Carbohydrate Polymers 80 (2010) 401–407

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Maltotriose syrup preparation from pullulan using pullulanase

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ARTICLE INFO

Article history:
Received 7 July 2009
Received in revised form 20 November 2009
Accepted 25 November 2009
Available online 3 December 2009

Keywords:
Pullulan
Pullulanase
Maltotriose syrup
Exopolysaccharide
Aureobasidium pullulans

ABSTRACT

Pullulan production was carried out at shake-flask level and purified with isopropanol precipitation using single-step purification strategy. Purified pullulan obtained was used for preparing maltotriose syrup using pullulanase. Pullulanase was subjected to kinetic and thermodynamic characterization before its use for pullulan hydrolysis. Pullulanase exhibited considerable activity between pH 5 and 6, with an optimum pH of 5.0. It remained stable (100%) for 6 h at optimum temperature of 50 °C. Mn^{2+} and Ca^{2+} showed 1.8 and 2.1 times increase in pullulanase activity. Pullulanase effectively hydrolyzed pullulan, soluble starch and dextran. Decimal reduction time (D value) was 76.77 h at 50 °C and *Z* value was 12.5 °C for pullulanase. Thermodynamic parameters (ΔH^*, ΔG^*, ΔS^*) for irreversible inactivation of free pullulanase at different temperatures (50–70 °C) were also determined. In a batch system, pullulanase hydrolyzed 94.25 ± 1.83% of purified pullulan and the resultant syrup contained 3.77 ± 0.07 mg/mL of maltotriose. Thus far, there is no report on thermodynamics of irreversible inactivation of pullulanase for the hydrolysis of pullulan.

1. Introduction

Recently, the microbial polysaccharides have received increasing interest because of their useful physiochemical properties and uncomplicated biodegradability in the natural environment. The repeating units of these exopolysaccharides are very regular, branched or unbranched, and interconnected by glycosidic linkages. Pullulan is one of such polymers synthesized by the yeast-like fungus *Aureobasidium pullulans*. It is a linear α-D-glucan built of maltotriose subunits, i.e., α-(1 → 4)Glu*p*-α-(1 → 4)Glu*p*-α-(1 → 6)Glu*p*-, connected by (1 → 6)-α-D-glucosidic linkages (Leathers, 2003). However, other structures particularly the tetramer or maltotetraose, α-(1 → 4)Glu*p*-α-(1 → 4)-Glu*p*-α-(1 → 4)Glu*p*-α-(1 → 6)Glu*p*-, may be present in the pullulan polymeric chain (Wallenfels, Keilich, Bechtler, & Freudenberger, 1965). The maximum extent to which maltotetraose subunits have been detected so far is 7% (Catley, Ramsay, & Servis, 1986). Generally, pullulan is viewed as a succession of α-(1 → 6)-linked (1 → 4)-α-D-triglucosides, i.e., maltotriose (G3) but it may also occur as a polymer of isopanose (Singh, Saini, & Kennedy, 2008a). The regular alteration of α-(1 → 4) and α-(1 → 6) bonds results in two distinctive properties of structural flexibility and enhanced solubility. The unique linkage pattern endows pullulan with distinctive physical traits along with adhesive properties. Pullulan and its derivatives have numerous potential for food, pharmaceutical and other industrial applications (Shingel, 2004; Singh et al., 2008a).

Pullulanase (EC 3.2.1.41, pullulan 6-glucanohydrolase) is a debranching enzyme which hydrolyses the α-1,6-glucosidic linkages in pullulan and other amylaceous polysaccharides, and belong to a family of 13 glycosyl hydrolases, also called the α-amylase family (Janecek, Svensson, & Henrissat, 1997; Matzke, Herrmann, Schneider, & Bakker, 2000). Pullulanases are widely distributed among animals, plants, fungi and bacteria. Detailed enzymatic mechanisms of substrate degradation and the resulting final products are different in each case (Domań-Pytka & Bardowski, 2004). Pullulan undergo enzymatic hydrolysis by both (1–6)-α-D- and (1–4)-α-D-pullulanases. The (1–6)-α-D-pullulanases cleave the (1–6)-α-D-glucopyranosidic linkages. Complete hydrolysis of pullulan using (1–6)-α-D-pullulanase yields maltotriose as major product along with traces of maltotetraose. The (1–4)-α-D-pullulanases act on (1–4)-α-D-glucosidic linkages at their reducing ends adjacent to (1–6)-α-D linkages. Complete hydrolysis of pullulan with (1–4)-α-D-pullulanase gives isopanose as the main product. Products of enzymatic pullulan degradation are used in food and pharmaceutical industry.

