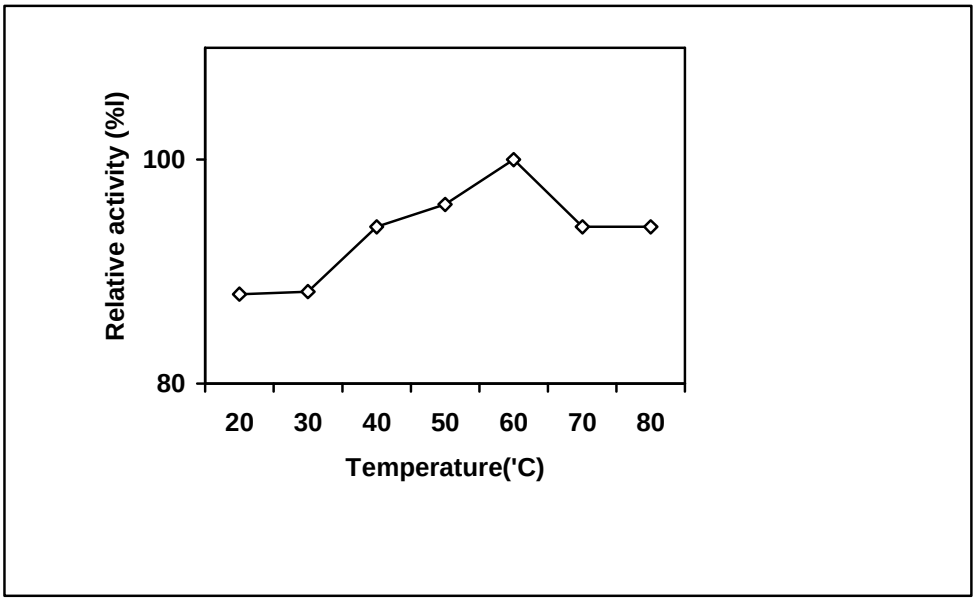

Fig 8. Effect of temperature on activity of polygalacturonase from *Bacillus* sp AD 1

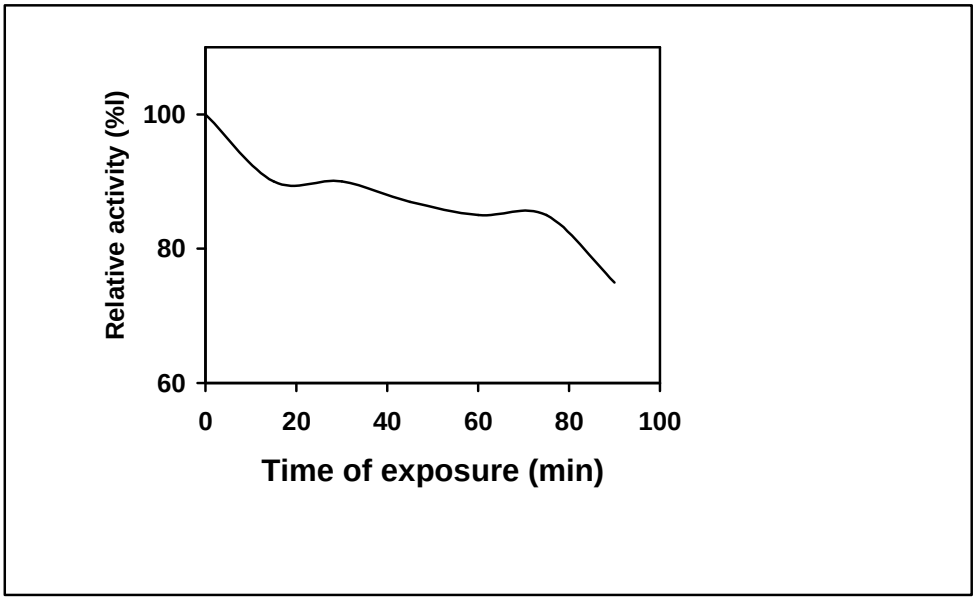

Fig 9. Thermo inactivation kinetics of polygalacturonase from *Bacillus* sp AD 1 exposed at 80°C.

The working strain being a rapid producer of polygalaturonase from citrus wastes might be used for the commercial production of the enzyme. Substitution of pure pectin by citrus wastes could not only reduce the cost of the enzyme, but open an avenue for successful waste utilization. The high thermostability and the broad pH stability of the enzyme made it convenient for application in food processing, pharmaceutical and distillery industries.

