[BIO09] Pullulanase type II from a local *Bacillus* isolate H1.5 : isolation, production and characterization of the enzyme

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Introduction

Enzymes that specifically break down the branch points – debranching enzymes – have been known for more than a decade. Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase), which was first reported in *Aerobacter aerogenes* splits the α-1,6-glucosidic linkages in pullulan and give rise finally to maltotriose (Yuzuru and Masaharu, 1983). In most cases, pullulanase can also cleave the α-1,6 link of other branched polysaccharides, such as starch, glycogen and maltodextrin Some novel pullulanase preparations display the unique property of not only degrading α-1,6-glucosidic linkages in pullulan but also degrading the α-1,4-glucosidic of soluble starch, amylose and amylopectin (Yang and Coleman, 1987; Gantelet and Duchiron, 1998).

The present study was undertaken to screen and to isolate pullulanase-producing-microorganism. One of the strains isolated from soil, which was pullulan- and starch-degrading mesophiles *Bacillus* spp. (denoted as strain H1.5), has been characterized. Some characterization and properties of this type II pullulanase was also reported.

Materials and methods

Culture Medium

The basal medium as proposed by Katsutoshi *et. al.* (1995) was slightly modified and used in this study. The composition of the medium is as follows; (%w/v): yeast extract, 0.1; tryptose, 0.2; K_2HPO_4, 0.03; $MgSO_4.7H_2O$, 0.02; $(NH_4)_2SO_4$, 0.1; $CaCl_2.2H_2O$, 0.02; $FeSO_4.7H_2O$, 0.001; $MnCl_2.4H_2O$, 0.0001; technical agar, 2.0; soluble starch, 0.25 (for starch agar); phenol red, 0.018 plus soluble starch, 0.25 (for phenol red agar); pullulan, 0.25 (pullulan agar) and red pullulan, 0.1 (red pullulan agar). For the preparation of liquid medium, technical agar was removed from the formulation. All media was adjusted to pH 5.5 with 1 M HCl prior to sterilization at 121°C for 20 min.

Sample Collection, Screening and Isolation

One hundred samples were collected from various sources and locations (soils samples from cassava, corn, paddy farm fields and Port Dickson beach, decayed samples from different types of fruits, vegetables and tubers and water samples for hotsprings and sea water). Four different detection methods were used to isolate pullulanase positive bacterium.

Fermentation in shake flasks

For further evaluation of the potential isolates, the positive isolates collected from red pullulan agar was grown in liquid medium in shake flasks culture (100 mL) for further evaluation of growth and enzyme production.

Characterization and identification of the isolates

The morphological and taxonomical studies of the isolates was performed according to the methods as described in Bergey Manual and examined by API kits.

Purification of pullulanase from isolate H1.5

Ammonium sulfate was added up to 80% saturation into the supernatant and kept for overnight precipitation. The precipitate formed was collected by centrifugation at 20, 000 rpm for 5 min and the pellet was suspended and dissolved in 0.02 M sodium phosphate buffer, pH 6.9. The sample was dialysed overnight against the same buffer until complete removal of ammonium salts was achieved. All steps of enzyme purification were carried out at or below 4°C.

Thermal and pH influences

The effect of temperature on the reaction was examined at various temperatures for 30 min at pH 6.9. Thermal stability was determined by incubating the enzyme preparation 30 min at various temperatures, in the absence or presence of $CaCl_2$. The effect of pH on enzyme activity was determined by measuring the activity at 55°C using buffers with different pHs. For determination of pH stability, the partially purified enzyme solution was diluted with the respective buffers and incubated at room temperature for 24 h. The residual pullulanase activities were assayed at 55°C and pH 6.9.

End Product Analysis

The partially purified enzyme was incubated at 55°C with 1% (w/v) of different carbon sources separately. The samples were collected at different time for the product analysis through HPLC on Rezex RKP Potassium column. The products were eluted with distilled water at 0.5 ml/min flow rate and detected by refractometer. In order to determine its nature, the DP3 product obtained after pullulan hydrolysis, was incubated with commercially available Maltogenase®L, a maltogenic α-amylase (from *Bacillus stearothermophilus* expressed in and produced by a genetically modified strain of *Bacillus subtilis*) and then analyzed by HPLC on the same column. The Maltogenase®L hydrolyzed α-1,4-glucosidic linkages in maltotriose and maltose units were removed in a stepwise manner from the non-reducing chain ends. The maltose released had the α-configuration. As Maltogenase®L was able to hydrolyze maltotriose, some D-glucose was formed.

Pullulanase Assay

Enzyme activity was determined by measuring the enzymatic release of reducing sugars from pullulan. The reaction mixture contained 1% pullulan in 0.02M sodium phosphate buffer, pH 6.9 and a sample of the enzyme, in a final volume of 1 mL. After incubation at 40°C for 30 min, the concentration of the reducing sugar was determined by using dinitrosalycilic acid (DNS) method. Sample blank were used to correct for the non-enzymatic release of reducing sugar. One unit of pullulanse activity is defined as the amount of enzyme required to produce 1 μmol reducing sugar (equivalent to glucose) per minute under the assay conditions.