Maltotriose rich syrups are being produced from maltose syrup by cation exchange resin chromatography and maltose solution of 98% purity is obtained as a by-product. Maltotriose syrup is also produced by enzymatic hydrolysis of the polysaccharide 'pullulan' using the debranching enzyme, pullulanase. This syrup possess many excellent properties as low freezing point depression, mild sweetness, keeps in moisture, prevention of retrogradation of starch in foodstuffs, less color formation compared with maltose syrups, glucose syrups or sucrose, good heat stability, low solution

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doi:10.1016/j.carbpol.2009.11.040

viscosity, high fermentability and favoring glassy states. These properties are useful in food and pharmaceutical industries (Zoebelein & Böllert, 2001). High maltotriose syrup may be applied in the food industry for the manufacturing of desserts, baking and brewing, as well as in the pharmaceutical industry for replacing glucose in intravenous feeding.

In the present work, the pullulan was produced at shake-flask level. The purified pullulan having maltotriose as repeating units was subjected to hydrolysis using debranching enzyme "pullulanase" for the preparation of maltotriose syrup. Current investigations have novelty as this is the first report on thermodynamic characterization and irreversible inactivation of pullulanase for the hydrolysis of pullulan.

2. Materials and methods

2.1. Organism and pullulan production

Aureobasidium pullulans FB-1 used in the present study has been isolated, identified and maintained as reported earlier (Singh & Saini, 2008). The culture has been assigned an Accession No. MTCC 6994 by Microbial Type Culture Collection, Chandigarh, India. The production of pullulan was carried out in shake-flasks as described earlier (Singh, Singh, & Saini, 2008b).

2.2. Recovery and purification of pullulan

Pullulan recovery and purification was done as described by current authors (Singh, Saini, & Kennedy, 2009). Briefly, pullulan from the fermentation broth was purified by isopropanol precipitation using a single-step purification strategy.

2.3. Procurement of pullulanase

Pure pullulanase (Source: *Bacillus acidopullulyticus*) procured from Sigma, USA was subjected to kinetic and thermodynamic characterization before its use for the hydrolysis of pullulan.

2.4. Characterization of free pullulanase

2.4.1. Optimum pH and pH stability

To identify the optimum pH for pullulanase, assays were carried out at different pH (3.5–7.0) using sodium acetate buffer (0.1 M, pH 3.5–5.0) and phosphate buffer (0.1 M, pH 6.0–7.0). The enzyme solution was incubated at different pH (3.5–7.0) at 50 °C to study the effect on the enzyme activity. Samples were withdrawn periodically and the residual activity measured in terms of relative activity, which was calculated as the percentage ratio of activity at a given pH to the activity at the optimum pH.

2.4.2. Optimum temperature, thermal stability, activation energy and temperature quotient (Q_{10})

Optimum temperature and activation energy (E_a) of pullulanase were determined by incubating appropriate amount of the enzyme with 0.1% pullulan at various temperatures ranging from 40 to 70 °C in 0.1 M phosphate buffer for 20 min at pH 5. The thermal stability was determined without any substrate or additives. The enzyme solution was kept at 40, 45, 50, 55, 60, 65 and 70 °C in a 0.1 M phosphate buffer (pH 5.0) in a temperature-controlled water bath and the residual activity was measured at regular time intervals. The residual activity measured in terms of relative activity, which was calculated as the percentage ratio of activity at a given temperature to the activity at the optimum temperature.

Activation energy (E_a) has been calculated from the slope ($-E_a/2.3R$) of linear representation of log V_{max} versus $1/T$, using Arrhenius equation ($k = Ae^{-E_a/RT}$). Temperature is expressed in Kelvin, the gas constant ($R = 8.314$) in $JK^{-1}mol^{-1}$ and the activation energy (E_a) in $kJmol^{-1}$.

The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to rise in the temperature by 10 °C. Q_{10} was calculated as follows:

$$E_a = \frac{2.3RT_2T_1 \log Q_{10}}{10}$$

2.4.3. Thermal inactivation

Enzyme inactivation often follows first-order kinetics. Under isobaric-isothermal conditions, the decrease of enzyme activity as a function of treatment time can be described by:

$$A = A_0 \exp(-kt)$$

This can be linearized by a logarithmic transformation as:

$$\ln A = \ln A_0 - kt$$

In food processing, it is common to express first-order reactions in terms of *D*- and Z-values. The decimal reduction time, or *D*-value, is defined as the time, at a given temperature and pressure, needed for a 90% reduction of the initial activity. For first-order reactions, the *D*-value is inversely proportional to the inactivation rate constant, $D = 2.303/k$. The decimal reduction time at a certain temperature or pressure was estimated from the slope of the regression line of $\log(A/A_0)$ versus treatment time at constant temperature:

