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Characterization of Pullulanase Type II from *Bacillus cereus* H1.5

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Abstract: Problem statement: Pullulanase is one of the important enzymes in starch industry. Search for the pullulanase with distinct features, possibly from easily grown bacterium, is of interest for industrial applications **Approach:** The extracellular pullulanase produced by *Bacillus cereus* HI.5 was purified by chromatographic method of DEAE-Sepharose, followed by Superdex gel filtration. The enzyme was characterized in terms of the optimal pH and temperature for activity as well as substrate specificity. **Results:** The enzyme showed optimal activity at 55°C and pH 6.0. The thermostability and the thermoactivity of the enzyme were increased considerably in the presence of Ca^{2+}. In the present of 2 mM Ca^{2+}, the enzyme had half-life duration of more than 2 h at 50°C. Almost all metal ions had a strong inhibitory effect, except Ca^{2+} and Mn^{2+}. The Ca^{2+} had a very strong stimulating effect on the enzyme, increasing its activity by 170%. The enzyme was activated by 2-mercaptoethanol and dithiothreitol, where as N-bromosuccinimide and Schardinger dextrins were inhibitors, suggesting that tryptophan and thiol residues may be important for the activity. The apparent K_m and V_{max} value for pullulan was 1.1 mg mL^{-1} and 0.275 µmol min^{-1}, respectively. A relative substrate specificity for hydrolysis of pullulan, amylopectin and soluble starch by this pullulanase was 100%, 28.5% and 20.4%, respectively. **Conclusion:** The enzyme was able to attack specifically the α-1,6 linkages in pullulan to generate maltotriose as the major end product, as well as the α-1,4 linkages in amylopectin and soluble starch leading to the formation of a mixture of maltose and glucose and therefore be classified as a type II pullulanase or an amylopullulanase.

Key words: Pullulanase, amylopullulanase, *Bacillus cereus*, starch, substrate specificity

INTRODUCTION

Pullulan is a linear glucan of maltotriosyl units (Glcα1-4Glcα1-4Glc) linked through α-1,6 glucosidic linkages. The α-1,6 linkages are considered partially to mimic the α-1,6 branch points of amylopectin and pullulan has been widely employed as a model substrate for starch-debranching enzymes[1]. Enzymes that hydrolyze pullulan have been classified into five groups based on the substrate specificity and end product[2,3]. They are: (i) pullulan hydrolase type I (neopullulanase, EC 3.2.1.135) which hydrolyzed α-1,4 glycosidic linkage of pullulan to form panose; (ii) pullulan hydrolase type II (isopullulanase, EC 3.2.1.57) which acts on pullulan to form isopanose; (iii) pullulan hydrolase type III which attacks α-1,4 as well as α-1,6 glycosidic linkages in pullulan forming a mixture of maltotriose, panose and maltose; (iv) pullulanase type I (EC 3.2.1.41) specifically hydrolyses α-1,6 glycosidic linkages in pullulan or branched substrates such as amylopectin forming maltotriose and (v) pullulanase type II (amylopullulanase) attacks α-1,6 linkages in pullulan and branched substrates in addition to α-1,4 links in polysaccharides other than pullulan[2,4].

Among pullulanase type II, amylopullulanases are interesting in starch processing industry due to the specific debranching capacity of hydrolyzing either α-1,6 and α-1,4 glycosidic linkages. A number of pullulanase with dual specificities have been investigated, including pullulanase from *Bacillus subtilis*[5],

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Thermoanaerobium brockii[6], *Clostridium thermohydrosulfuricum*[7,8], *Bacillus circulans*[9], *Bacillus* sp.[10], *Clostridium thermosulfurogenes*[11], *Thermus aquaticus*[12], *Thermoanaerobacterium saccharolyticum*[13], *Pyrococcus furiosus* and *Thermococcus litoralis*[14], an alkalophilic *Bacillus* sp.[15] and *Bacillus* sp.[3].

In this study, pullulanase produced by *Bacillus cereus* HI.5, isolated from Malaysian soil, was purified and characterized. In addition, substrate specificity and the hydrolysis products were also analyzed.

MATERIALS AND METHODS

Microorganism: *Bacillus cereus* H1.5, obtained from a culture collection unit at the Laboratory of Industrial Biotechnology, Institute of Bioscience, Universiti Putra Malaysia, was used in this study. This pullulanase producing strain was isolated from soil using red pullulan agar method.

