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Covalent immobilization and thermodynamic characterization of pullulanase for the hydrolysis of pullulan in batch system

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ABSTRACT

Pullulanase showed significant improvement in thermal stability after covalent immobilization on Duolite XAD761. Optimum temperature of immobilized enzyme was 60 °C, which was 10 °C higher than the free enzyme. Apparent K_m values for pullulan, soluble starch and dextran were 4.4, 20 and 50 mg mL^{-1}, respectively. Mn^{2+} and Ca^{2+} showed 2.0- and 2.2-fold increase in enzyme activity. Activation energy (*Ea*) of immobilized biocatalyst was 22.38 kJ mol^{-1}. Thermodynamic parameters (ΔH^*, ΔG^*, ΔS^*) for irreversible inactivation of immobilized pullulanase at different temperatures (60–70 °C) were also determined. *D*-value was maximum (95.95 h) at 60 °C and temperature quotient (Q_{10}) was 1.29. Immobilized biocatalyst was effectively used for pullulan hydrolysis in a batch system. Stationary phase in hydrolysis (95.70 ± 1.36%) was reached after 300 min at 125 rpm. Pullulan hydrolysis yielded 4.21 ± 0.06% reducing sugars under optimal conditions. Immobilized biocatalyst was successfully recycled for 33 batches, but the enzyme activity was reduced to half after 25th cycle.

1. Introduction

Exopolysaccharides produced by a wide variety of microorganisms, are generally water soluble gums which have novel and unique physical properties. Due to their diversity in structure and unique properties, they have a wide range of food, pharmaceutical and industrial applications. Some of these applications include their usage as emulsifiers, stabilizers, binders, gelling agents, coagulants, lubricants, film formers, thickening and suspending agents. The biopolymers are rapidly emerging as a new and industrially important source of polymeric materials which are gradually becoming economically competitive with natural gums produced from marine algae and other plants. Pullulan is a linear α-D-glucan built of 'maltosyl units', 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). 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, Saini, & Kennedy, 2008).

Pullulanase (EC 3.2.1.41) is an enzyme whose primary specificity is to hydrolyze the (1 → 6)-α-D-glycosidic linkages in pullulan to yield maltotriose. It is an industrially important enzyme, which is generally used in combination with other amylolytic enzymes (α-amylase, β-amylase, glucoamylase) in the starch processing industry for the production of sugar syrups (Abdulla & French, 1966). Moreover, it has gained significant attention as a useful tool for structural studies of carbohydrates. Pullulan serves as model substrate for amylolytic enzymes, especially those specific for α-1,6 bonds. Since the discovery of *Klebsiella pneumoniae* pullulanase by Bender and Wallenfels (1961), a number of microbial pullulanases have been purified and characterized from thermophilic bacteria by many investigators (Anju, Gupta, & Patnaik, 1992; Saha & Zeikus, 1989). Pullulanases are widely distributed among animals, plants, fungi and bacteria. Many mesophilic, thermophilic and hyperthermophilic bacteria and archae have been reported to produce pullulanase (Gomes, Gomes, & Steiner, 2003). In food industry, pullulan degrading enzymes are used in brewing process and starch hydrolysis together with β-amylase to produce starch syrup high in maltose content (Belitz & Grosch, 1999).

The specificity of enzymes and their ability to catalyze reactions make them appealing for many applications in biochemical and industrial fields. However, the recovery and re-usability of free enzymes as catalysts is quite limited and this has resulted in the development of a wide variety of immobilization techniques. Immobilization also offers some other operational advantages

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over free enzymes, such as choice of batch or continuous process, rapid termination of reactions, controlled product formation, ease of removal from the reaction mixture and adaptability to a variety of engineering designs (Brahim, Narinesingh, & Guiseppi-Elie, 2002). The economic consideration dictates the use of cheap and simple, yet effective immobilization method with carefully characterized system when scale-up is foreseen. Improvement in the stability of pullulanase as well as ability to reuse along with consecutive hydrolysis cycle or continuous mode, are the targets of considerable importance. This prompted us to investigate the immobilization of pullulanase on a suitable support. In the present investigation, commercially available pullulanase from *Bacillus acidopullulyticus* (Sigma, USA) has been immobilized on Duolite XAD761, characterized for thermodynamic and kinetic parameters and evaluated for the hydrolysis of pullulan in a batch system. Literature survey reveals no report on thermodynamic characterization of immobilized pullulanase and its operational stability for the hydrolysis of pullulan in batch system.

2. Materials and methods

2.1. Pullulan production

A. pullulans FB-1 used in the present study was maintained as reported earlier (Singh & Saini, 2008). The culture has been deposited in Microbial Type Culture Collection, Chandigarh, India and assigned Accession No. MTCC 6994. Pullulan production was carried out in shake-flasks as described earlier (Singh, Singh, & Saini, 2009).