$$\log(A/A_0) = -t/D$$

The *Z*-value is defined as the temperature increase necessary to obtain a 10-fold decrease of the *D*-value. The *Z*-value was calculated from the negative reciprocal slope of the regression of log *D* versus temperature:

$$\log D = \log D_{ref} - \frac{T - T_{ref}}{Z}$$

2.4.4. Thermodynamics of irreversible thermal inactivation

Thermal inactivation of native pullulanase was determined by incubating the enzyme solutions in 0.1 M phosphate buffer (pH 5.0) at varying temperatures (50, 55, 60, 65 and 70 °C) in the absence of substrate. Aliquots were withdrawn at different times, cooled and assayed for pullulanase activity. The thermodynamic parameters for thermostability were calculated by rearranging Eryings absolute rate equation derived from the transition state theory (Erying & Stearn, 1939; Stearn, 1949) as described by Siddiqui, Shemsi, Anwar, Rashid, and Rajoka (1999).

$$K_d = (k_b/h)e^{(-\Delta H^*/RT)} \cdot e^{(-\Delta S^*/R)}$$

where h = Planck's constant = 6.63×10^{-34} Js; k_b = Boltzman's constant (R/N) = 1.38×10^{-23} JK^{-1}; R = gas constant = 8.314 $JK^{-1}mol^{-1}$; N = Avogadro's No.= 6.02×10^{23} mol^{-1}; T = Absolute temperature.

$$\Delta H^* = E_a - RT$$

where ΔH^* is the Enthalpy of activation of denaturation and E_a is the activation energy for denaturation.

$$\Delta G^* = -RT \ln(K_d h/K_b.T)$$

where, ΔG^* is the free energy of activation of denaturation.

$$\Delta S^* = (\Delta H^* - \Delta G^*)/T$$

where ΔS^* is the entropy of activation of denaturation.

2.4.5. Substrate specificity and kinetic constants

Affinity of pure pullulanase towards pullulan, soluble starch and dextran was investigated by incubating the enzyme with these substrates and assaying the enzyme activity. The kinetic constants (V_{max}, K_m and V_{max}/K_m) were determined by incubating pullulanase with varied concentrations of different substrates, i.e., pullulan (0.25–1.75%, w/v), soluble starch (0.25–1.75%, w/v) and dextran (0.25–1.75%, w/v) at 50 °C and pH 5.0 using a Lineweaver–Burk plot.

2.4.6. Effect of metal ions and chelating agent

To analyze the effect of metal ions, pullulanase activity was also checked in the presence of various metal ions (Zn^{2+}, Ca^{2+}, Cu^{2+}, Fe^{2+}, Ni^{2+},Cd^{2+}, Ba^{2+}, Mg^{2+}, Mn^{2+}, Co^{2+}, Hg^{2+}, K^{+}, and Na^{+}) and EDTA, individually at varied concentrations (2–10 mM).

2.4.7. Effect of surfactants

The effect of surfactants on pullulanase activity was tested by using different concentrations (0.025–0.10%, v/v) of various surfactants like sodium dodecyl sulphate (SDS), Tween 80, Triton X-100 and Brij-35. Relative activity in the presence of surfactants was calculated as percentage of the activity in the control set (without any surfactant) which was taken as 100%.

2.5. Hydrolysis of pullulan for the preparation of maltotriose syrup

Purified pullulan produced from *A. pullulans* FB-1 was used for hydrolysis. Operational parameters like agitation, hydrolysis time and stability were studied for the preparation of maltotriose syrup in a batch system. To investigate the optimal hydrolysis condition, 0.4% pullulan was incubated with pullulanase (4U) under agitation (50–200 rpm) at 50 °C for different time intervals (0.5–6 h). For comparison, the hydrolysis of 0.4% commercial pullulan (Sigma, USA) solution was also carried out at 50 °C under optimal conditions (150 rpm agitation and 6 h hydrolysis time). The maltotriose produced was quantified in terms of reducing sugars released and the extent of hydrolysis (%) was calculated as under:

$$\text{Hydrolysis}(\%) = \frac{\text{Amount of reducing sugars released}}{\text{Amount of pullulan}} \times 100$$

2.6. Pullulanase activity

A reaction mixture (3 mL) consisting 0.5 mL of pullulan (1%, w/v), 0.5 mL of appropriately diluted enzyme and 2.0 mL of 0.1 M phosphate buffer (pH 5.0) was taken in a test tube and incubated at 50 °C in a water bath for 20 min. After incubation, the test tube was kept at 100 °C for 10 min to inactivate the enzyme. The reaction mixture was assayed for reducing sugars by the DNSA method (Miller, 1959). One unit of enzyme is defined as amount of enzyme that produces one micromole of reducing sugars (as maltotriose equivalents) per minute under standard assay conditions.