Production of pullulanase: The *B. cereus* H1.5 culture was initiated in 1 L shake flasks. The culture medium consisted of (w/v) 1% gelatinized sago starch, 0.5% yeast extract, 0.03% K_2HPO_4, 0.02% $MgSO_4.7H_2O$, 0.1% $(NH_4)_2SO_4$, 0.02% $CaCl_2.2H_2O$, 0.001% $FeSO_4.7H_2O$ and 0.0001% $MnCl_2.4H_2O$. The pH of all media was adjusted to 5.5 with 1 M HCl prior to sterilization at 121°C for 20 min. After incubation of the cultures at 30°C for 24 h, the cells were removed from the culture medium by centrifugation at 10,000 rpm for 15 min.

The supernatant was used for further purification. The supernatant was treated with $(NH_4)_2SO_4$ at 80% saturation and the flask was continuously stirred for overnight precipitation. The precipitate formed was collected by centrifugation at 20, 000 rpm for 5 min and the pellets were suspended and dissolved in 0.02 M sodium phosphate buffer, pH 6.9 and dialyzed against the same buffer for 24 h at 4°C with two buffer changes.

Enzyme purification: All chromatographic steps were performed at 4°C using Akta Prime system (Pharmacia Amersham Biotech, Sweden). The dialyzed enzyme preparation was applied to a DEAE-Sepharose C1 6B FF column (2.5×30 cm) with 0.02 M sodium phosphate buffer, pH 6.9. The column was washed with the same buffer and bound proteins were eluted by applying a linear gradient of 0-1.0 M NaCl in the same buffer at a flow rate of 1.8 mL min^{-1}. Fractions exhibiting pullulanase activity were pooled and concentrated by freeze-drying. Samples were dissolved in a minimal volume of 0.02 M sodium phosphate buffer, pH 6.9. and dialyzed against the same buffer for 24 h at 0.02 M sodium phosphate buffer, pH 6.9 and 4°C, with two buffer changes.

For further purification of the enzyme, gel filtration was performed onto a Superdex 75 (10/300 GL) gel filtration column (1.0×30 cm), equilibrated with 0.02 M sodium phosphate buffer, pH 6.9. Elution at a flow rate of 0.5 mL min^{-1} was performed using the same buffer. The active fraction pooled and concentrated by ultrafiltration was used as the purified enzyme for further analysis. The purity and molecular mass of the enzyme were determined using SDS-PAGE, using Bio-Rad Mini protean II apparatus at 100 V. Protein standards were co-migrated and stained with silver staining kid (Amersham Biosciences).

Assay of pullulanase activity: Enzyme activity was determined by measuring the enzymatic release of reducing sugar from pullulan [16]. In this assay, 0.5 mL of enzyme sample was added into 0.5 mL of 1% (w/v) pullulan in 0.02 M sodium phosphate buffer, pH 6.9. The reaction mixture was incubated at 40°C for 30 min and the amount of reducing sugar released at the end of the reaction was determined by Dinitrosalisyilic Acid (DNS) method. Sample blank was 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) min^{-1} under the assay conditions.

Effect of pH on enzyme activity and stability: The pH optimum for the enzyme activity was studied over a range from pH 1-10 with pullulan as a substrate. The pH stability of the enzyme was determined by incubating the enzyme in buffers with different pHs for 24 h and incubated at 40°C. The following buffer systems were used: 0.02 M potassium chloride-HCl buffer (pH 1.0-pH 2.0); 0.02 M glycine-HCl buffer (pH 3); 0.02 M acetate buffer (pH 4-pH 5); 0.02 M phosphate buffer (pH 6-pH 8); and 0.02 M glycine-NaOH buffer (pH 9-pH 10).

Thermal influence on enzyme activity: To determine the temperature optima on the enzyme activity, the reaction mixture was incubated at different temperatures for 30 min with and without the present of Ca^{2+} ion. Thermal stability was determined by incubating the enzyme sample in different buffers (0.02 M, pH 6.9) with and without the present of Ca^{2+}. The reaction mixtures were pre-incubated at various temperatures for 30 min and then residual pullulanase activities were assayed.

Thermal inactivation was examined by incubating the enzyme at 50, 60 and 70°C for 150 min with and without the present of Ca^{2+}. Aliquots were withdrawn at desired time interval to test the remaining activity at standard conditions. The enzyme incubated at 4°C in the absence of an additive was used as a control and was assumed to have 100% activity.