2.2. Recovery and purification of pullulan

Pullulan recovery and purification was carried out as described previously (Singh, Saini, & Kennedy, 2009) and further used for the hydrolysis using immobilized pullulanase.

2.3. Optimization of immobilization technique

Commercially available pullulanase (Source: *Bacillus acidopullulyticus*) procured from Sigma, USA was immobilized on Duolite XAD761 (SD Fine-Chem. Ltd., India). Immobilization technique was standardized in order to get the maximum activity yield of pullulanse. Briefly, Duolite XAD761 is a hydrophobic synthetic macroporous matrix made of phenol–formaldehyde copolymer having phenolic functional groups. The resin was thoroughly and successively washed with ethanol, water, diluted hydrochloric acid (10%), diluted lye (NaOH, 1 M) and then equilibrated with sodium phosphate buffer (0.1 M, pH 5.5). To optimize the glutaraldehyde concentration and treatment time to derivitize the resin, it was gently stirred in aqueous solutions of glutaraldehyde (0.50–1.75%) at room temperature for 30–180 min. The derivitized resins were then filtered and washed thoroughly with deionised water to remove the traces of glutaraldehyde. After modification to optimize enzyme loading and enzyme coupling-time, activated resin was incubated with varied concentrations (5–11 U g^{-1} of wet resin) of pullulanase in sodium phosphate buffer (0.1 M, pH 5.5) at 30 °C, under gentle stirring for 6–32 h. Immobilized biocatalyst was recovered by filtration, thoroughly washed with deionised water and stored at 4 °C in wet state, until its further use. Immobilization yield (%) was calculated as amount of immobilized protein/amount of protein loaded × 100. Activity yield (%) of the immobilized enzyme was determined as $\{C/(A-B)\} \times 100$, where A is the pullulanase activity loaded, B is the pullulanase activity in the supernatant after immobilization and C is the pullulanase activity of the immobilized enzyme.

2.4. Characterization of immobilized pullulanase

Immobilized pullulanase was subjected to thermodynamic and kinetic characterization before its use for the hydrolysis of pullulan.

2.4.1. Effect of pH

To find out pH optima of the immobilized pullulanase, assays were carried out at different pH, using sodium acetate buffer (0.1 M, pH 3–5) and phosphate buffer (0.1 M, pH 6–7). Immobilized enzyme has also been investigated for pH stability by preincubating it at different pH (4–8) at 60 °C. Samples were withdrawn periodically and residual activity was measured in terms of relative activity, which was calculated as the percentage ratio of activity at given pH to the activity at optimal pH.

2.4.2. Effect of temperature, activation energy and temperature quotient (Q_{10})

To investigate the temperature optima, assays were carried out at different temperatures (30–70 °C). Thermal stability was examined without substrate and any additives. Immobilized enzyme was kept at 40, 45, 50, 55, 60, 65 and 70 °C in sodium phosphate buffer (0.1 M, pH 5.5) in a temperature controlled water bath and residual activity was measured from time to time in terms of relative activity.

Activation energy (Ea) has been calculated from the slope ($-Ea/2.3R$) of linear representation of $\log V_{max}$ versus $1/T$, using Arrhenius equation ($k = Ae^{-Ea/RT}$). Temperature was expressed in Kelvin, the gas constant ($R = 8.314$) in J K^{-1} mol^{-1} and the activation energy (Ea) in kJ mol^{-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 a raise in the temperature by 10 °C. Q_{10} was calculated as follows:

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

2.4.3. Thermal inactivation of immobilized biocatalyst

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. 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:

$$\log\left(\frac{A}{A_0}\right) = -\frac{t}{D}$$

Z-value is the temperature increase necessary to obtain a 10-fold decrease of the D-value. It 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 of immobilized biocatalyst

Thermal inactivation of immobilized pullulanase was determined by incubating the immobilized biocatalyst in 0.1 M phosphate buffer (pH 5.5) at varying temperatures (60, 65 and 70 °C) in the absence of substrate. Immobilized biocatalyst was withdrawn at different times, cooled and assayed for pullulanase activity. Thermodynamic parameters for thermostability were calculated by rearranging Erying's absolute rate equation derived from the transition state theory (Erying & Stearn, 1939; Stearn, 1949) as described by Siddiqui, Shemsi, Anwar, Rashid, and Rajoka (1999).