2.7. Analytical techniques

The reducing sugars were determined by the method of Miller (1959).

2.8. Statistical analysis

Experiments were carried out in triplicates and the mean values were calculated. One-way analysis of variance (ANOVA) and pair wise multiple comparison procedures (Tukey's test) were carried out using the statistical software SigmaStat, version 2.0 (Jandel Corp., San Rafael, CA, USA). Values are expressed as the mean ± SEM. The level of significance was set at $P < 0.001$.

3. Results and discussion

Pullulan production was carried out at shake-flasks in a fermentation medium containing (g/L) sucrose, 50.0; K_2HPO_4, 5.0; NaCl, 1.0; $MgSO_4{\cdot}7H_2O$, 0.2; yeast extract, 2.0 and adjusted to pH 6.5 as described earlier (Singh et al., 2008b). Pullulan from the fermentation broth was purified with isopropanol precipitation using a single-step purification strategy as reported earlier by current authors (Singh et al., 2009). The purified pullulan having maltotriose as repeating units was subjected to hydrolysis for the preparation of maltotriose syrup using pullulanase.

3.1. Characterization of pullulanase

To develop an efficient process, characterization of enzyme is required. Therefore, the thermodynamic and kinetic characterization of the pullulanase was carried out to develop an efficient process for the preparation of maltotriose syrup.

3.1.1. Effect of pH

Pure pullulanase showed a pH optima of 5.0. Though maximum activity was observed at pH 5.0, enzyme exhibited appreciable activity up to pH 6.5. Thus, an enzyme with a broad pH range is always preferable for application in the food industry. Enzyme retained more than 89% relative activity between pH 5.0 and 6.5 whereas, at pH 4.5 more than 75% relative activity was observed (data not shown). Decline in activity above and below the optimal pH may be attributed to the formation of an improper ionic form of the substrate or the enzyme, or inactivation of the enzyme or a combination of these effects. Various workers reported optimum pH of pullulanase from *Bacillus acidopullulyticus* in the range of 4.0–5.5 (Jensen & Norman, 1984; Stefanova, Schwerdtfeger, Antranikian, & Scandurra, 1999). However, pH optima of pullulanase reported from *Klebsiella pneumoniae* 6.5 (Bender & Wallenfels, 1961), *Micrococcus* sp. 8.0 (Kimura & Horikoshi, 1990), *Thermococcus* sp. 5.5 (Gantelet, Ladrat, Godfroy, Barbier, & Duchiron, 1998) and *Rhodotehrmus marinus* 6.5–7.0 (Gomes, Gomes, & Steiner, 2003).

The enzyme preparation was incubated at different pHs for different time intervals (0–360 min) and found to remain quite stable over a range of pH 4.5–6.5, and showed 100% stability at pH 5.0 (Fig. 1). More than 75% relative residual activity was found at pH 4.5 after 6 h incubation at 50 °C. There was a decrease in enzyme activity at pH 4.0 and 7.0. However, long term (24 h) pH stability (retaining 100% activity) of pullulanase from *Rhodothermus marinus* at 50 °C and pH 6.0 has been reported (Gomes et al., 2003).

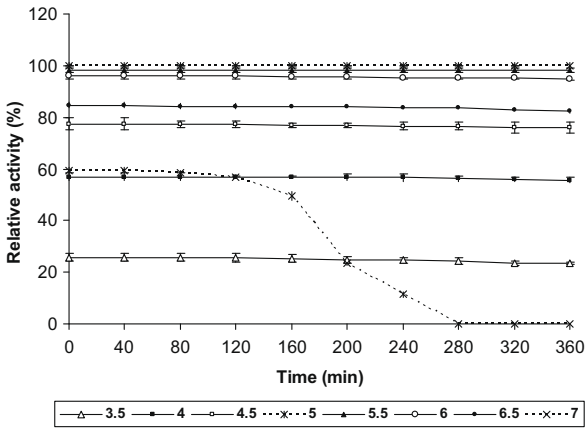

Fig. 1. Stability of pullulanase at different pH.