Effect of metal ions, chemical reagents, carbohydrates and protein stabilizer salts on enzyme activity: The purified pullulanase was pretreated with various metal ions at two different concentrations (0.2 mM and 2 mM), various types of small carbohydrate (glycerol, mannitol, sorbitol, myo-inositol, sucroce) and other protein stabilizer salts (Na_2SO_4 and glycine). The reaction mixtures containing the enzyme sample and pullulan as a substrate were incubated at 55°C for 30 min in 0.02 M sodium phosphate buffer, pH 6.9. The enzyme sample without any additives was considered as control (100%).

Action of pullulanase on different concentrations of pullulan: The purified enzyme was also assayed for pullulanase activity at different concentrations of pullulan. Reaction rate (v) is expressed as µmol of reducing sugar (as glucose) formed per mg of protein and substrate concentration (s) as mg mL^{-1}. The kinetic parameters, K_m and V_{max}, were determined by Eadie-Hofstee plot.

Substrate specificity: The ability of the purified enzyme to hydrolyze various carbohydrates was examined at 55°C and pH 6.9 in 0.02 M sodium phosphate buffer. The carbohydrates tested were pullulan, amylopectin (from potato), amylopectin (from maize), soluble starch (from potato), amylose, dextran and gelatinized sago starch at a concentration of 1% (w/v).

End product analysis: The pullulanase sample (10 mL) was added into different substrates (pullulan, soluble starch and amylopectin) at a concentration of 1% (w/v) in 0.02 M sodium phosphate buffer, at pH 6.9. The reaction mixtures were incubated at 55°C. In order to determine the nature of the DP3 product of pullulan hydrolysis, Maltogenase®L, a maltogenic α-amylase (from *Bacillus stearothermophilus* expressed in and produced by a genetically modified strain of *Bacillus subtilis*), product of Novozymes, was added to the reaction medium after 12 h of the action of pullulanase from *B. cereus* H1.5 on pullulan (at this time, the formation of DP3 product was maximum). The Maltogenase®L hydrolyzed α-1,4-glucosidic linkages in maltotriose and maltose units are removed in a stepwise manner from the non-reducing chain ends. The maltose released had the α-configuration. As Maltogenase®L is able to hydrolyze maltotriose, some D-glucose is formed.

During the reaction, samples were collected at time intervals for analysis using HPLC (Waters, USA). The Rezex RKP Potassium (300×7.8 mm ID) analytical column was used as a stationary phase while filtered distilled water was used as a mobile phase. The products were eluted with a mobile phase at a flow rate of 0.5 mL min^{-1} and detected with a Refractive Index (RI) detector (Waters 996 Photodiode Array Detector). The column temperature was maintained at 80°C and the injection volume of each sample was 10 µL and the running time was 15 min. Standard sugar mixtures containing pure glucose, maltose, maltotriose, up to maltoheptaose (DP7) were used for the generation of calibration curves; DP denotes degree of polymerization.

RESULTS

Purification of pullulanase enzyme: Pullulanase produced by *B. cereus* H1.5 was purified 23.6-fold with a 8.5% yield and a final specific activity of 14.250 U mg^{-1} protein (Table 1). The homogeneity of the purified pullulanase was ascertained by SDS-PAGE. A single band of protein was obtained at approximately 93 kDa (data not shown).

Effect of pH on the activity and stability of the enzyme: Effect of pH on the activity and stability of pullulanase from *B. cereus* H1.5 is shown in Fig. 1. The optimum pH for the activity of pullulanase was observed at pH 6.0. The pullulanase activity was apparent over the acidic ranges, with more than 50% of the maximal activity was detected at pH ranging from 3-6. The enzyme activity was greatly reduced at pH of above 7. The enzyme was stable over a wide pH range, from 2-10.

