Effects of Nitrogen Sources on the Production of Exo-Pectinase

Özlem Tepe and Arzu Y. Dursun

***Abstract*—** In this study, production of exo-pectinase by a gram-positive bacterium, *Bacillus pumilus* was studied in a batch system and effects of nitrogen sources, on microbial growth and exo-pectinase activity were investigated. Ammonium sulphate, yeast extract and peptone were used as different nitrogen sources and the highest enzyme activity was determined as 8.0 U/mL for 0.05 % (w/v) ammonium sulphate.

***Index Terms*—** *Bacillus pumilus*, exo-pectinase activity, nitrogen sources.

I. Introduction

Pectin is the descriptive name for a diverse group of complex heteropolymers present in the middle lamella of the primary plant cell wall of dicotyledonous plants. Pectinases (pectinolytic enzymes) comprise a group of enzymes able to catalyze the breakdown of pectin-containing substrates [1-6]. There are several types of pectinolytic enzymes according to the activities shown toward the pectic regions found in pectin chain. Basically, there are three types of pectinases: depolymerizing enzymes (hydrolases and lyases), de-esterifying enzymes (pectinesterases) and protopectinases. Pectinase is extensively used in food processing industry, souring of cotton, degumming of plant fibers, waste water treatment, vegetables oil extractions, tea and coffee fermentation, bleaching of paper, in the alcoholic beverage [1,4-6]. The microbial production of pectinolytic enzymes can be achieved in solid-state fermentation (SSF) or submerged fermentation (SmF), where free and immobilized fungal cells can be used. However, in SSF there are technological problems such as; controlling the temperature and pH, and process monitoring due to the nutrient gradients in large scale. Hence, industrial production of enzymes is performed predominantly by SmF [7,8]. The pectinolytic enzymes have been industrially produced by the moulds [9,10]. Like all pectinases, exo-pectinase is mainly produced by fungal genera; *Aspergillus, Penicillium* and *Fusarium* [11,12]. But there is still lack of the studies focusing on using bacteria in exo-pectinase fermentation.

In this study, production of exo-pectinase by a gram-positive bacterium, *Bacillus pumilus* was studied in a batch system and effects of nitrogen sources, on microbial growth and exo-pectinase activity were investigated.

Ozlem Tepe is with Firat University, Elazig, 23119 Turkey
Arzu Y. Dursun is with Firat University, Elazig, 23119 Turkey

II. Experimental

A. *Microorganism and Culture Conditions*

Bacillus pumilus (NRRL B-212) was obtained from National Center for Agricultural Utilization Research (Microbial Genomics & Bioprocessing Research Unit, lllinois, USA) in lyophilized form. Firstly, It was activated in potato dextrose agar at 30 ^{o}C and then transferred to the microorganism growth medium (enrichment medium) which contained (amounts given per L); glucose, 3 g; yeast extract, 2 g; peptone, 2 g; KH_2PO_4, 1 g; K_2HPO_4, 1 g; $(NH_4)_2SO_4$, 1 g; $MgSO_4.7H_2O$, 0.05 g. The pH of the medium was adjusted to 7 and it was sterilized by autoclaving at 1.1 atm, 121 ^{o}C for 20 min. The microorganism was incubated at 30^{o}C in an agitated shaker (100 rpm) for 24 h. Then it was transferred (in 1:10 ratio) into the enzyme production medium.

B. Enzyme production

Exo-pectinase was produced under submerged fermentation in 500 mL Erlenmeyer flasks containing 150 mL of enzyme production medium (%w/v: apple pectin, 1; $(NH_4)_2SO_4$, 0.14; KH_2PO_4, 0.2; K_2HPO_4, 0.6; $MgSO_4.7H_2O$, 0.01) at 150 rpm shaking conditions. After sterilization by autoclaving (pectin solution was autoclaved separately), the pH of the medium was adjusted to the desired value by using sterilized H_2SO_4 or NaOH solutions. Samples were taken out at fixed time intervals and crude exo-pectinase was harvested by centrifuging and the clear supernatant was used as the enzymes source for further studies.

C. Analytical methods

The biomass concentration was determined by measuring the absorbance at 400 nm using a standard curve of absorbance against dry cell weight.