3.1.2. Optimum temperature, thermal stabiltiy, activation energy and temperature quotient (Q_{10})

The optimum temperature of pullulanase at pH 5.0 was found to be 50 °C. Enzyme exhibited 92.51% and 82.52% relative activity at 55 °C and 60 °C, respectively, and there was a rapid decrease at 70 °C (data not shown). Pullulanases from *Bacillus acidopullulyticus* and *Klebsiella pneumoniae* have been reported to have temperature optima of 60 °C and 55 °C, respectively (Jensen & Norman, 1984). However, Stefanova et al. (1999) reported temperature optima of 55 °C for pullulanase from *Bacillus acidopullulyticus*. Thermal activity was then checked at above the optimal temperature. Enzyme remained 100% stable at 50 °C (Fig. 2). Pullulanase from *Bacillus acidopullulyticus* showed good thermostability and retained 55% of activity after incubation at 60 °C for 30 min (Stefanova et al., 1999). Gomes et al. (2003) reported long-term thermostability for pullulanase from *Rhodothermus marinus* at 75 °C with very little loss of activity after 66 h.

An Arrhenius plot (Fig. 3) in a temperature range of 25 °C to the optimum (50 °C) was linear and indicated the inactivation of the enzyme at higher temperatures. E_a of the enzyme for pullulan hydrolysis was 34.29 kJ/mol. The energy of activation for pullulanase from *Bacillus acidopullulyticus* for pullulan has been reported 21 kJ/mol (Kusano, Shiraishi, Takahashi, Fujimoto, & Sakano, 1989) and 30.16 kJ/mol (Stefanova et al., 1999). The effect of temperature on the rate of reaction was expressed in terms of temperature quotient (Q_{10}), which is the factor by which the rate increases due to rise in temperature by 10 °C. The temperature quotient for pullulanase was 1.51.

3.1.3. Thermal inactivation

Thermal inactivation of pullulanase was studied in the range 50–70 °C indicating that the inactivation constants (k values) increased with increasing temperature (Table 1). The decimal reduction time (D value) is the time, at a given temperature and pressure, needed for 90% reduction of the initial activity. D values of pullulanase ranged between 1.43 and 76.77 h at the temperatures studied. Pullulanase presented low thermal deactivation rate indicating highest stability at 50 °C with a D-value of 76.77 h. The temperature dependence of the D values is given by the Z value. The Z value equals the temperature increase necessary to obtain a 10-fold decrease of the D value. Z value (extrapolated from D value) was found to be 12.5 °C for free pullulanase, i.e., the temperature has to be increased with 12.5 °C to obtain a 10-fold decrease of the D value. Higher temperatures resulted in higher rates of enzyme inactivation as indicated by lower D values and higher k values.

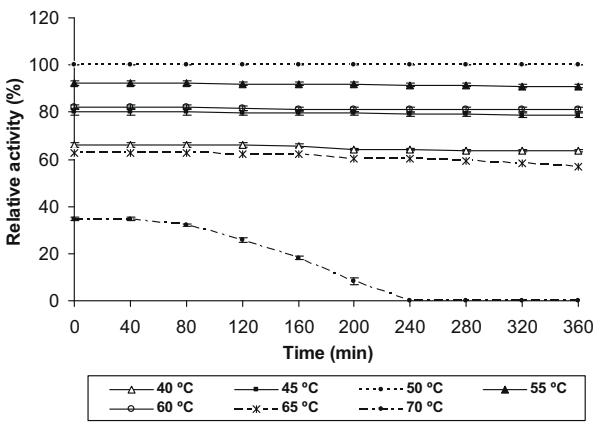

Fig. 2. Stability of pullulanase at different temperatures.

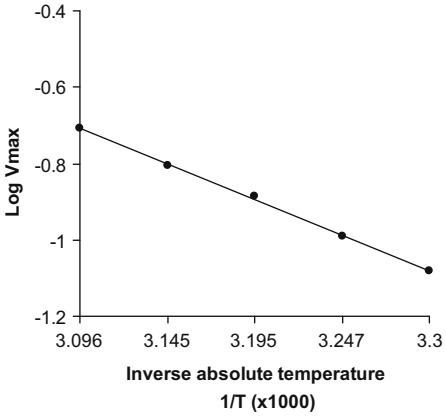

Fig. 3. Arrhenius plot for the determination of activation energy (E_a) of pullulanase for the hydrolysis of pullulan.

Table 1
Summary of kinetic parameters for thermal inactivation of pullulanase.

T (°C)	A[a]	K[b] (h^{-1})	D[c] (h)	$t^{1/2}$ [d] (h)
50	0.196	0.030	76.77	23.1
55	0.182	0.141	16.33	4.91
60	0.162	0.314	7.33	2.21
65	0.123	0.714	3.22	0.97
70	0.068	1.610	1.43	0.43

[a] Remaining pullulanase activity after heating for 40 min.
[b] First order rate constant for inactivation.
[c] Decimal reduction time.
[d] Half life of pullulanase.

