Enhancement of Polygalacturonase Production

from Enterobacter Aerogenes NBO2

by Submerged Fermentation

I. Darah, M. Nisha and *S. H. Lim

Industrial Biotechnology Research Laboratory
School of Biological Sciences
Universiti Sains Malaysia
11800 Minden, Pulau Pinang, Malaysia

* Corresponding author
e-mail: limshehhong77@gmail.com

Copyright © 2013 I. Darah et al. This is an open access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Abstract

The bacterium, Enterobacter aerogenes NBO2 was cultivated in 250 ml Erlenmeyer flask under submerged fermentation and physiochemical characteristics were studied in order to improve the pectinase production. Fermentation condition improved were pH 6.5, cultivation temperature of 37°C, inoculums size of 3% (v/v) 7.4x10^8 cell/ml and agitation speed of 250 rpm, together with pectin concentration of 1.50% (w/v) and 0.26% of yeast extract as carbon and nitrogen sources, respectively had produced the highest pectinase production of about 18.54 U/mL and 0.43 g/L cell growth at 24 hours incubation time. The enzyme production by this bacterial isolate was found not growth dependent. There were increment in enzyme production and cell growth after improvement of physical and chemical parameters.

Keywords: Enterobacter aerogenes, pectinase, submerged fermentation
1. Introduction

Pectinase is an enzyme which can be found in the pith of citrus fruits and it functions to hydrolyze pectin into polygalacturonic acids and finally to galacturonic acid. Pectinase can be divided into pectin lyase (EC 4.2.2.10), pectin esterase (EC 3.1.1.11), pectate lyase (EC 4.2.2.2) and polygalacturonase (EC 3.2.1.15) based on their mode of action of hydrolyzing glycosidic linkages in the pectic polymers [1]. However, polygalacturonase is the most-studied and widely used pectinase. It exists in two forms that is Endo-polygalacturonase and Exo-polygalacturonase, where both of them act only on pectin with a degree of esterification of less than 50-60%. Endo-polygalacturonase acts randomly at the 1, 4-polygalacturonase backbone and results in a pronounced decrease in viscosity whereas Exo-polygalaturonase acts at the non-reducing ends of the chain. Exo-polygalacturonase releases small fragments from the chain and does not significantly reduce the viscosity [2].

Pectinases are extensively used in the feed and drink industries, chiefly in fruit juice clarification [3] because they are capable of reducing the viscosity of liquors during the clarification process [4, 5]. Besides that, they also have other application including extraction of vegetable oils [6], curing of coffee and cocoa beans [7], refinement of vegetable fibers and manufacturing of pectin-free starch [8]. In fact, in the textile industry pectinases are sometimes used in the treatment of natural fibers, such as cotton and ramie fibers [9] as well as biopulping of papers [10]. Therefore, it is important to discover new pectinase producing microbes and optimized their enzyme production conditions in order to meet the increasing demand.

Microbial pectinases have penetrated the industrial market and have become one of the most promising enzymes recently due to their economical value. A few recent reviews have highlighted that almost all the pectinase enzymes in industrial use now, are produced by fungi such as Aspergillus sp., Aspergillus japonicus, Rhizopus stolonifer, Alternaria mali, Fusarium oxysporum, Neurospora crassa and Penicillium italicum ACIM F-152 [11, 12]. However, there are also some bacterial species producing pectinases, namely Agrobacterium tumefaciens, Bacteroides thetaiotamicron, Ralstonia solanacearum, and Bacillus sp. [13].

Generally for the production of high-priced materials and for the study of biochemical and physiological aspects of the microbial metabolites formation, submerged fermentation system is very useful [14]. The usage of submerged fermentation is technically easier than solid state fermentation [8]. The objective of this work was to study the production of polygalacturonase in bacterial strains E. aerogenes NBO2 isolated from rotten oranges and improving the culture conditions and medium composition to maximize the enzyme production.
2. Materials and Methods

Microorganisms, culture maintenance and inoculum preparation

_E. aerogenes_ NBO2 which was isolated from rotten oranges was supplied by the Industrial Biotechnology Research Laboratory, School of Biological Sciences, Universiti Sains Malaysia, Penang, Malaysia. The bacterial culture was maintained on nutrient agar slant supplemented with 1.0\% citrus pectin (w/v) and incubated at 30°C for 24 hours aerobically before storing them at 4°C for further use. The subculturing was performed every month to ensure its survival.