Enzyme assay

Exo-pectinase activity was assayed by measuring the amount of D-galacturonic acid liberated from pectin. The reaction mixture containing 0.25 mL appropriately diluted enzyme and 0.25 mL of 1 % pectin (apple pectin in glycine-NaOH buffer, pH 10) (70-75% degree of esterification, Sigma-Aldrich) was incubated for 15 min at 50 ^{o}C, and the end products were quantitated using dinitrosalicylic acid (DNSA) reagent [13]. An equal volume of 1% DNS (0.5 mL) was added to the tube, and the mixture was incubated for 5 min in a boiling water bath and immediately cooled in an ice bath. Then, the O.D.530 nm was read on a spectrophotometer (Jenway 6105 UV/Vis.Spec.) by comparison with a blank (control) containing only the substrate and DNS. One unit of exo-pectinase was defined as the amount of enzyme required for liberating l µmol of D-galacturonic acid

mL^{-1} min^{-1} under the assay conditions, using a standard curve obtained from the galacturonic acid (Sigma-Aldrich).

III. Results And Discussion

A. Effects of nitrogen sources

The selection of appropriate sources of carbon, nitrogen and other nutrients is one of the most critical stages in the development of an efficient and economic enzyme production process. It is also known that 30–40% of the enzyme production cost is attributed to the fermentation medium. Besides other nutrient sources, nitrogen constituent has a profound effect on production of pectinolytic activity in culture medium. In this study, the effects of nitrogen sources, on microbial growth and exo-pectinase activity were investigated. Initial studies showed that maximum of exo-pectinase activity was observed at an initial pH=8 and 1% (w/v) pectin concentration. Ammonium sulphate, yeast extract and peptone were used as different nitrogen sources. Three various concentrations of these materials 0.05, 0.14 and 0.3 % (w/v) were tested while the pectin (1% (w/v)) and other components' concentrations kept constant.

Yeast extract has been reported to be one of the most widely used nitrogen source for pectinase production by *Bacillus subtilis* [14]. In this study, firstly yeast extract was tested as nitrogen source. Figure 1 shows the variations in cell concentration. Figure 1 indicates that the adaptation phase (lag phase) for the bacterial growth lasted for around 10 h for the culture in three concentrations. The exponential phase (log phase) of the culture lasted for around 30-34 h. The bacterial growth reached the stationary phase after 40-44 h of cultivation. The highest bacterial growth (11.48 g/L after 84 hours) of liquid culture was obtained at yeast extract concentration of 0.3% (w/v). Exo-pectinase activity was not obtained during fermentation.

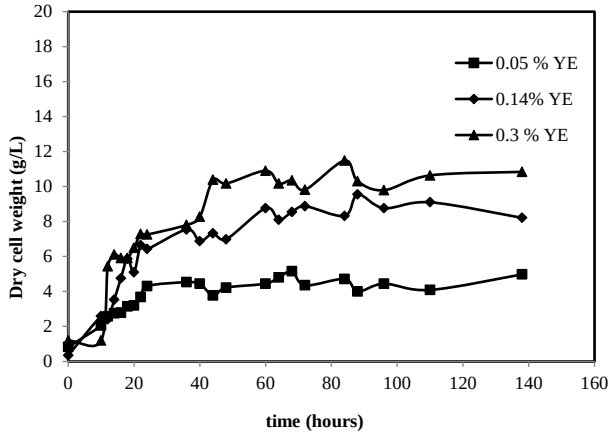

Fig. 1 Effect of yeast extract concentrations on growth curves of *Bacillus pumilus* (pH=8, T:30 °C, agitation rate: 150 rpm, C_{pectin}*: 1%)*

In order to determine the best nitrogen source, ammonium sulphate was used as inorganic nitrogen source. Effect of ammonium sulphate concentrations on growth curves of *Bacillus pumilus* are presented in Figure 2. The organism showed slower growth with lag phase of around 24 h and the late induction of stationary phase. The highest bacterial growth (11.91 g/L after 96 hours) of liquid culture was obtained at ammonium sulphate concentration of 0.05% (w/v). Enzyme activity followed the same trend with bacterial growth. As seen Figure 3, the maximum exo-pectinase activity (8.0 U/mL at 72 h) was also reported at 0.05% ammonium sulphate concentration and higher concentrations of ammonium sulphate caused substrate inhibition.

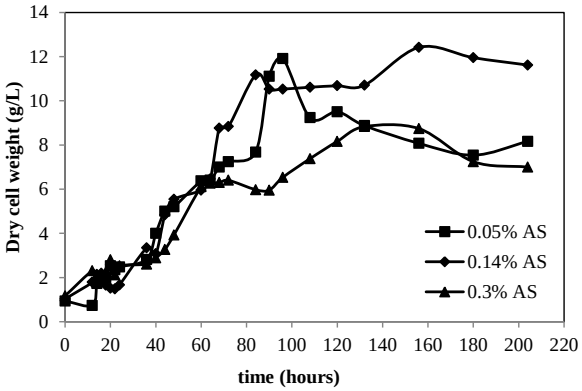

Fig 2 Effect of ammonium sulphate concentrations on growth curves of *Bacillus pumilus* (pH=8, T:30 °C, agitation rate: 150 rpm, C_{pectin}: 1%)