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

where h = Planck's constant = 6.63×10^{-34} J s; k_b = Boltzman's constant $(R/N) = 1.38 \times 10^{-23}$ J K^{-1}; R = gas constant = 8.314 J K^{-1} mol^{-1}; N = Avogadro's no. = 6.02×10^{23} mol^{-1}; T = absolute temperature.

$$\Delta H* = Ea - RT$$

where ΔH^* is the enthalpy of activation of denaturation and Ea is the activation energy for denaturation.

$$\Delta G* = -RT \ln\left(\frac{K_d h}{K_b T}\right)$$

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

$$\Delta S* = \frac{DH* - DG*}{T}$$

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

2.4.5. Determination of kinetic constants of immobilized biocatalyst

Affinity of immobilized pullulanase towards pullulan, soluble starch and dextran was investigated by incubating the immobilized biocatalyst with these substrates and analysing the enzyme activity. Kinetic constants (V_{max}, K_m and V_{max}/K_m) were determined from Lineweaver–Burk plots by incubating the immobilized 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 60 °C and pH 5.5.

2.4.6. Effect of metal ions, chelating agent and surfactants

To investigate the effect of metal ions, pullulanase activity of immobilized biocatalyst was 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 varying concentrations (2–10 mM).

The influence of surfactants was also investigated 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 (without any surfactant) which was taken as 100%.

2.5. Hydrolysis of pullulan using immobilized biocatalyst

Purified pullulan produced from *A. pullulans* FB-1 was used for hydrolysis. Operational parameters like agitation, hydrolysis time and stability were studied for the hydrolysis of pullulan in a batch system. Purified pullulan (0.44%) was incubated with immobilized pullulanase (4 U) under agitation (50–150 rpm) for different time intervals (30–300 min) at 60 °C. The reducing sugars released were quantified and the extent of hydrolysis (%) was calculated as under:

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

2.5.1. Operational stability of immobilized biocatalyst in batch system

Immobilized pullulanase (4 U) was added to 50 ml of 0.44% pullulan and incubated at 60 °C, under shaking (125 rpm) for 5 h. After the completion of each batch, immobilized biocatalyst was recovered, washed thoroughly with sodium phosphate buffer (0.1 M, pH 5.5) and recycled for a new bioconversion run. Spent broth from each cycle was analyzed for reducing sugars and the extent of hydrolysis was also calculated for each run.

2.6. Pullulanase activity

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. 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. Statistical analysis

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

3. Results and discussion

3.1. Pullulanase immobilization

Commercially available pullulanase (Source: *Bacillus acidopullulyticus*) was successfully immobilized on Duolite XAD761 via Schiff's base (aldimine) linkage between carbonyl activated support and free amino groups on the protein. Glutaraldehyde (0.50–1.75%) was used for the pre-activation of the resin. It is clear from Fig. 1a, as the concentration of the cross-linking agent was increased up to 1.25%, corresponding activity yield was also increased and after this, a decline in this function was observed. At this concentration, the activity yield and immobilization yield was 41.99 ± 1.0% and 86.13 ± 1.42%, respectively. It has been reported that aldimine (Schiff's base) bonds between enzyme and glutaraldehyde are irreversible and survive extremes of pH and temperature (Kennedy & Cabral, 1987). The most important advantage of the covalent binding of immobilization is that one bi- or multi-functional reagent can be utilized to activate the resin. Glutaraldehyde being one of the most extensively used cross-linking agents was selected for the activation of the resin. The decrease in activity yield and immobilization yield beyond 1.25% concentration of glutaraldehyde used for activation of resin may be attributed to the reaction with the active site or group that directly affects it. Moreover, glutaraldehyde is a denaturant for enzymes and its concentration beyond a certain level for activation of a resin may cause toxic effect, thus inhibiting enzyme activity. The glutaraldehyde concentration in the