Table 1: Protocols used to purify pullulanase from *Bacillus cereus* H1.5

Step	Total activity (Units)	Total protein (mg)	Specific activity (U mg^{-1})	Yield (%)	Purification (fold)
Crude	115.57	951.46	0.121	100	1.00
$(NH_4)_2SO_4$ fractionation	20.46	14.63	1.399	17.7	11.5
DEAE sepharose	6.74	0.75	8.987	10.9	18.4
Superdex 75	4.56	0.32	14.250	8.5	23.6

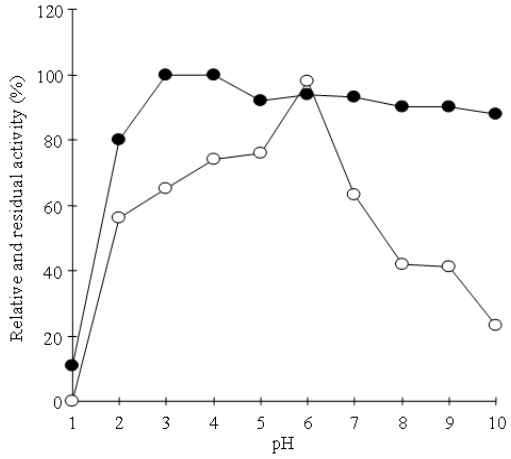

Fig. 1: Effect of pH on the activity and stability of pullulanase from *Bacillus cereus* H1.5. Symbols represent: (open circles) activity; (closed circles) stability

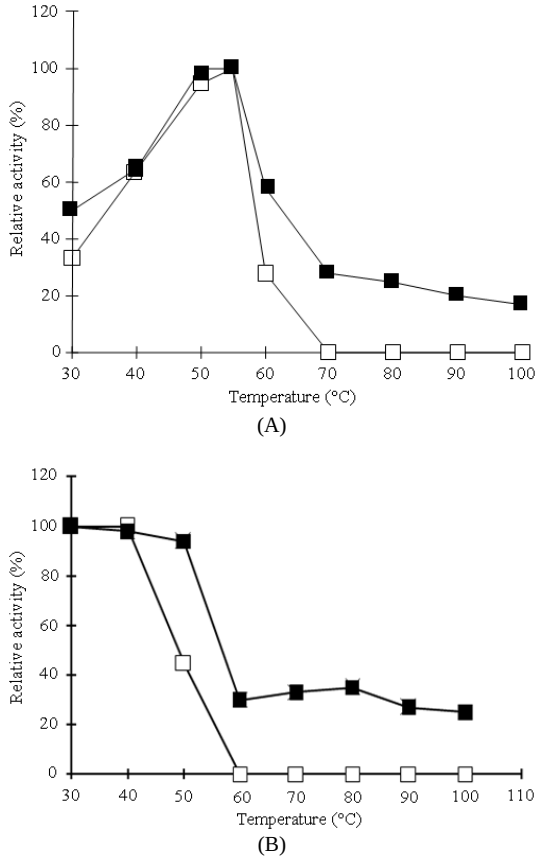

Fig. 2: Effect of temperature on activity (A) and stability (B) of pullulanase from *Bacillus cereus* H1.5. Symbols represent: (open squares) control; (closed squares) with 2 mM Ca^{2+}

Effect of temperature on the activity and stability of the enzyme: The effect of temperature on the activity and stability of pullulanase from *B. cereus* H1.5 is shown in Fig. 2. The activity of pullulanase was optimal at 55°C, for reaction with and without the present of Ca^{2+}. For the enzyme without Ca^{2+}, the activity was not detected at temperature of above 70°C (Fig. 2A). On the other hand, about 20% of relative activity was still maintained at temperature of above 70°C for pullulanase with Ca^{2+}.

Fig. 2B shows that the enzyme was stable up to 40°C, with essentially no loss of activity in 30 min. The activity was decreased rapidly with increasing temperature of above 50°C. Incubation for over 30 min at 60°C and above 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 retained at temperature of above 60°C.

Inactivation of pullulanase at two different temperatures, 50 and 60°C, with and without the present of Ca^{2+} is shown in Fig. 3. The half life of pullulanase was more than 2 h for the enzyme with the present of 2 mM Ca^{2+} and incubated at 50°C. At the same temperature, the half-life was reduced to 20 min for the enzyme without 2 mM Ca^{2+}, suggesting that Ca^{2+} stabilized and protect the enzyme from thermal inactivation at 50°C. However, the stabilization effect of Ca^{2+} on pullulanse from *B. cereus* H1.5 was not observed at higher temperature (60°C).