Results and Discussion

Isolation and identification of the pullulan-degrading bacteria

Based on the area of pullulan degradation, the strain designated H1.5 was selected as it exhibited a comparactively high activity (0.05 U/mL – 0.1 U/mL). Furthermore, red pullulan agar seemed to be the most directly method, which can be used to detect the presence of the pullulanase producing bacterium directly from the agar plate used in the isolation experiment.

Microscopic studies revealed rods, single cell or pairs or chains of 3-5 cells in culture. Physiological test showed the strain was $Gram^+$, non-motile, and spores formation. From the identification using API kits, it was found that the isolate H1.5 belong to *Bacillus* spp.

Nutrients requirement for growth of isolate H1.5 and pullulanase production

All starches were able to enhance growth of isolate H1.5 and the pullulanase synthesis. In fermentation using potato starch, the activity was 0.112 U/mL compared with fermentation using pullulan as the sole carbon source (0.03 U/mL). Pullulanase synthesis was inhibited by EDTA, β-cyclodextrins, sucrose and glycerol, with the pullulanase activity less than 0.001 U/mL. The strain could grow between 30 to 43°C with 37°C as optimum temperature. During optimization of pH in culture medium, it was observed that the strain could grow only in pH 5.5 – 9.0 with optimum growth at pH 7.0.

Characterization of enzyme activity

Maximum activity of the enzyme was observed at 55°C and above 70°C the enzyme was denatured completely (Figure 1). The reported temperature of pullulanase obtained from plants or other microorganisms was normally in the range of 40°C to 60°C (Scandurra *et al.,* 1999; Bejar *et al.*, 2003). The enzyme was stable up to 40°C, with essentially no loss of activity in 30 min, as shown in Figure 2. The activity was decreased rapidly with temperature above

50°C. Incubation for over 30 min at temperature at and above 60°C destroyed the activity completely. However, in the presence of Ca^{2+} the activity was stable up to 50°C and around 30% of its activity was retained at temperature of above 60°C (data not shown).

From Figure 2, the half life of pullulanase was more than 2 h at 50°C when 2 mM Ca^{2+} was present in the absence of substrate compared to the half-life duration of the enzyme was only 20 min at the same temperature for the reaction without any stabilizers, suggesting that Ca^{2+} stabilized and protect the enzyme from thermal inactivation at 50°C.

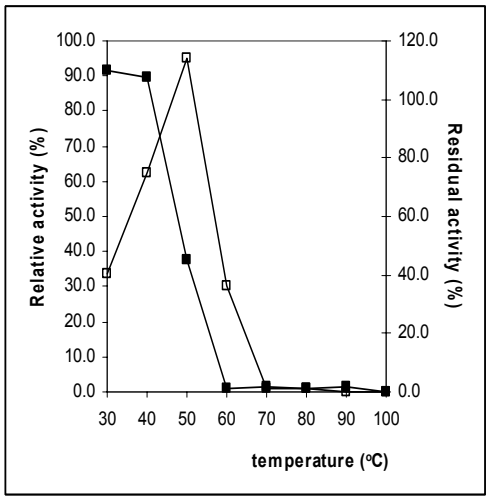

FIGURE 1 Thermal influences on pullulanase of *Bacillus* spp. H1.5
Symbol : □, Relative activity; ■, Residual activity

The enzyme was active between pH 3.0 and 6.0 with an optimum of 6 (Fig. 3). This proved the enzyme to be acidic in nature. The enzyme was not active at pH above 7.0. The enzyme was stable over a broad pH range (pH 2 - pH 10). The optimal pH for activity and the pH range for stability of pullulanase produced by *Bacillus* sp. was in the same range as for other pullulanases from other sources as reported in the literature (Brown and Kelly, 1993; Canganella *et al.*, 1994; Gantelet and Duchiron, 1998; Bertoldo *et al.*, 1999; Antranikian *et al.,* 2000; Bejar *et al.,* 2003).

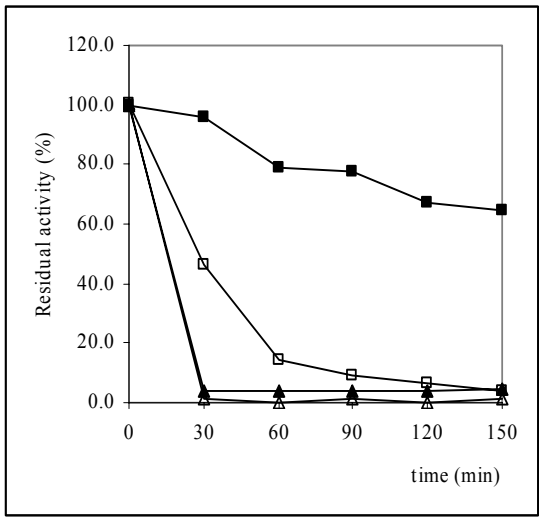

FIGURE 2 Inactivation of pullulanase from *Bacillus* sp, H1.5 at different temperatures. Symbols : open, control; solid, with 2 mM Ca2+; □, ■, 50°C; □, ▲, 60°C.