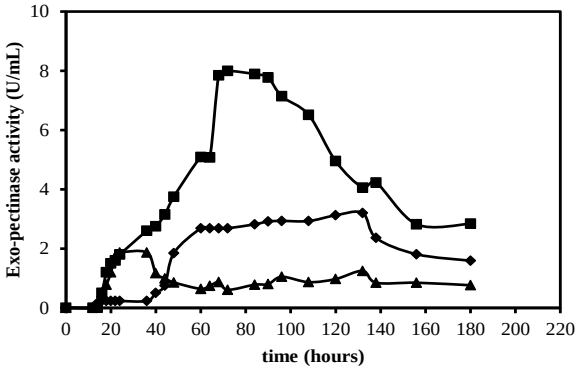

Fig 3 Effect of different ammonium sulphate concentrations on pectin lyase production by *Bacillus pumilus* (pH=8, T:30 °C, agitation rate: 150 rpm, C_{pectin}: 1% (w/v), ammonium sulphate concentrations; ■: 0.05%,♦: 0.14%, ▲: 0.3% (w/v))

Peptone is another most widely used organic nitrogen source for pectinase production. Effect of peptone concentrations on growth curves of *Bacillus pumilus* are presented in Figure 4. The organism when grown at peptone concentration of 0.05% (w/v) showed faster growth with the early induction of stationary phase after 40 h of cultivation. At peptone concentration of 0.14% (w/v), the adaptation phase (lag phase) for the bacterial growth lasted for around 68 h, the exponential phase (log phase) of the culture lasted for around 100 h. The bacterial growth reached the stationary phase. At peptone concentration of 0.3% (w/v), the growth patterns show a fast exponential phase (log phase) followed by a stationary phase (after 18 h of cultivation). As known, peptone is used not only as a nitrogen source but also as a carbon source by microorganisms. In addition, some amino acids and vitamins in the peptone

formula also play an important role as growth factors for microorganisms [15]. In the present study, the maximum dry cell concentrations were determined as 4.17 g/L, 8.77 g/L and 8.00 g/L with the peptone concentrations of 0.05, 0.14 and 0.3 % (w/v), respectively. As seen from the results, the bacterial growth increased to peptone concentration of 0.14%, then decreased. Figure 5 showed that, peptone concentration highly affected the exo-pectinase activity. As seen from the figure, exo-pectinase activity firstly increased with increasing peptone concentration. The highest enzyme activity (5.26 U/mL at 110 h) was noted when % 0.14 (w/v) concentration of peptone was used and exo-pectinase activity was not observed for % 0.3 (w/v) peptone concentration. Among all the different nitrogen sources tested, ammonium sulphate was reported as the best nitrogen source.

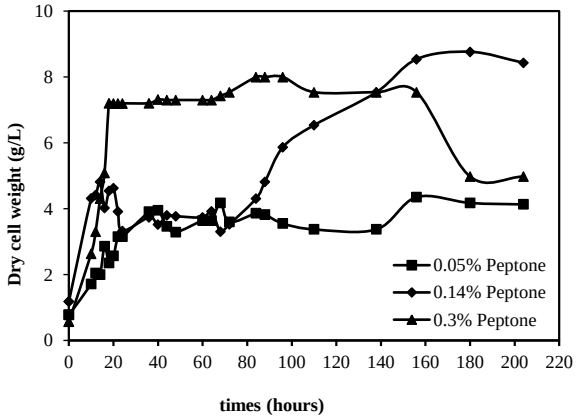

Fig 4 Effect of peptone concentrations on growth curves of *Bacillus pumilus* (pH=8, T:30 ^{o}C, agitation rate: 150 rpm, C_{pectin}: 1%)

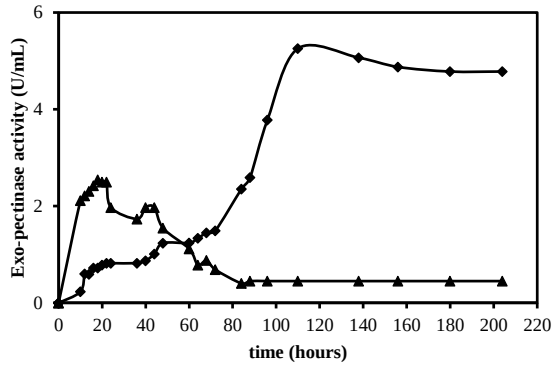

Fig 5 Effect of different peptone concentrations on exo-pectinase production by *Bacillus pumilus* (pH=8, T:30^{o}C, agitation rate: 150 rpm, C_{pectin}: 1% (w/v), peptone concentrations; ■: 0.05%, ♦: 0.14%, ▲: 0.3% (w/v))

IV. Conclusion

This study evaluated the growth and exo-pectinase activity of *Bacillus pumilus* bacteria grown with various nitrogen sources. Ammonium sulfate, yeast extract and peptone were used as nitrogen sources and the highest bacterial growth and enzyme activity was observed at ammonium sulfate concentration of 0.05%.