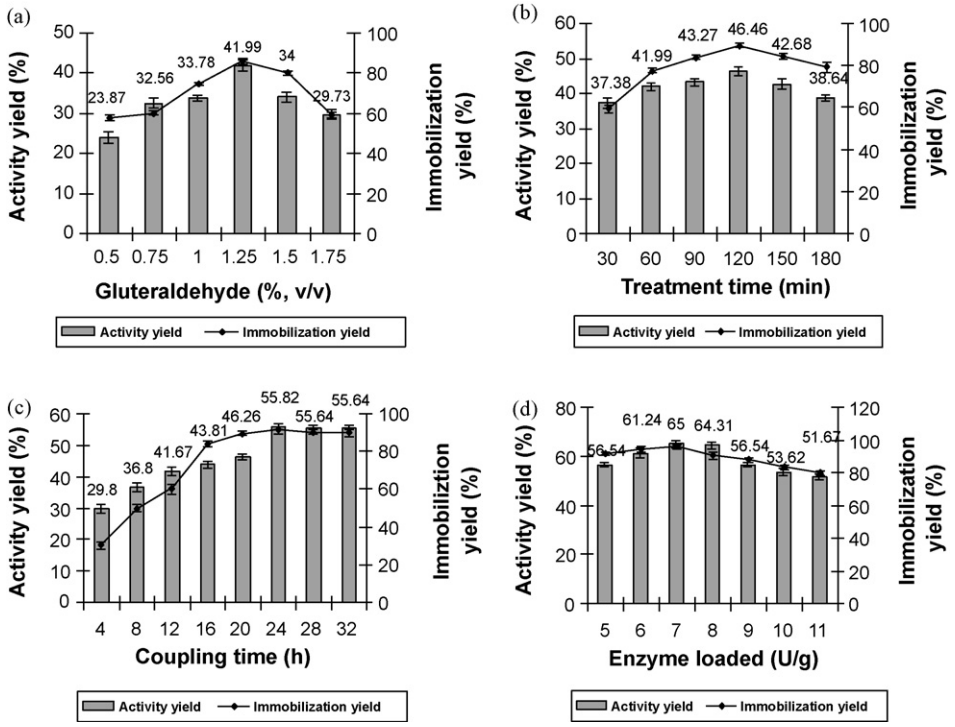

Fig. 1. Activity yield and immobilization yield of pullulanase immobilized on Duolite XAD761 with (a) glutaraldehyde concentration (b) glutaraldehyde treatment time (c) enzyme coupling-time and (d) enzyme loading as a function.

reaction during activation of resin increases the yield of immobilization activity to a maximum and then declines. Generally, best activation yields are reported with glutaraldehyde concentration of 0.30–0.60% (Kennedy & Cabral, 1987). The decrease in activity yield and immobilization yield after the treatment of resin with glutaraldehyde for 120 min may be due to the severe toxic effects left after long time treatment of the matrix. The results depicted in Fig. 1b shows the activation time of 120 min were optimal for immobilization in terms of activity yield and immobilization yield. At this treatment time, the activity yield was $46.46 \pm 1.32\%$ and the corresponding immobilization yield was $89.65 \pm 1.06\%$. An increase in activity yield was observed with increase in coupling-time up to 24 h and after this a slight decrease was recorded (Fig. 1c). Activity yield and immobilization yield obtained after 24 h of coupling-time were $55.82 \pm 0.88\%$ and $91.38 \pm 1.92\%$, respectively. No improvement in activity yield after 24 h of coupling-time may be due to saturation of the resin with enzyme. All the binding sites of the resin may be occupied by the enzyme. Different amounts (5–11 U) of enzyme were added to every portion of the pre-activated resin and incubated for 24 h. The activity yield of immobilized pullulanase increased with increase in concentration of enzyme up to 7 U (Fig. 1d). Activity yield as well as immobilization yield decreased beyond this concentration. Activity yield of $65.0 \pm 1.65\%$ and immobilization yield $95.82 \pm 1.38\%$ were recorded at this optimal concentration. Generally, the immobilization of higher concentration of the enzymes on supporting materials is not recommended. The higher amount of enzyme fixed on the support may result in intermolecular space hindrance which restrains the diffusion of substrate and product (Liang, Zihua, Tong, Qiang, & Jia, 2003). After standardization of immobilization technique, the final immobilized system contained $3.1\ U\ g^{-1}$ (wet weight) with $65.00 \pm 1.65\%$ recovery yield. It is observed that activity recovery was reduced after immobilization. The reduction in the activity may be attributed to mass diffusion limitations due to high molecular weight of pullulan, conformational changes of enzyme molecule or modification of amino acids present in the active sites (Nakamura, Ogata, Shitara, Nakamura, & Ohta, 1995). *Bacillus acidopullulyticus* pullulanase has been reported to be immobilized by adsorption on porous glass (PG-Pase), covalent binding on chitosan beads treated with glutaraldehyde (GA-CB-Pase) and ionic binding on Amberlite IRC-50 (IRC-Pase) with high pullulanase activities (Kusano, Shiraishi, Takahashi, Fujimoto, & Sakano, 1989).

3.2. Characterization of immobilized pullulanase

3.2.1. Effect of pH

Immobilized pullulanase showed the pH optima of 5.5 and was slightly higher than that of free pullulanase. Furthermore, pH stability studies showed that the enzyme retained more than 85% of its initial activity after its incubation in pH 5.0–6.5 for 6 h. pH activity profiles of a biocatalyst reflect the pH at which important proton donating or proton accepting groups in the enzyme catalytic site are in their required state of ionization. The stability of immobilized pullulanase over a wide range of pH is better especially when there is variation in pH during processing. An increase in pH stability after immobilization has been attributed to the electrostatic interaction between positively charged support and the protein resulting in intramolecular salt bridges responsible for maintenance of active conformation of enzyme (Kilinç, Önal, & Telefoncu, 2002).