The inoculums was prepared by transferring one loopful of 24 hour old colonies into 4.0 mL of sterile distilled water and then shaken vigorously to get a uniform cell suspension. The cell concentration was adjusted to absorbance equivalent to 1.0 at 600 nm which contained approximately 5.4x10\(^7\) cells/mL.

Medium composition and cultivation conditions

Cultures were grown in 250 ml Erlenmeyer flasks containing 50 mL of medium (w/v) [15]: (NH\(_4\))\(_2\)SO\(_4\) 0.14\%; KH\(_2\)PO\(_4\) 0.2\%; K\(_2\)HPO\(_4\) 0.6\%; MgSO\(_4\).7H\(_2\)O 0.01\% and citrus pectin 1.0\% (as a sole carbon source). The pH of the medium was adjusted to pH 6.0. The initial experiment was conducted to determine the optimal incubation or cultivation time that produced the highest pectinase production. Therefore, experiments containing the medium with the addition of 2.0\% (v/v; 7.4x10\(^8\) cells/mL) inoculum agitated at 150 rpm and incubated at 30°C was carried out for 96 hours. The samples were withdrawn at every 6 hourly intervals and were assayed for polygalacturonase activity and cell growth determination.

Improvement of fermentation process for enzyme production

Various process parameters influencing enzyme production during submerged fermentation were optimized. The strategy followed was to optimize each parameter (polygalacturonase activity and cell growth), independent of the others and subsequently optimal conditions were employed in all experiments.

Physical parameters

Improvement of cultural conditions in shake flask system for a maximal pectinase production involves various physical parameters. They were initial medium pH in the range between pH 5.5 to 7.2, temperature (30, 37, 40, 45 and 50°C), inoculums sizes [1, 2, 3 and 4\% (v/v) of 5.4x10\(^7\) cells/mL] and agitation speed (0, 50, 100, 150, 200, 250, 260 and 270 rpm). The cultivations were carried out for 24 hours before determining the enzyme activity and cell growth determination. Time course profile was carried out for 96 hours before and after the enhancement
of physical parameter conditions. The samples were withdrawn at every 6 hourly intervals and were assayed for polygalacturonase activity and cell growth determination. All the experiments were performed in triplicate and the values were reported as standard deviations.

**Carbon source**

Pectinase production was enhanced with different carbon sources. Glucose, arabinose, sucrose, lactose, carboxymethyl cellulose (CMC) and soluble starch at the concentration of 1.0% (w/v) were tested as sole carbon source substituting pectin. Polygalacturonase production was also tested with combination of pectin-starch, pectin-sucrose and pectin-carboxymethyl cellulose (CMC). The effect of concentration of improved carbon source was studied for its maximal enzyme production with concentrations of (0.5%, 1%, 1.5%, 2%, 2.5% and 3% w/v) in the production media for 36 h of incubation period based on the previous time course results (after enhancement of physical parameters). All the experiments were performed in triplicate and the values were reported as standard deviations.

**Nitrogen source**

The production medium was then supplemented with inorganic and organic nitrogen sources. Organic nitrogen sources used were yeast extract, urea and peptone whereas inorganic nitrogen sources were ammonium nitrate, sodium nitrate and ammonium hydrogen phosphate. The effect of various concentrations (0.02%, 0.08%, 0.14%, 0.20%, 0.23%, 0.26%, 0.29% and 0.32% w/v) of the selected nitrogen sources in the pectinase production was studied with incubation period of 36 h. After the parametric enhancement, pectinase production was carried out under improved nutritional and fermentation conditions for a maximum yield.

**Improvement of different cultural conditions on polygalacturonase production and fungal growth**

Time course profile was carried out for 96 hours after the improvement of physical (initial medium pH, temperature, inoculums size and agitation speed) and chemical (carbon and nitrogen sources) parameter conditions. The samples were withdrawn at every 6 hourly intervals and were assayed for polygalacturonase activity and cell growth determination. All the experiments were performed in triplicate and the values were reported as standard deviations.