3.1.4. Thermodynamics of irreversible thermal inactivation

Thermostability represents the capability of an enzyme molecule to resist against thermal unfolding in the absence of substrate, while thermophilicity is the ability of an enzyme to work at elevated temperatures in the presence of substrate (Georis et al., 2000). Irreversible thermal inactivation occurs in two steps as shown below:

$$N \leftrightarrow U \rightarrow I$$

N is the native, U is the unfolded enzyme which could be reversibly refolded upon cooling and I is the inactivated enzyme formed after prolonged exposure to heat, and therefore, cannot be recovered on cooling. The thermal denaturation of enzymes is accompanied by the disruption of non-covalent linkages, including hydrophobic interactions, with concomitant increase in the enthalpy of activation (Daniel, 1996). The opening up of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation (Vieille & Zeikus, 1996). This is the first report on thermodynamics of irreversible thermal inactivation of pullulanase for the hydrolysis of pullulan.

Activation energy for irreversible inactivation '$E_a(d)$' of the pullulanase was determined by applying Arrhenius plot and Gibbs free energy (ΔG^*) for activation of thermal unfolding of enzyme was 110.72 kJmol^{-1} at 50 °C. With an increase in temperature, a decrease in free energy was observed. The decrease in ΔG^* value with increasing temperature makes the enzyme thermally unstable. The enthalpy of activation of thermal unfolding (ΔH^*) of the enzyme at 50 °C was 31.79 kJmol^{-1}. Its value remained almost same up to 70 °C. The entropy of activation (ΔS^*) for unfolding of transition state of the pullulanase was found to be -244.36 Jmol^{-1} (Table 2). When enthalpy and entropy values for inactivation were calculated at each temperature, ΔS^* had negative values. This suggested that there was negligible disorderness as was that of β-glucosidase

Table 2
Kinetics and thermodynamics of irreversible thermal denaturation of free pullulanase.

T (°C)	T (K)	k_d[a] (h^{-1})	k_d (sec^1)	$t^{1/2}$ (h)	ΔH^*[b] (kJ/mol)	ΔG^*[c] (kJ/mol)	ΔS^* (J/mol)
50	323	0.030	8.33×10^{-6}	23.1	31.79	110.72	−244.36
55	328	0.141	3.89×10^{-5}	4.91	31.79	108.28	−233.20
60	333	0.314	8.61×10^{-5}	2.21	31.71	107.77	−228.41
65	338	0.714	1.97×10^{-4}	0.97	31.67	107.11	−223.19
70	343	1.610	4.47×10^{-4}	0.43	31.63	106.39	−217.96

[a] First order rate constant for inactivation.
[b] E_a (34.29 kJ/mol) $-RT$, where E_a is activation energy.
[c] $-RT \ln \{(k_d.h)/(k_B.T)\}$.

from *A. wentii* or the transition state of α-amylase from *Bacillus licheniformis* was found to be more ordered as revealed by its negative ΔS^* at high temperature of 80 °C (Declerck et al., 2003).

3.1.5. Substrate specificity and kinetic constants

Pullulanase was found to hydrolyze pullulan, soluble starch and dextran. Enzyme showed 61.93% activity on soluble starch with respect to activity on pullulan, which was taken as 100%. Pullulanase from *Desulfurococcus mucosus* hydrolyzed pullulan (100%) and soluble starch (62%), but not cleaved dextran and glycogen (Duffner, Bertoldo, Andersen, Wagner, & Antranikian, 2000).

The effect of different concentrations of pullulan, soluble starch and dextran on pullulanase activity was investigated and the results were extrapolated from Lineweaver and Burk plots. Apparent Michaelis constant (K_m) and V_{max} for pullulan, soluble starch and dextran were found to be 4.0 mg/mL and 0.23 U/min, 11.1 mg/mL and 0.20 U/min and 40.0 mg/mL and 1.25 U/min, respectively. The ratio between V_{max} and K_m were estimated to be 0.57, 0.18 and 0.31, respectively (Table 3). The highest V_{max}/K_m ratio for pullulan showed that it is the best substrate for pullulanase. The apparent K_m of pullulanase from *Bacillus acidopullulyticus* for pullulan has been reported as 3.46 mg/mL (Stefanova et al., 1999).