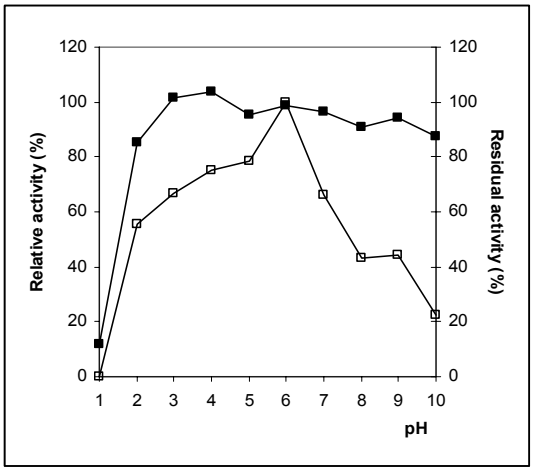

FIGURE 3 Effect of pH on activity and stability of pullulanase of *Bacillus* H1.5. Symbol : □, Relative activity; ■, Residual activity

End Product Analysis

Maltotriose was the only trimeric (degree of polymerization, DP3), product of pullulan hydrolysis after prolonged incubation (up to 13 hours) (Fig. 4B), indicating that the enzyme hydrolysis is specific for the α-1,6 glucosidic linkages of pullulan. Furthermore, when this DP3 product was incubated with commercial Maltogenase®L for 30 min, it was disappeared and led to form a DP2 (maltose) and glucose peaks (Figure 4C). This observation suggested that the DP3 product of pullulan hydrolysis had been a straight chain maltotriose with no α-(1,6) linkage and not panose or isopanose. In contrast, if the DP3 had been panose (α-D-Glc-[1→6]- α-D-Glc-

[1→4]-D-Glc) or isopanose (α-D-Glc-[1→4]-α-D-Glc-[1→6]-D-Glc), the commercial enzyme could not have degraded it at all. Accordingly, the pullulanase attacks pullulan and release maltotriose, indicating that the enzyme had an absolute specificity directed at the (1→6) glucosidic linkages of pullulan. In addition to the α-1,6-glucosidic linkages in pullulan, the enzyme was able to cleave the α-1,4-glucosidic linkages in other polysaccharides, such as soluble starch and amylopectin – which contain both types of glucosidic linkage (data not shown). However, the action of pullulanase on both soluble starch and amylopectin was very slow, demonstrating its low affinity for α-1,4-glucosidic linkages.

Since pullulanase H1.5 attacks pullulan (producing maltotriose) and other polysaccharides such as starch and amylopectin, it can be classified as a pullulanase type II or amylopullulanase.

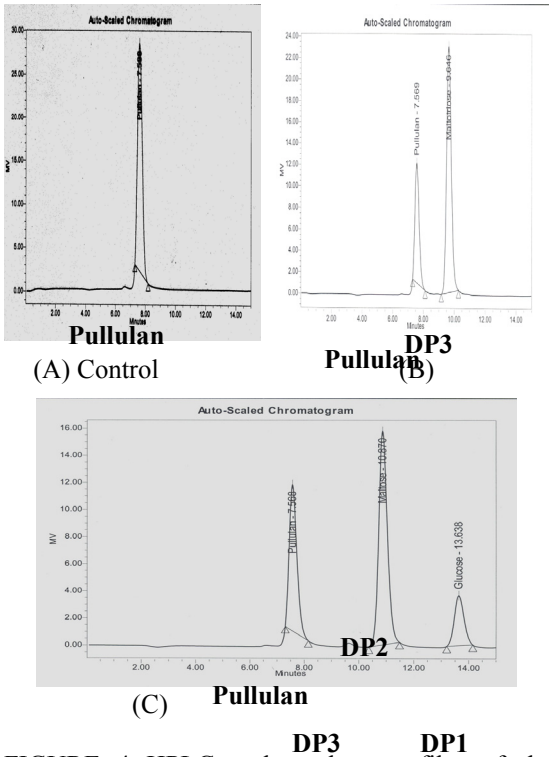

FIGURE 4 HPLC end product profiles of the action of pullulanase H1.5 on pullulan.
(A) Pullulan without enzyme as control, (B) 13 hours action on pullulan, (C) DP3 product degraded by Maltogenase®L.

Acknowledgments

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