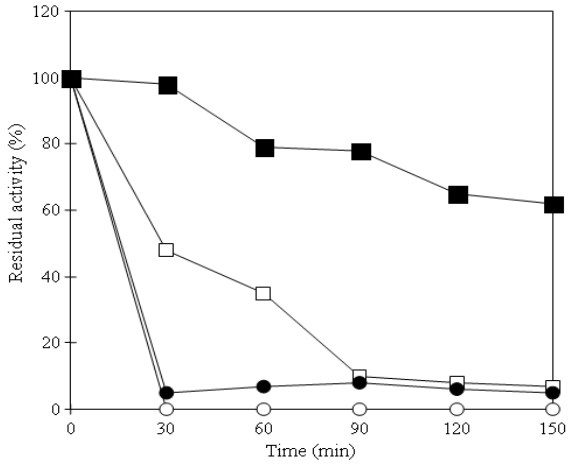

Fig. 3: Inactivation of pullulanase from *Bacillus cereus* H1.5 at different temperatures. Symbols represent: (open squares) control at 50°C; (closed squares) with 2 mM Ca^{2+} at 50°C; (open circles) control at 60°C; (closed circles) 2 mM Ca^{2+} at 60°C

Table 2: Comparison of metal ions as inhibitors for pullulanase from *Bacillus cereus* H1.5

	Relative activity at two different concentrations	
Reagents	0.2 mM	2 mM
$CaCl_2$	132.0	172.5
$NiCl_2$	5.2	4.8
$CoCl_2$	6.5	6.5
CdCl2	18.9	5.8
$MnCl_2$	17.5	64.5
$MnSO_4$	18.4	65.8
$FeCl_3$	66.2	4.9
$MgCl_2$	8.5	6.4
$MgSO_4$	6.7	19.5
$CuSO_4$	3.8	0.5
$ZnCl_2$	3.9	3.5
NaCl	12.3	5.8
EDTA	3.9	1.1
Control	100.0	100.0

Effect of metal ions on enzyme activity: The inhibition effect of various metal ions on the activity of pullulanase from *B. cereus* H1.5 is shown in Table 2. The pullulanase activity was inhibited strongly by Ni^{2+}, Co^{2+}, Cd^{2+}, Mg^{2+}, Cu^{2+}, Zn^{2+} and Na^{+} ions. The activity was slightly inhibited by 0.2 mM Fe^{3+} ions but pullulanase activity was completely eliminated at higher concentration (2 mM). In contrast to Fe^{3+}, the activity of pullulanase was only slightly reduced at high concentration of Mn^{2+}. Among the metal ions tested, Ca^{2+} had a very strong stimulating effect on the enzyme, increasing its activity by around 170%.

Effect of various chemical reagents on enzyme activity: Effect of various chemical reagents on the activity of *B. cereus* H1.5 pullulanase is shown in Table 3. Strong inhibition on the pullulanase activity by N-bromosuccinimide (NBS) was observed at low concentration, suggesting a crucial involvement of tryptophan residues at the active site of the enzyme.

The suphydryl inhibitors, such as 4-chloromercuribenzoate, had no inhibitory effect on the pullulanase activity. The reducing agents, dithiothreitol and 2-mercaptoethanol, increased the pullulanase activity slightly, indicating the involvement of a thiol-group in the catalytic action. The enzyme was not inhibited by phenylmethanesulphonyl fluoride (PMSF), suggesting that histidine and serine residues were not participate in the catalysis by the enzyme, as in general, amylolytic enzymes are not serine enzyme.

The enzyme activity was inhibited by the chelating agent (EDTA), with more than 92% lost in activity. However, the pullulanase activity was slightly activated with Ca^{2+}. This result indicates that Ca^{2+} is not required for its activity but is probably important for the stability and may maintain the conformation of the enzyme.

Table 3: Influence of various chemical reagents on the activity of pullulanase from *Bacillus cereus* H1.5

Chemical reagents	Concentration	Relative activity (%)
Control	-	100
Phenylmethysulfonyl fluoride (PMSF)	1 mM	95
N-Bromosuccinimide	0.1 mM	6
	0.3 mM	0
4-chloromercuribenzoate	0.1 mM	102
EDTA	10 mM	8
EDTA + Ca^{2+}	10+2 mM	21
Urea	0.1 M	109
	3 M	76
	7 M	29
α-cyclodextrin	0.10%	71
	0.50%	25
β-cyclodextrin	0.10%	21
	0.50%	6
γ-cyclodextrin	0.10%	53
	0.50%	22
Dithiothreitol	2 mM	122
2-mercaptoethanol	2 mM	102

Table 4: Effect of polyols on thermostability of pullulanase from *Bacillus cereus* H1.5