3.1.6. Effect of metal ions and chelating agent

Metal ions have long been known to stabilize/activate/inhibit enzymes. They function as enzyme cofactors and can participate with an enzyme to accelerate the rate of reaction through one of several recognized mechanisms including general acid–base catalysis, covalent catalysis, the approximation of the reactants, or the induction of the strain in the enzyme or substrate. Thus, the effect of potential metal ions and enzyme inhibitor on pullulanase activity was investigated at varied concentrations. The maximum increase in pullulanase activity was observed with the addition of Ca^{2+} (2.1 times) and Mn^{2+} (1.84 times), when compared with the control with no metal ions, suggesting that the enzyme required these ions for a better catalytic action (Data not shown). In comparison, Cu^{2+}, Ni^{2+} and Hg^{2+} were found potent inhibitors. Cu^{2+} and Hg^{2+} caused complete inactivation of the activity even at a lower concentration of 4 mM. Even Ni^{2+} at a very low concentration of 1 mM completely inhibited the enzyme activity. The rest of the metal ions studied had no significant effect at lower concentrations (Data not shown). Ethylenediaminetetraacetate (EDTA) was also not inhibiting, suggesting that this chelating agent did not chelate a possible divalent cation(s) required for the activity of pullulanase. The stimulatory effect of Ca^{2+} and Mn^{2+} has also been reported earlier (Kunamneni & Singh, 2006).

Table 3
Kinetic parameters of pullulanase.

Substrate	K_m (mg/mL)	V_{max} (U/min)	V_{max}/K_m
Pullulan	4.0	0.23	0.57
Soluble starch	11.1	0.20	0.18
Dextran	40.0	1.25	0.31

3.1.7. Effect of surfactants

The effect of various surfactants (SDS, Tween 80, Triton X-100 and Brij-35) on the pullulanase activity was investigated at varying concentrations (0.025–0.10%, v/v). None of the surfactants had shown enhancement of pullulanase activity (data not shown). Incubation of purified pullulanase from *Pyrococcus woesei* with 5 mM SDS caused a decrease in activity to 13%, whereas Triton X-100 (0.1 and 1%) caused activation of the enzyme (Rüdiger, Jorgensen, & Antranikian, 1995). Gantelet and Duchiron (1998) reported no effect of Triton X-100 on pullulanase from *Thermococcus hydrothermalis*.

3.2. Hydrolysis of pullulan for the preparation of maltotriose syrup

To prepare maltotriose syrup, hydrolysis of purified pullulan (0.4%, w/v) in sodium phosphate buffer (0.1 M, pH 5.0) was carried out with pullulanase (4 U) under stationary and agitation (50–200 rpm) at 50 °C for 6 h. Less hydrolysis under stationary condition was recorded which may be due to the inhibition of enzyme activity caused by accumulation of reducing sugars in the system. Better interactions between the substrate and the enzyme can be achieved in agitation mode. A comparative analysis of the percent hydrolysis at different agitation speeds reveal that maximum pullulan hydrolysis was achieved at 150 rpm (Table 4). At higher agitation, there was less conversion which may be attributed to enzyme denaturation or less enzyme substrate contact time. Therefore, the hydrolysis was studied as a function of time using this mode of agitation. The results depicted that the hydrolysis of purified pullulan after 120, 240 and 360 min of incubation was 48.25 ± 1.53, 72.00 ± 2.02 and 94.25 ± 1.83%, respectively. Maximum hydrolysis was observed after 360 min and after this time there was no change in this function. At this optimal hydrolysis time, 3.77 ± 0.07 mg/mL of maltotriose was present in the syrup produced from purified pullulan (Fig. 4). Similar results were obtained when for comparison purified pullulan was replaced with commercially available pullulan (Sigma, USA).

Pullulan hydrolysis using pullulanase has been studied by various researchers. Hydrolysis of pullulan by pullulanase at 30 °C yielding maltotriose as the only product has been reported (Drummond, Smith, Whelan, & Tai, 1969). Bertoldo, Duffner, Jorgensen, and Antranikian (1999) reported more than 98% pullulan hydrolysis after 1 h incubation at 80 °C by thermostable pullulanase from *Fervidobacterium pennavorans* Ven5. Similar hydrolysis rate of pullulan by pullulanase aqueous solution at 25 °C for each molecular weight of pullulan has been reported (Ohtani, Ishidao, Iwai, & Arai, 1999). Random decomposition of pullulan has been suggested by aqueous solution of pullulanase with the proceeding time.

Table 4
Effect of agitation on hydrolysis of pullulan by free pullulanase in batch system.