3.2.2. Effect of temperature, activation energy and temperature quotient (Q_{10})

The optimum temperature for immobilized pullulanase at pH 5.5 was found to be 60 °C, which was 10 °C higher than that of free enzyme (50 °C). After immobilizing pullulanase covalently onto Duolite XAD761, significant improvement in thermal stability was observed. Comparing the enzyme in its free state (Singh, Singh, et al., 2009), immobilized biocatalyst has shown a remarkable difference. It retained almost 85% of its original activity after incubation at 65 °C for 6 h (Fig. 2). At 70 °C, it was found to be 32.38% active till the end of 3 h, but its counterpart was rapidly inactivated. Immobilized pullulanase showed higher temperature

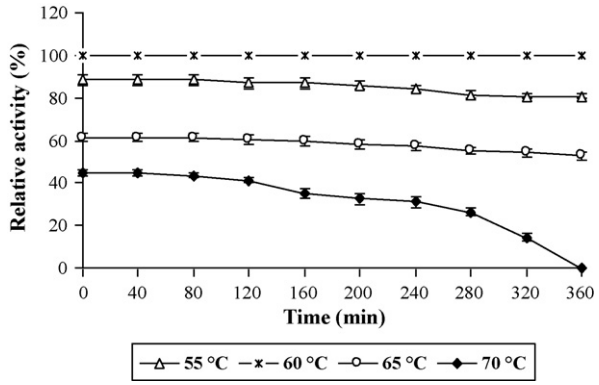

Fig. 2. Thermal stability of immobilized pullulanase.

optima (60 °C) and considerably higher thermal stability. Pullulan hydrolysis at higher temperature is advantageous to increase the reaction velocity. Immobilization of pullulanase on Duolite XAD761 caused an increase in enzyme rigidity which is commonly reflected by increase in stability towards denaturation by raising the temperature. The covalent linking methods usually lead to enzyme preparations with improved thermal stabilities. This may be attributed to the fact that immobilization limits the thermal movement of the enzyme as temperature increases, consequently resulting in a decrease in denaturation. The enhancement in thermal stability of pullulanase due to immobilization has been reported earlier also (Kuroiwa, Shoda, Ichikawa, Sato, & Mukataka, 2005; Kusano et al., 1989).

Activation energy for immobilized pullulanase was estimated using Arrhenius equation and plotting a graph between log V_{max} and $1/T$ (K) whose slope equals to $-Ea/R$. Arrhenius plot was linear (Fig. 3). Activation energy calculated for immobilized pullulanase was 22.38 kJ mol^{-1}, which was lower than that of free enzyme (34.29 kJ^{-1} mol) as reported earlier (Singh, Saini, et al., 2009), indicating that the reaction is kinetically controlled. Hence, immobilization improved the quality of pullulanase by lowering down the energy required to make the activated complex (ES). This has also been observed earlier (Kusano et al., 1989).

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. Temperature quotient for immobilized pullulanase was 1.29.

3.2.3. Thermal inactivation of immobilized biocatalyst

Thermal inactivation of immobilized pullulanase was studied in the range of 55–70 °C and the inactivation parameters are summarized in Table 1. Thermal inactivation process of immobilized enzyme corresponds well to the simple first-order reaction. *D*-values of immobilized pullulanase ranged between 1.92 and 95.95 h at the temperatures studied. Pullulanase presented low thermal deactivation rate indicating highest stability at 60 °C with a *D*-value of 95.95 h. *Z*-value (extrapolated from *D*-value) was found to be 5.88 °C for immobilized 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.

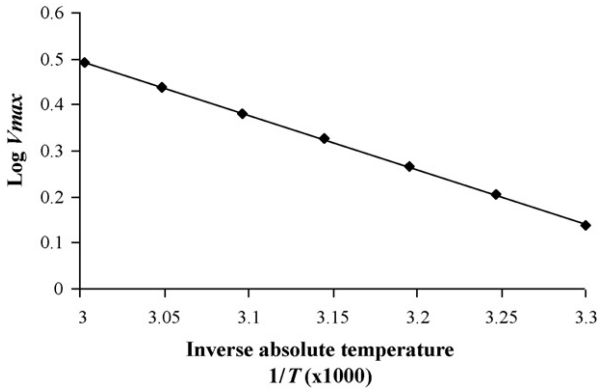

Fig. 3. Activation energy of immobilized pullulanase.

Table 1
Kinetic parameters of thermal inactivation for immobilized pullulanase.