Polyols	Relative activity (%)
Control	100.0
Glycerol	58.1
Mannitol	72.4
Sorbitol	75.6
Myo-inositol	158.8
Na_2SO_4	76.2
Glycine	0.4
Sucrose	172.6

Effect of polyols on the enzyme activity: Thermostability of enzyme could also be enhanced by modification of the environment, such as with the addition of various polyols or simple sugar. Effect of polyols on the thermostability of pullulanase from *B. cereus* H1.5 is shown in Table 4. Thermostability of pullulanase at 55°C was significantly improved with the addition of myo-inositol and sucrose into the reaction media. The highest improvement in stability (relative activity of 172.6%) was obtained with sucrose. On the other hand, reduced thermostability of the enzyme was observed with the addition of other polyols. The enzyme was almost denatured with the present of glycine. The experiment was also conducted to investigate the effect of sucrose concentration (0.5-4 M) on pullulanase thermostability at 55°C (data not shown). The protective effect of sucrose on thermostability was optimal for sucrose concentration ranging from 1-2 M.

Kinetic properties of pullulanase activity: The activity of pullulanase from *B. cereus* H1.5 on pullulan as a substrate showed Michaelis-Menten kinetics,

where the maximal activity was reached at pullulan concentration of 10 mg mL^{-1} (data not shown). The plot of Eadie-Hofstee for the determination of enzyme kinetics is shown in Fig. 4. The apparent Michaelis-Menten constant (K_m) value for pullulan was 1.1 mg mL^{-1}, while the value of V_{max} was 0.275 µmol min^{-1}.

Substrate specificity and analysis of hydrolysis product by HPLC: A number of different α-glucans were incubated with pullulanase from *B. cereus* H1.5 in order to ascertain the substrate specificity. Among the carbohydrate tested as substrate, the highest hydrolysis was observed with pullulan (Table 5). Amylopectin (potato) and soluble starch were hydrolysed to some extent, while amylose was cleaved at very low rate. The enzyme did not cleave dextran which containing only α-1,6 glycosidic linkages.

Individual polysaccharides and oligosaccharides were incubated with pullulanase and the products were separated and identified by HPLC analysis. Figure 5 shows the time course of enzymatic hydrolysis of pullulan by *B. cereus* H1.5 pullulanase, while the end product profile is shown in Fig. 6.

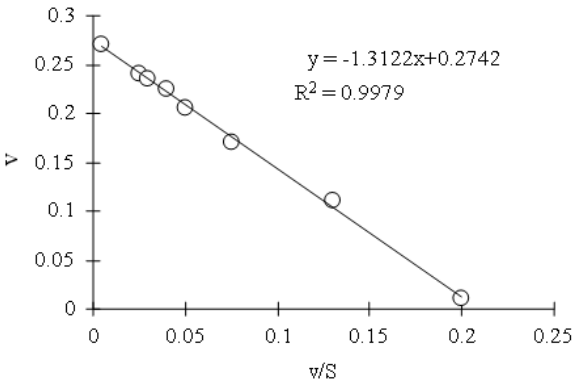

Fig. 4: Eadie-Hofstee plot for the determination of the kinetics of pullulanase from *Bacillus cereus* H1.5

Table 5: Substrate specificity of pullulanase from *Bacillus cereus* H1.5

Substrate (1%, w/v)	Specific activity (U mg^{-1})	Relative activity (%)
Pullulan	1.397	100.0
Amylopectin (potato)	0.398	28.5
Amylopectin (maiza)	0.076	5.5
Amylose	0.040	2.9
Dextran	0.003	0.2
Soluble starch (potato)	0.285	20.2
Gelatinized sago	0.013	1.0

After 3 h incubation at 55°C, the pullulan was converted into a major identifiable DP3 product. The final product after prolonged incubation (up to 13 h) showed that DP3 was the only trimeric (degree of polymerization, DP) product of pullulan hydrolysis (Fig. 6B).

Furthermore, when this DP3 product was incubated with commercial Maltogenase®L for 30 min, it disappeared and led to form a DP2 (maltose) and glucose peaks (Fig. 6C). This result suggests 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.

Incubation of soluble starch, amylose and amylopectin with pullulanase for more than 24 h resulted in the formation of low level of maltose and glucose (data not shown) and the action of pullulanase on both soluble starch and amylopectin was very slow, demonstrating its low affinity for α-1,4-glucosidic linkages. These results indicate that the enzyme acts on pullulan as a true pullulanase and has an endo-acting α-amylase activity.