**Crude enzyme extraction**

The culture broth was filtered through a Buchner funnel containing a filter paper (Whatman No. 4) to separate the cells. The cell-free culture filtrate containing the crude enzyme was centrifuged at 5000 rpm at 4°C for 10 minutes and the supernatant was then assayed for polygalacturonase activity.

**Cell growth determination**
Biomass was determined as dry cell weight calibrated by optical density at 600 nm using a spectrophotometer (Genesys 10 uv, Spectronic Unicam, USA).

**Assay for polygalacturonase activity**

Polygalacturonase activity was determined by measuring the release of reducing sugar from citrus pectin using arsenomolybdate method of Nelson [16] and Somogyi [17] which was modified. The reaction mixture containing 0.1 ml of 1.0% citrus pectin in 0.2M citrate-phosphate buffer, pH 6.0 and 0.9 ml of culture supernatant, was incubated at 40°C for 10 minutes. Enzyme activity was measured spectrophotometrically at 760 nm. One unit of enzymatic activity (U) was defined as the amount releasing one micromole of galacturonic acid per minute under standard assay conditions.

**3. Results and Discussion**

**Incubation time for highest polygalacturonase production**

Figure 1 shows a rapid increased in polygalacturonase production by *E. aerogenes* NBO2 until it achieved maximal production at 24 hours of cultivation (0.61 U/mL). The enzyme production decreased slowly after achieving its maximal production. This might be due to sugar utilization or enzyme denaturation as a result of interaction of other compounds in the medium [18]. Therefore, incubation time of 24 h was used to improve other physical parameters. Growth was observed to be highest at the 18 hours of cultivation (0.12 g/L) and became constant thereafter.

![Figure 1: Time course profile of polygalacturonase production and cell growth by *E. aerogenes* NBO2 in a shake flask system before improvement of cultural conditions.](image)
Effect of incubation temperature on polygalacturonase production

Figure 2 shows the effect of temperature on polygalacturonase activity. The optimum temperature for the production of polygalacturonase was 37°C with the enzyme activity of 3.52 U/mL. However, the highest growth achieved was at 30°C. Therefore, the data obtained indicated the enzyme production was not growth dependent. This result was in agreement with pectinase production by *Bacillus subtilis* SS [19], *Bacillus* sp. DT7 [20] and *Bacillus firmus*-I-10104 [18] which also had the optimum temperature of 37°C. The enzyme activity decreased gradually as the temperature was raised to 40, 45 and 50°C. This condition could be due to the effects of high temperature on the growth rate of *E. aerogenes* NBO2, since this bacteria initially was a mesophilic bacteria. Another possible reason could be the breaking down of enzyme at higher temperatures as enzyme denature above the temperature of 40°C.

![Figure 2: Effect of temperature on polygalacturonase production and cell growth by *E. aerogenes* NBO2.](image)

Effect of pH on polygalacturonase production

Figure 3 shows the effect of pH on enzyme activity. The pH for the maximum production of pectinase was found to be 6.5 with 4.52 U/mL. This study revealed that *E. aerogenes* NBO2 produce acidic polygalacturonase. This could also be explained by the fact that *E. aerogenes* NBO2 was isolated from the soil of decaying orange, which was an acidic fruit by nature. Moreover, the optimal initial medium pH for pectinase production by bacteria in submerged fermentation
Enhancement of polygalacturonase production

has also been reported in the acidic range of 6.0 [15] and 6.8 [21]. Soares et al [15] reported that *Bacillus* P4.3 produced highest polygalacturonase at the optimal pH between 6.5 and 7.0. The enzyme production was low at pH values out of the optimum level. The cell growth also maximal at pH 6.2 (0.03 g/L) and became constant until pH 7.2. This condition reflected that increased in pH affected the cell growth which finally reduced enzyme production. Again the results obtained reflected that enzyme production was not growth dependant.

Figure 3: Effect of initial medium pH on polygalaturonase production and cell growth by *E. aerogenes* NBO2.