T (°C)	A[a] (U g^{-1})	k[b] (h^{-1})	D[c] (h)	$t^{1/2}$ (h)[d]
55	2.74	0.184	12.52	3.77
60	3.05	0.024	95.95	28.87
65	1.90	0.731	3.15	0.95
70	1.39	1.197	1.92	0.58

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

3.2.4. Thermodynamics of irreversible thermal inactivation of immobilized biocatalyst

Activation energy for irreversible inactivation '*Ea*(*d*)' of the immobilized pullulanase as determined by applying Arrhenius plot and Gibbs free energy (ΔG^*) for activation of thermal unfolding of immobilized enzyme was 114.85 kJ mol^{-1} at 60 °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 60 °C was 19.61 kJ mol^{-1}. Its value remained almost same up to 70 °C. The entropy of activation (ΔS^*) for unfolding of transition state of pullulanase was found to be −286.01 J mol^{-1} (Table 2). With an increase in temperature, a decrease in free energy was observed. The enthalpy of activation of thermal unfolding (ΔH^*) of the enzyme at 60 °C was 19.61 kJ mol^{-1}. Its value remained almost same up to 70 °C. The entropy of activation (ΔS^*) for unfolding of transition state of pullulanase was found to be −286.01 J mol^{-1}. Similar results have been documented for α-amylase from *Bacillus licheniformis* (Violet & Meunier, 1989) and carboxymethylcellulase from *Aspergillus niger* (Siddiqui, Saqib, Rashid, & Rajoka, 2000) possessing negative entropy indicating that the transition states of these two enzymes were more ordered than the ground states. The stabilization of enzyme upon immobilization is accompanied by lower ΔS^* and ΔH^* values. This shows that the increased thermostability of immobilized enzyme is due to a conformational change at higher temperatures and the conformation of the modified enzyme was altered in the direction of partially unfolded transition state or hydrophobic core of immobilized proteins decreases with increase in thermostability. 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 (Nosoh & Sekiguchi, 1990). The opening up of the enzyme structure is accompanied by an increase in the disorder, randomness or entropy of activation (Vieille & Zeikus, 1996).

3.2.5. Substrate specificity and kinetic constants

Immobilized pullulanase was active on pullulan, soluble starch and dextran. It showed 59.45% and 23.26% relative activity on soluble starch and dextran with respect to pullulan, which was considered 100%. There was no pronounced effect on substrate specificity after immobilization. Effect of different concentrations of pullulan, soluble starch and dextran was further investigated and results were plotted using Lineweaver–Burk plot. Minimum

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

T (°C)	T (K)	k_d[a] (h^{-1})	k_d[a] (s)	$t^{1/2}$ (h)	ΔH^*[b] (kJ mol^{-1})	ΔG^*[c] (kJ mol^{-1})	ΔS^*[d] (J mol^{-1})
60	333	0.024	6.67×10^{-6}	28.87	19.61	114.85	−286.01
65	338	0.731	2.03×10^{-4}	0.95	19.57	107.00	−258.67
70	343	1.197	3.32×10^{-4}	0.58	19.53	107.22	−255.65

[a] K_d = first-order rate constant for inactivation.
[b] ΔH^* (kJ mol^{-1}) = Ea (22.38 kJ/mol) − RT, where Ea is activation energy.
[c] ΔG^* (kJ mol^{-1}) = $-RT\ln\{(k_d h)/k_b T)\}$.
[d] ΔS^* (J mol^{-1}) = entropy of irreversible inactivation and was calculated from $\Delta S = (\Delta H^* - \Delta G^*)/T$.

apparent K_m was observed for pullulan (4.40 mg mL^{-1}) as compared to soluble starch (20.0 mg mL^{-1}) and dextran (50 mg mL^{-1}), suggesting greater affinity of enzyme for this substrate. Immobilized pullulanase was active on pullulan, soluble starch and dextran. No change in the substrate specificity of pullulanase from *Bacillus acidopullulyticus* has been reported after immobilization (Kusano et al., 1989). Minimum apparent K_m was observed for pullulan (4.4 mg mL^{-1}) as compared to soluble starch (20 mg mL^{-1}) and dextran (50 mg mL^{-1}), suggesting greater affinity of enzyme for this substrate. It was observed that K_m for immobilized pullulanase was slightly changed from their respective values in the free counterpart, which may be ascribed to the conformational changes of the enzyme molecules or steric hindrances due to immobilization. The observed differences between free (Singh, Saini, & Kennedy, 2010) and immobilized pullulanase kinetics can be explained in terms of the structural change caused by binding of the enzyme onto the polymeric support. The maximum reaction velocities (V_{max}) of immobilized enzyme were 4.0 (U g^{-1}), 4.17 (U g^{-1}) and 5.00 (U g^{-1}) for pullulan, soluble starch and dextran, respectively. These values were higher than the free enzyme (Singh et al., 2010), which suggests that the immobilized pullulanase was more active than the native form. Ratios between V_{max} and K_m for pullulan, soluble starch and dextran were 9.09, 2.08 and 1.00, respectively. The highest V_{max}/K_m ratio for pullulan shows that it is the best substrate for pullulanase followed by soluble starch and dextran. The increase in K_m value after immobilization may be partially due to distortion of the active site causing the bonds between substrate and the active sites of the enzyme to be less efficient (Dessouki, Issa, & Atia, 2001).