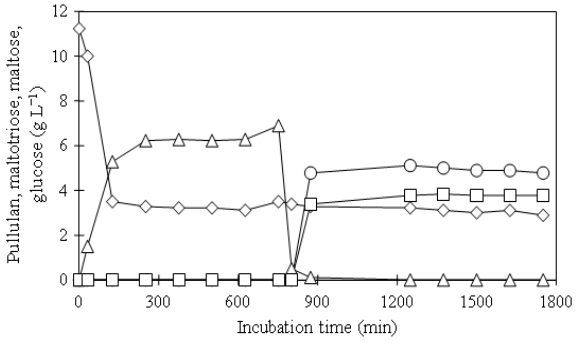

Fig. 5: Time course of enzymatic hydrolysis of pullulan by the pullulanase from *Bacillus cereus* H1.5. Symbols represent: (open diamonds) pullulan; (open triangles) maltotriose; (open circles) maltose; (open squares) glucose

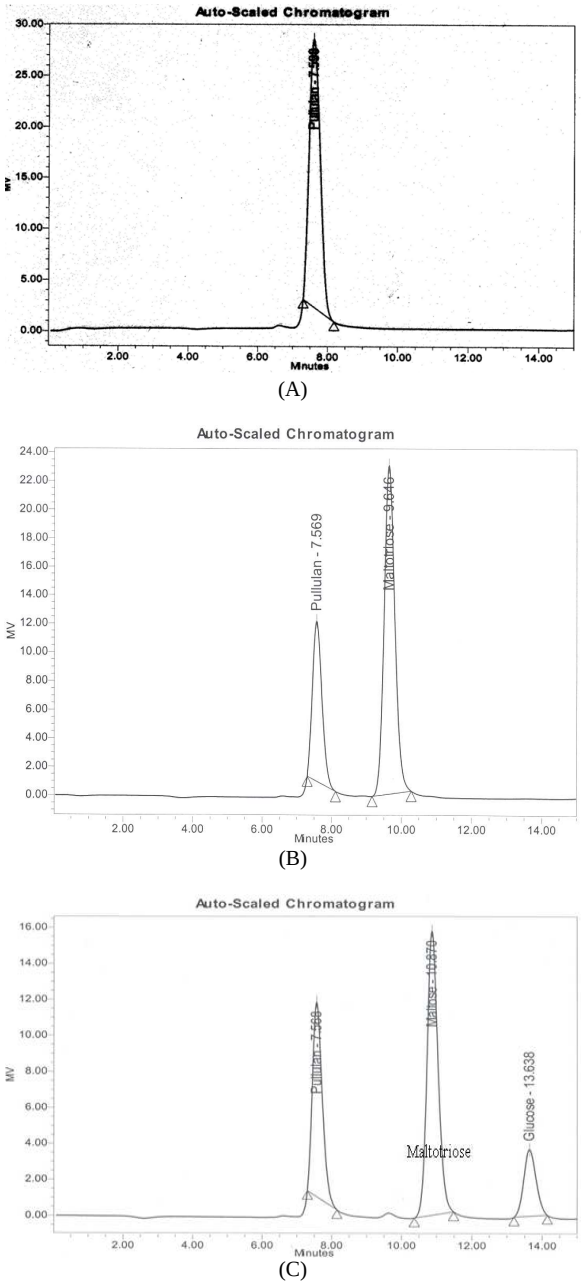

Fig. 6: End product profile of the action of pullulanase *Bacillus cereus* H1.5 on 1% pullulan as analyzed by HPLC. DP denotes degree of polymerization. (A) pullulan without enzyme as control; (B) after 3 h action on pullulan; (C) DP3 product degraded by Maltogenase®L

DISCUSSION

The optimal pH for pullulanase activity and the range of pH for pullulanase stability of observed in this study was in agreement with those reported for other sources of pullulanase[3,4,14,18-19]. For example, the pH optima for most pullulanases were at pH of around 5.0 to 5.8. Likewise, pullulanase from other sources was also stable at pH ranging from 4-9[4,21-23], except for some alkaline pullulanases which were active and stable within the alkaline range of pH[23,24].