Time (min)	Stationary	Hydrolysis (%) under agitation (rpm)						
		50	75	100	125	150	175	200
30	6.25 ± 1.00	9.50 ± 1.08	13.25 ± 1.21	12.27 ± 1.80	15.50 ± 1.15	18.75 ± 1.10	14.50 ± 1.15	9.75 ± 1.19
60	15.00 ± 1.28	21.25 ± 1.91	24.00 ± 1.57	24.75 ± 1.30	26.75 ± 1.27	29.50 ± 1.10	27.25 ± 2.08	23.75 ± 1.50
90	24.25 ± 1.52	29.18 ± 1.41	31.00 ± 1.84	33.75 ± 1.50	36.51 ± 1.63	37.50 ± 1.82	33.25 ± 1.98	29.25 ± 1.37
120	30.25 ± 1.39	36.00 ± 2.33	37.25 ± 2.08	41.75 ± 1.13	43.25 ± 1.39	48.25 ± 1.53	39.25 ± 1.18	36.00 ± 2.33
150	37.00 ± 1.73	42.00 ± 2.31	40.25 ± 1.56	48.50 ± 1.77	49.50 ± 1.54	54.75 ± 2.02	48.50 ± 1.37	40.00 ± 1.53
180	41.00 ± 1.25	47.25 ± 2.08	46.75 ± 1.47	59.50 ± 1.08	57.25 ± 2.21	61.25 ± 1.74	57.13 ± 1.57	47.25 ± 2.08
210	44.97 ± 1.28	51.25 ± 1.45	51.00 ± 1.19	62.50 ± 1.75	60.00 ± 1.84	67.25 ± 2.33	60.75 ± 1.35	54.00 ± 2.32
240	49.75 ± 1.12	56.75 ± 1.47	57.25 ± 2.08	68.25 ± 2.09	66.50 ± 1.64	72.00 ± 2.02	65.00 ± 2.33	58.75 ± 0.95
270	52.66 ± 1.52	61.00 ± 1.16	66.75 ± 1.71	71.50 ± 1.44	71.00 ± 1.07	75.25 ± 1.15	72.75 ± 1.18	64.75 ± 2.02
300	59.50 ± 1.27	65.75 ± 1.09	73.75 ± 0.93	77.00 ± 1.79	76.25 ± 1.62	80.25 ± 1.27	78.25 ± 1.09	72.25 ± 1.30
330	65.00 ± 1.79	72.75 ± 1.84	78.25 ± 1.09	81.75 ± 1.13	83.00 ± 1.68	89.75 ± 1.27	84.25 ± 1.52	75.50 ± 1.39
360	73.25 ± 1.51	77.25 ± 1.41	82.75 ± 1.90	85.25 ± 1.12	89.25 ± 1.27	94.25 ± 1.83	90.75 ± 1.51	83.50 ± 1.64

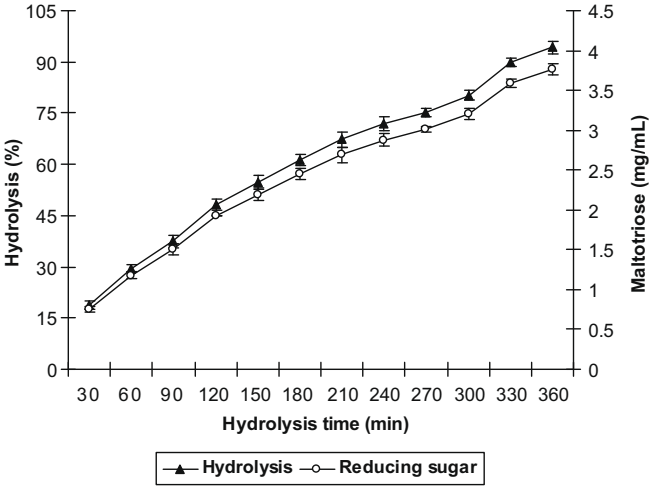

Fig. 4. Time course of sugar release from purified pullulan under agitation (150 rpm) at 50 °C by pullulanase.

4. Conclusions

More than 94% hydrolysis of pullulan by pullulanase yielding 3.77 ± 0.07 mg/mL of maltotriose in the syrup suggest it a potent substrate for the preparation of maltotriose syrup. Pure pullulanase was thoroughly characterized in terms of optimum pH and stability, temperature (thermal stability, activation energy, temperature quotient, thermal inactivation, thermodynamics of irreversible thermal inactivation), substrate affinity, kinetic characterization, effect of metal ions and effect of surfactants. Due to lower values of K_m and ΔG^*, the enzyme can prove to have greater affinity to catalyze pullulan hydrolysis. Kinetic and thermodynamic parameters of the enzyme suggested that it may be used for the preparation of maltotriose for its utilization in food processing industry.

Acknowledgement

Authors are thankful to Department of Biotechnology, Punjabi University, Patiala for providing necessary laboratory facilities to execute this work.

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