3.2.6. Effect of metal ions, chelating agent and surfactants

The effect of metal ions on immobilized enzyme system was studied using different ions at varying concentration (data not shown). Maximum increase in enzyme activity of 2.2 times and 2.0 times was observed on the addition of Ca^{2+} and Mn^{2+}, respectively as compared to control, where no metal ions were added. A strong inhibitory effect on activity was observed in the presence of Zn^{2+}, Ba^{2+} and EDTA, they inhibited the activity of immobilized enzyme by 22%, 21% and 43%, respectively of its original activity. Hg^{2+} and Ni^{2+} were found to be potent inhibitors and these ions completely inhibited the activity even at the lower concentrations of 2 mM. The increase in pullulanase activity by the addition of Ca^{2+} and Mn^{2+} indicates that these metal ions are required for better catalytic action. Metal ions like mercury, cadmium and lead react with the protein thiol groups (converting them to mercaptides), as well as with histidine and tryptophan residues. Moreover, silver and mercury are responsible for hydrolytic degradation of disulfide bonds responsible for maintenance of protein structure (Kumar, Tiwari, & Jany, 1999). None of the surfactants investigated had shown enhancement of pullulanase activity (data not shown).

3.3. Hydrolysis of pullulan in batch system using immobilized biocatalyst

Hydrolysis of purified pullulan (0.44%, w/v) in sodium phosphate buffer (0.1 M, pH 5.5) was carried out in a batch system using immobilized pullulanase (4 U) under stationary and agitation (50–200 rpm) at 60 °C and the results are presented in Table 3. The hydrolysis of pullulan was recorded better under agitation mode as compared to the stationary condition. Low level of pullulan hydrolysis observed at stationary mode may be attributed to the more diffusional limitations in this mode of operation. Agitation had shown a greater impact on hydrolysis with immobilized pullulanase. A comparative analysis of percent hydrolysis at each agitation speed showed that maximum hydrolysis was attained at 125 rpm and 300 min of hydrolysis time. Furthermore, reducing sugars produced corresponding to the percent hydrolysis at this mode of agitation was observed with respect to time (Fig. 4). A stationary phase in hydrolysis by immobilized pullulanase was reached after 300 min at 125 rpm; hence it was considered optimal hydrolysis time. Pullulan hydrolysis yielded 4.21 ± 0.06% reducing sugars as maltotriose equivalents, under these optimized conditions. Agitation had a greater impact on hydrolysis with immobilized pullulanase. A comparative analysis of percent hydrolysis at each agitation speed showed that

Table 3
Hydrolysis of pullulan by immobilized pullulanase at 60 °C with agitation and time-course as functions.

Time (min)	Control[a]	Hydrolysis (%) under agitation (rpm)						
		50	75	100	125	150	175	200
30	5.85 ± 1.41	13.49 ± 1.45	15.25 ± 1.41	17.00 ± 2.52	18.88 ± 1.46	16.23 ± 1.35	13.27 ± 2.05	12.04 ± 2.27
60	15.29 ± 1.43	25.00 ± 1.28	28.00 ± 2.78	30.66 ± 1.20	29.43 ± 1.66	24.46 ± 2.16	20.40 ± 1.75	16.14 ± 2.38
90	26.00 ± 2.09	36.90 ± 1.62	39.94 ± 1.61	41.31 ± 1.42	42.00 ± 2.08	38.51 ± 1.78	34.37 ± 1.84	23.61 ± 2.81
120	32.68 ± 1.98	43.44 ± 2.44	45.39 ± 1.56	49.90 ± 1.21	49.94 ± 1.55	46.60 ± 1.61	41.69 ± 1.16	33.41 ± 0.87
150	43.14 ± 2.49	48.60 ± 1.46	51.00 ± 2.09	56.70 ± 1.57	54.17 ± 1.17	50.75 ± 1.13	49.90 ± 1.03	40.68 ± 1.67
180	49.23 ± 0.52	54.25 ± 2.12	55.82 ± 4.31	61.45 ± 1.83	59.99 ± 1.55	57.07 ± 2.29	57.21 ± 1.94	51.14 ± 1.97
210	53.00 ± 2.78	62.07 ± 1.19	63.91 ± 1.82	68.00 ± 2.63	67.33 ± 1.46	63.82 ± 1.61	61.89 ± 1.19	58.39 ± 3.92
240	60.31 ± 2.41	66.11 ± 2.07	72.68 ± 0.89	76.52 ± 1.77	76.29 ± 1.00	72.37 ± 1.81	69.00 ± 1.41	67.73 ± 1.41
270	69.75 ± 1.28	72.24 ± 2.01	77.23 ± 2.07	85.91 ± 1.02	83.50 ± 0.87	80.00 ± 1.82	80.22 ± 2.36	78.64 ± 1.19
300	75.00 ± 2.54	80 27 ± 2.10	84.00 ± 1.45	90.33 ± 1.16	95.70 ± 1.36	92.25 ± 2.39	89.45 ± 0.79	84.54 ± 1.91