Table 1. Effect of various additives on the activity of the polygalacturonase from *Bacillus* sp AD 1

Additives (10mM)	Relative activity (%)
None	100
Na^{+}	72.7
K^{+}	68.2
Ca^{2+}	72.7
Mn^{2+}	136.4
Hg^{2+}	22.7
Cu^{2+}	58.2
Mg^{2+}	68.2
Fe^{2+}	86.4
DTT	131.8
pCMB	65.5
GSH	131.8
Cystine	90.9
Cysteine	118.2
Tween 20	72.7
Tween 40	90.0
Tween 80	81.8
EDTA	50
Beta Marcaptoethanol	ND

100% activity: 1030U/ml

REFERENCES

[1]R.P. de Vries; J. Visser. *Microbiology and Molecular Biology Reviews***,2001**, 65,4, 497-552.
[2]G.S.Hoondal; R.P.Tiwari; R.Tewari; N .Dahiya; O.K.Beg. *Applied Microbiology and Biotechnology* **,2002**, 59, 409-418.
[3]E.G. da Silva; M.de.Fatima Vorges; C.Medina; R.H. Piccoli; R.F. Schwan. FEMS *Yeast Research.* **2005**, 5, 859-865.
[4]A.G.Chapin; J.V.Santos; G.A. de la Riva; A. Antunes; T.G.Villa; E. Uriarte;H. G.Dıaz. *Journal of Proteome Research*, **2009**, 8,4,2122–2128
[5] R. S. Jayani; S. Saxena;R. Gupta. *Process Biochemistry* ,**2005** ,40, 2931-2944
[6] M. R. Swain; S Kar; R. C. Ray. *Brazilian Journal of Microbiology*, **2009**,40,3.
[7]R.S. Jayani ; S.K. Shukla , R. Gupta. *Enzyme Research*, **2010**.
[8] N.Noisommit-Rizzi; A. Kastle;A. Pritschow; M. Reuss;M. Rizzi. *Biotechnology Letter*,**1996**, 10, 173-178.
[9] P. Bernfeld. *Methods of Enzymology*, **1955,** 1,149-150
[10]B. Trejo-Aguilar; J.Visser; G.Aguilar-Osorio. Proceedings of Pectin Pectinases Symposium, **1996,** 915-920.
[11] P.H.A.Sneath; N.S. Mair; M.E. Sharpe;J.G. Holt. Bergey' s Manual of Systematic Bacteriology. Williams and Wilkins Co., Baltimore, **1986,** 1104-1139.
[12] M.M.C.N. Soares; R. Da Silva; E.Gomes. *Revista de Microbiologia*,**1999**,30,4,299–303.
[13] A.Thakur; R.Pahwa;S. Singh; R. Gupta. *Enzyme Research*, **2010,** 7.
[14] M. L. Buga;S.Ibrahim; A. J. Nok. *African Journal of Biotechnology*, **2010**, 9 , 52, 8934-8943.

[15] M. Galiotou-Panayotou; M .Kapantai; O. Kalantzi. *Applied Microbiology and Biotechnology*, **1997**,47,4,425–429.
[16] D.R. Kashyap; S.K. Soni; R.Tewari . *Bioresource Technology*,**2003,**88,3,251–254
[17] O. Loera;J. Aguirre; G. Viniegra-González. *Enzyme and Microbial Technology*, **1999**,25, 1-2, 103–108,
[18] P.M. de Freitas; N.Martin; D.Silva; R. da Silva; E.Gomes. *Brazilian Journal of Microbiology*, **2006** , 37,302-306.
[19] E. Ta$kın; E. Rengin. *Food Biotechnology*, **2008**, 22,203 – 217.
[20] D.B. Pedrolli ; E. Gomes ; R .Monti ; E.C. Carmona. *Applied Biochemistry and Biotechnology*, **2008** ,144, 2,191-200.
[21] C. Tari; N.Dogan ;N. Gogus. *Food Chemistry*, **2008,** 111, 4, 824-829.
[22] N.Patil; K.Patil; B. Chaudhari;S. Chincholkar. *Indian Journal of Microbiology*, **2011**, 1-7.
[23]G.Kaur; S.Kumar; T.Satyanarayana. *Bioresource Technology*, **2004**,94, 3, 239-243.