**Effect of inoculum size on polygalacturonase production**

Figure 4 shows the effect of inoculum sizes on the enzyme activity. The best inoculum size was found at 3% (v/v) 5.4x10^7 cell/mL which produced 4.77 U/mL polygalacturonase. Further increased in inoculum size decreased the enzyme production. This condition could be due to a competition for the nutrients among the bacterial cells. Similar observations are explicitly reported by Ahlawat et al [19] (2009) in the case of pectinase production by *Bacillus subtilis* SS. The enzyme production by inoculums size of 1% (v/v) was much higher compared to 2% (v/v). Adequate nutrient supply could be the reason of the higher enzyme production with optimum inoculums size.
I. Darah, M. Nisha and S. H. Lim

Figure 4: Effect of inoculum size on polygalacturonase production and cell growth by *E. aerogenes* NBO2.

**Effect of agitation speed on polygalacturonase production**

Figure 5 shows the effect of agitation speed on the enzyme production. The enzyme production increased as the agitation speed increased. Maximum polygalacturonase production of 7.96 U/mL was achieved at agitation speed of 250 rpm. It was observed that increased in mixing help the microbial synthesis of enzyme [22] which consequently increased the assimilation of sugars. However, at higher agitation speeds (beyond 250 rpm) the enzyme production dropped. This could be due to the higher shear stress when the cells were agitated at higher speeds [23]. Although culture grown on static condition was not supplied with enough aeration (dissolved oxygen), but low enzyme production (1.72 U/mL) still achieved this implying that enzyme production could also occur at oxygen limited conditions. This result showed that *E. aerogenes* NBO2 is a facultative anaerobic bacterium where they can survive with little oxygen tension. According to Obi, [24], there were anaerobic and facultative anaerobic bacteria which produced pectinolytic enzyme and this included strains of *Erwinia carotovora*, *Clostridium butyricum* and *Clostridium felsineum*. Similarly, Gummadi et al [25] reported the production of enzyme by *Debaryomyces nepalensis* under oxygen limitation conditions. Lower agitation speed than 250 rpm resulted in lower enzyme production could be due to the minimum level of dissolved oxygen in the cultivation medium. According to Kao et al [23], incomplete mixing and/or oxygen transfer might be the reason of low production at lower agitation speeds. Thus, mixing is crucial for better oxygen and nutrient transfer rate in microbial enzyme production. The agitation speed was also affected the cell growth. As
shown in the Figure 5, the cell growth was slightly increased from 0 to 100 rpm with 0.024 to 0.026 g/L, respectively, but increased when agitated at 150 rpm (0.054 g/L) and achieved its maximal value at 200 rpm (0.058 g/L). The cell growth started to decline after achieving the maximal production. The results showed that the enzyme production was not growth dependant. This condition could be due to shear forces derived from higher agitation speed where collision among the cells occurred and damaging them. Darah et al [26] explained that at lower agitation speed, the inadequate mixing of the broth towards the later stages of growth affected the enzyme synthesis, while the drastic dropped in enzyme activity at higher agitation speeds was due to shearing effect on the cells. Darah et al [27] found that changes in the morphology of microorganisms caused by agitation speeds were also influenced enzyme production and growth of the microorganisms.

![Figure 5: Effect of agitation rate on polygalacturonase production and cell growth by E. aerogenes NBO2](image)

**Effect of carbon sources on polygalacturonase production**

Among the various carbon sources tested for polygalacturonase production (Figure 6), the maximum enzyme production was observed in the medium supplemented with pectin (Figure 6a) as a sole carbon source. Similar result was reported by Reda et al [18], in accordance to this study where the pectin produces the highest polygalactuonase compared to other carbon sources substituted (12.19 U/mL). However, soluble starch and CMC showed the significant value of polygalacturonase production. A drastic decreased in enzyme production was observed when sucrose, soluble starch and CMC were added to the pectin
There was an inhibitory effect on the synthesis of polygalacturonase. This result is in agreement with the study of Solis-Pereira et al. [14] where the production of polygalacturonase was lower when free sugars were added to the medium compared to the presence of pectin as the sole carbon source in submerged fermentation. According to Ahlawat et al. [19], low enzyme production with other carbon sources is maybe because of catabolite repression. It was also evidenced in the study of Solis-Pereira et al. [14], that there was a catabolite repression of pectic enzymes in the presence of glucose and other sugars. Polygalacturonase production was further increased up to 13.47 U/mL when the concentration of pectin (Figure 6b) used was 1.5% (w/v) and the enzyme production decreased with further increased in substrate concentration (2-3%). Probably, this is due to the presence of a high galacturonic acid concentration from pectin degradation.
Figure 6: Effects of carbon sources (a) and pectin concentrations (b) on polygalacturonase and cell growth production by *E. aerogenes* NBO2