[a] Hydrolysis (%) with respect to time-course under stationary condition.
Immobilized biocatalyst having 4 U of pullulanase was used for hydrolysis of pullulan (0.44%, w/v) in sodium acetate buffer (0.1 M, pH 5.5).
Data is expressed as mean ± S.E.M.

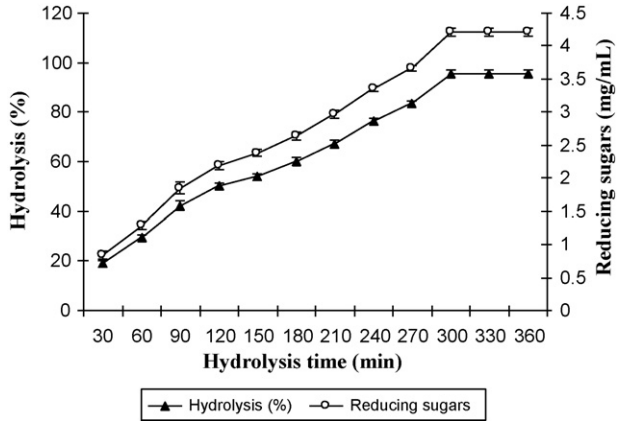

Fig. 4. Profile of hydrolysis (%) of pullulan and reducing sugars produced by immobilized biocatalyst with respect to time-course at 60 °C under agitation (125 rpm).

maximum hydrolysis was attained at 125 rpm and 300 min hydrolysis time. Pullulan hydrolysis yielded 4.21 ± 0.06% reducing sugars under optimized conditions. Ohtani, Ishidao, Iwai, and Arai (1999) reported pullulan hydrolysis by pullulanase immobilized on NIPA gel and the hydrolysis rate by immobilized pullulanase decreased with increasing molecular weight of pullulan. Pullulan, which can enter the gel, is hydrolyzed randomly. However, pullulan, which cannot enter the gel, is hydrolyzed from the end groups, which can partly enter the gel.

3.3.1. Operational stability of immobilized biocatalyst in batch system

The reutilization of the immobilized biocatalyst has been investigated using purified pullulan. The profiles of percent hydrolysis, residual enzyme activity and reducing sugars produced after each batch are given in Fig. 5. There was approximately 10% loss of immobilized pullulanase activity at 5th cycle and approximately 20% loss was recorded after 12th cycle. After this, the system was stable to a good extent. The immobilized system was repeatedly used for 33 batches and the enzyme activity was reduced to half after 25th batch. The decrease in hydrolysis observed in the initial batches may be due to the loss of loosely bound enzyme especially during washing of the biocatalyst. No report in literature has been cited till date on operational stability of immobilized pullulanase in a batch system.

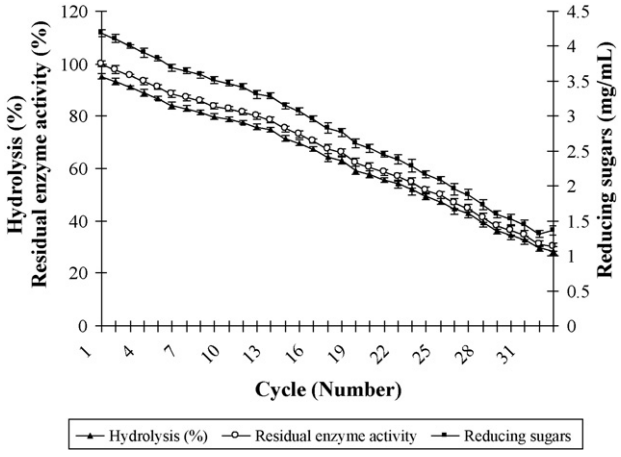

Fig. 5. Operational stability of immobilized biocatalyst for the hydrolysis of pullulan at 60 °C in a batch system.

4. Conclusions

Covalently linked pullulanase to Duolite XAD761 via glutaraldehyde had good capacity and stability. Improvement in optimum temperature as well as thermal stability has been achieved after immobilization of pullulanase on Duolite XAD761. Immobilization also increased pH stability of pullulanase, which is an essential characteristic of an enzyme to be employed in food industry. The recycling of immobilized biocatalyst has shown a good operational and mechanical stability suggesting it an effective system for pullulan hydrolysis.

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