Acknowledgements

The authors wish to thank FUBAP (The research Foundation of Firat University), for the financial support of this study (Project No: FÜBAP 1674).

References

[1] D.R. Kashyap, P.K. Vohra, S. Chopra, and R. Tewari, "Applications of pectinases in the commercial sector: a review," *Bioresour Technol.*, 77, pp. 215–227, 2001. https://doi.org/10.1016/S0960-8524(00)00118-8

[2] I. Alkorta, C. Garbisu, M.J. Llama, and J.L. Serra, "Industrial applications of pectic enzymes: a review," *Process Biochem.*, 33, pp. 21–28, 1998. https://doi.org/10.1016/S0032-9592(97)00046-0

[3] E. Taşkın, and R. Eltem, "The enhancement of polygalacturonase and polymethylgalacturonase production on solid-state conditions by Aspergillus foetidus," *Food Biotechnol.*, 22(3), pp. 203-217, 2008. https://doi.org/10.1080/08905430802262533

[4] H. Uhlig, "Processing of fruit, vegetables and wine. In: W. Gerhartz Editor, Enzymes in Industry; Production and Applications", New York, *VCH publishers.*, pp. 126-128, 1990.

[5] T.S. Chang, M. Siddiq, N.K. Sinha, and J.N. Cash, "Plum juice quality affected by enzyme treatment and fining," *J Food Sci.*, 59, pp. 1065-1069, 1994. https://doi.org/10.1111/j.1365-2621.1994.tb08191.x

[6] G.S. Hoondal, R.P. Tiwari, R. Tewari, N. Dahiya, Q.K. Beg, "Microbial alkaline pectinases and their industrial applications: a review," *Appl Microbiol Biotechnol*,. 59, pp. 409-418, 2002. https://doi.org/10.1007/s00253-002-1061-1

[7] S. Rodríguez Couto, and J.L. Toca-Herrera, "Laccase production at reactor scale by filamentous fungi," *Biotechnol. Adv.* 25, pp. 558–569, 2007. https://doi.org/10.1016/j.biotechadv.2007.07.002

[8] F.M. Cunha, M.N. Esperança, T.C. Zangirolami, A.C. Badino, and C.S. Farinas, "Sequential solid-stateand submerged cultivation of Aspergillus niger on sugarcane bagasse for the production of cellulose," *Bioresour. Technol*, 112, pp. 270-274, 2012. https://doi.org/10.1016/j.biortech.2012.02.082

[9] S. Yadav, P.K. Yadav, D. Yadav, and K. Deo Singh Yadav, "Pectin lyase: A review," *Process Biochem*, 44, pp. 1–10, 2009. https://doi.org/10.1016/j.procbio.2008.09.012

[10] A. Blandino, K. Dravillas, D. Cantero, S.S. Pandiella, and C. Webb, "Utilisation of whole wheat flour for the production of extracellular pectinases by some fungal strains," *Process Biochem*, 37, pp. 497-503, 2001. https://doi.org/10.1016/S0032-9592(01)00241-2

[11] W.C. Chen, H.J. Hsieh, and T.C. Tseng, "Purification and characterization of a pectin lyase from Pythium splendens infected cucumber fruits.," *Bot Bull Acad Sin*,. 39, pp. 181–186, 1998.

[12] A.O. Adejuwon, and P.O. Olutiola, "Pectin lyase activity in culture medium of Lasidioplodia theobromae," *J Plant Sci.*, 2, pp. 334-340, 2007. https://doi.org/10.3923/jps.2007.334.340

[13] G.L. Miller, "Use of dinitrosalicylic acid reagent for determination of reducing sugars," *Anal. Chem.*, 31, 426-428, 1959. https://doi.org/10.1021/ac60147a030

[14] A.S. Qureshi, M.A. Bhutto, Y. Chisti, I. Khushk, M.U. Dahot, and S. Bano, "Production of pectinase by Bacillus subtilis EFRL 01 in a date syrup medium," *African J Biotechnol*, 11 (62, pp. 12563-12570, 2012.

[15] A.K. Halkman, "Merck Gıda Mikrobiyolojisi Uygulamaları," Başak Matbaacılık Ltd. Şti, Ankara, 358 sayfa. 2005.