The optimal temperature (55°C) for activity of pullulanase from *B. cereus* H1.5 was comparable to pullulanase from other microbial sources, which were normally in the range from 40-60°C[3,23,25], except for thermophilic bacterium and extreme thermophiles, which has optimal activity at temperature ranging from 70-100°C[3,4,18,20-22]. The thermostability of pullulanase from *B. cereus* H1.5 was increased from 40-50°C in the present of Ca^{2+}. Enhanced thermostability of pullulanase in the present of Ca^{2+} have been reported by many researchers. For example, pullulanase from *T. litoralis* and *P. furiosus*[14], *Bacillus stearothermophilus*[26] and *Thermococcus hydrothermalis*[18]. On the other hand, activity and stability of pullulanase from *Desulfurococcus mucosus* was not enhanced with the present of Ca^{2+}[4].

Indeed metal ions often act as salt or ion bridges between two adjacent amino acids[19]. This tryptophan-specific oxidant has been reported to inhibit the activity of pullulanase from strains of alkalophilic *Bacillus* sp.[23,24], an anaerobic bacterium *Desulfurococcus mucosus*[4], *Clostridium thermohydrosulfuricum*[7] and a hyperthermophilic archaeon *Thermococcus hydrothermalis*[18]. It is well known that metal ions often act as salt or ion bridges between two adjacent amino acids[18]. Schardinger dextrins inhibited the pullulanase activity of *B. cereus* H1.5 and β-dextrin was a strongest inhibitor than α- and γ-dextrin. This is true with other pullulanases[7,19,25], except pullulanase from an archaeal *Desulfurococcus mucosus*[4] which was not inhibited by these possible competitive inhibitors of pullulanase.

From this study, it was found that the activity of pullulanase was quantitatively affected by the presence of small carbohydrates. Indeed, the catalytic efficiency of the enzymes depended on the additive present in the reaction medium and the influence was varied with its concentration. As a general rule, the catalytic efficiency increases with increasing sugar concentration to reach an optimum, after which it decreases. Addition of the highly soluble carbohydrates like polyols or simple sugars into enzymatic reaction media decreased the water activity, which in turn, the physicochemical properties will also be modified. These additives were also found relevant in regards to the thermal stability of the enzymes[27]. In addition, these protective effects

were explained by the strengthening of the hydrophobic interactions inside protein molecules and by indirect action of additives on water structure[28]. Furthermore, increased in the thermal stability of proteins may be due to increasing in rigidity of their tertiary structure[29].

The pullulanase of *B. cereus* H1.5 did not cleave dextran which containing only α-1,6 glycosidic linkages, as observed for many pullulanases[4, 21]. Since pullulanase from *B. cereus* 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. The molecular mass of pullulanase produced by *B. cereus* H1.5 was bigger than pullulanase produced by *A. niger* (69 kDA)[17] and almost similar to pullulanase produced by *Bacillus* sp. US149 (95 KDA)[3]. This enzyme has ability to hydrolyse pullulan to produce maltotriose as a final product. The enzyme was able to attack specifically the α-1,6 linkages in pullulan to generate maltotriose as the major end product, as well as the α-1,4 linkages in amylopectin and soluble starch. The enzyme also exhibits some distinct features in term of thermoactivity and thermostability as well as well as stability at wide range of pH (2-10). Pullulanase type II is normally produced by the extreme thermophilic bacteria where the cultivation is difficult. In this case, the producing microorganism (*B. cereus*) can be grown easily, thus the enzyme production process may have potential to be transferred directly into industrial applications without the need in the development of recombinant strains.

CONCLUSION

Pullulanase type II, produced by *B. cereus* H1.5, was capable to attack specifically the α-1,6 linkages in pullulan to generate maltotriose as the major end product, as well as the α-1,4 linkages in amylopectin and soluble starch leading to the formation of a mixture of maltose and glucose. A relative substrate specificity for hydrolysis of pullulan, amylopectin and soluble starch by this pullulanase was 100, 28.5 and 20.4%, respectively. The reaction of this pullulanase on pullulan has apparent K_m and V_{max} value of 1.1 mg mL^{-1} and 0.275 μmol min^{-1}, respectively. This pullulanase showed optimal activity at 55°C and pH 6.0 and its thermostability and thermoactivity were increased considerably in the presence of Ca^{2+}. The enzyme was activated by 2-mercaptoethanol and dithiothreitol and deactivated N-bromosuccinimide and Schardinger dextrins.

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