**Effect of nitrogen sources on polygalacturonase production**

Various organic and inorganic nitrogen sources were tried in enzyme production by *E. aerogenes* NBO2 (Figure 7). As shown in Figure 7a, the best nitrogen source was yeast extract (12.90 U/mL) followed by ammonium sulphate (12.20 U/mL), peptone (11.67 U/mL) and ammonium hydrogen phosphate (6.46 U/mL). On the other hand, urea, ammonium nitrate and sodium nitrate seem to inhibit polygalacturonase production. Urea was a very poor nitrogen source for pectinase production by *E. aerogenes* NBO2. The cell growth was highest when yeast extract was used as a nitrogen source with 0.23 g/L. Generally the cell growth were not affected by the type of nitrogen sources used, where they ranged between 0.12 to 0.23 g/L.
Increased in enzyme production was observed with yeast extract at 0.26% (Figure 7b) which produced about 14.69 U/mL polygalacturonase production. Further increased in the yeast extract concentration, resulted in slight decreased in enzyme activity. Yeast extract have been reported to give maximum pectinase yield by
Bacillus sp. DT7 [20] when it was combined with pectin. Phutela et al [28], found that ammonium sulphate stimulated pectinase production and lack of this nitrogen source resulted in absence of extracellular pectinases by Aspergillus fumigatus. This findings suggested that ammonium sulphate may be suitable for fungal pectinase whereas yeast extract was suitable for bacterial pectinase production.

Polygalacturonase production using improved parameters

After the improvement of the physical parameters (initial medium pH of 6.5, cultivation temperature of 37°C, inoculums size of 3% (v/v) 5.4x10^7 cell/mL and agitation speed of 250 rpm), 1.50% of pectin and 0.26% of yeast extract as carbon and nitrogen sources, a time-course profile was carried out for 96 hours. The samples were withdrawn at every 6 hour intervals and polygalacturonase production and cell growth were determined. Figure 8 shows the polygalacturonase production increased gradually and achieved its maximum production of 18.54 U/ml after 24 hours of cultivation. The enzyme production started to decline thereafter. The cell growth was also increased gradually and achieved its maximum of about 0.43 g/L at 36 hours of cultivation time and remained almost constant thereafter. There was increment of about 2939% in enzyme production compared to before improvement of culture conditions (0.61 U/ml). When compared to previous findings, the cultivation time by E. aerogenes NBO2 (24 hour) was shorter compared to cultivation time showed by Bacillus sp. DT7 and Bacillus firmus-I-10104 which yield maximum pectinase activity at 36 and 98 h of cultivation, respectively [18, 20]. Hence, the shorter fermentation cycle will make the strain cost effective for commercial exploitation.

Figure 8: Time course profile of polygalacturonase production and cell growth by E aerogenes NBO2 in a shake flask system after improvement parameters
4. Conclusion

The result of this study revealed that, the local bacterial isolate *E. aerogenes* NBO2 is a potential pectinase (polygalacturonases) producer. This is the first report of pectinase from *Enterobacter aerogenes*. The physiochemical conditions studied did enhance the polygalacturonase production in a submerged fermentation using shake-flask system. Thus, *E. aerogenes* NBO2 is expected to produce higher enzyme yield when cultivated in bioreactor and the results will be reported elsewhere.

Acknowledgement

The authors are grateful to Universiti Sains Malaysia for awarding Postgraduate Research Grant Scheme (PRGS) to Nisha, M. to support this work.

References


Received: December, 2012