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Hydrolysis of soluble starch using *Bacillus licheniformis* α-amylase immobilized on superporous CELBEADS

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Abstract—In the present work, indigenously prepared rigid superporous (pore size of approximately 3 μm) cross-linked cellulose matrix (CELBEADS) has been used as a support for the immobilization of *Bacillus licheniformis* α-amylase (BLA). Optimum pH and temperature, and Michaelis–Menten constants were determined for both free and immobilized BLA. Immobilized BLA was observed to produce a different saccharide profile than free BLA at any value of dextrose equivalent. It was observed that pH, temperature, and initial starch concentration has a significant effect on the saccharide profile of starch hydrolysate produced using immobilized BLA in the batch mode, whereas the ratio of concentration of enzyme units to initial starch concentration has no influence on the same. Hence immobilized BLA can be used as an additional tool for production of maltodextrins with different saccharide profiles. Immobilized BLA has better thermostability than free BLA. Immobilized BLA was found to retain full activity even after eight batches of hydrolysis, each of 8 h duration at 55 °C and 90 mg/mL initial starch concentration. A semi-empirical model has been used for the prediction of saccharide composition of starch hydrolysate with respect to time.

Keywords: Amylase; Immobilization; Hydrolysis; Starch; Maltodextrins

1. Introduction

Starch has become a very important biopolymer and is used in many industries as a feedstock material. The sweetener and fermentation industries are two of the main consumers of starch. Nutritive sweeteners are mainly starch hydrolysis products, namely maltodextrins,[†] high-maltose syrup, maltose, glucose syrup, and glucose (dextrose), all of which are used in food and pharmaceutical industry. The term maltodextrins is used for the saccharide mixtures of dextrose equivalent (DE) less than 20 that consist of maltose, malto-oligosaccharides, and linear or branched dextrins.[1] Maltodextrins with the same average DE value can have different saccharide composition.[2] The DE value of a maltodextrin has been shown to be inadequate to predict product performance in various applications.[3] The saccharide composition of maltodextrins determines both its physical and biological functionality, and there are different parameters (such as the type and source of the enzyme, the source of starch, starch concentration, temperature, organic solvents, immobilization of enzymes, and downstream processing) influencing the saccharide composition of maltodextrins.[4] Design of the desired saccharide composition and production possibilities for maltodextrins have been briefly discussed.[4] Maltodextrins are used as bulking agents, texture providers, spray-drying aids, flavor encapsulating aids, fat replacers, tablet excipients, film formers, sport beverages, as well as parenteral, and enteral nutrition products.[1]

Though partial hydrolysis of starch has traditionally been carried out using acids, acid hydrolysis is being replaced by enzymatic hydrolysis for the production of tailor-made maltodextrins.[4,5] The most widely used enzymes for production of maltodextrins using partial hydrolysis of starch are α-amylase from *Bacillus licheniformis*, *Bacillus stearothermophilus*, and *Bacillus*

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† The term maltodextrin (as well as dextrin) is not IUPAC-approved terminology but is used here to describe starch-derived materials commonly encountered in the starch processing industry.

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amyloliquefaciens. There are several attempts[6–11] to immobilize bacterial α-amylase and apply it to hydrolyze starch. Reasons for a different saccharide composition with immobilized enzyme compared to free enzyme can be attributed to diffusion limitations, which increase with the degree of polymerization (DP) of oligosaccharide, enhancing heterogeneous hydrolysis.[7] Immobilization alters the three-dimensional structure of the enzyme, which causes changes in its affinity toward the substrates, thus increasing its product specificity.[9] Small size pores (<0.1 μm) in typical matrix supports offer high diffusional resistance to the large starch molecules and limit their accessibility to active immobilized enzyme sites inside the pores, which leads to low reaction rates. However, these diffusional resistances can be overcome or significantly reduced by use of matrix supports with large pore diameters (70–80 nm; 7–550 nm; ~5000 nm)[6,12,10] for the immobilization of enzymes. Hence in the present work *B. licheniformis* α-amylase (BLA) is immobilized on superporous (pore diameter ~3 μm) CELBEADS, in order to minimize diffusional resistances.

It is reported[13] that there is no significant influence of pH (in the range of 5.1–7.6) and significant influence of temperature on saccharide profile of starch hydrolysate produced using free BLA (Maxamyl). But there is no literature available on the effect of pH, temperature, and initial starch concentration on the saccharide composition of starch hydrolysate produced using immobilized bacterial amylase. Hence, in the present work, the effect of different parameters like pH, temperature, initial starch concentration, and ratio of concentration of enzyme units to initial starch concentration on the saccharide profile of starch hydrolysate produced using immobilized BLA has been also studied in a batch mode. Thermostability and reusability of immobilized BLA was also studied. Also, a semiempirical model has been used for a priori prediction of saccharide composition of starch hydrolysate with respect to time.

2. Experimental

2.1. Materials

3,5-Dinitrosalicylic acid (DNSA), soluble starch, maltose, dextrose, MeCN for chromatography LiChrosolv and other chemicals were purchased from E. Merck Ltd (India). *B. licheniformis* α-amylase (BLA) (EC Number 3.2.1.1) was provided as a gift by Advance Enzyme Technologies Pvt Ltd (India). CELBEADS, a rigid superporous cross-linked cellulose matrix, were prepared indigenously according to a patent[14] and made available for the present work. Properties of the CELBEADS are given in Table 1.

2.2. Methods

2.2.1. Measurement of protein concentration and reducing sugar concentration. The protein concentration of the free enzyme was determined using the Folin–Lowry method[15] using BSA (0–0.6 mg/mL) as a standard. The reducing sugar concentration was measured using the DNSA method[16] with dextrose (0–1 mg/mL) as a standard. The theoretical dextrose equivalent (DE) of the starch hydrolysate is defined as the following:

$$\mathrm{DE} = \frac{\text{mol wt of anhyd glucose, that is, 180.6}}{\text{number-averaged mol wt of starch hydrolysate } (162 \times n + 18)} \times 100 \tag{1}$$

where n is the average degree of polymerization (DP) of starch hydrolysate, which can be calculated by the following formula:

$$n = \frac{\text{concn of reducing sugar (glucose equiv) after complete hydrolysis using amyloglucosidase}}{\text{concn of reducing sugar (glucose equiv) in starch hydrolysate}}$$

By using the above formula, the DE of dextrose, maltose, and starch can be calculated to be 100, 53, and 0, respectively. The values of DE reported later in the text are those calculated using Eq. 1.

2.2.2. HPTLC analysis. Oligosaccharide separation of starch hydrolysate samples was performed using 20 cm × 10 cm TLC sheets (Silica Gel 60, E. Merck Ltd, India). The samples were applied to the TLC sheet (prewashed with MeOH) using applicator AS 30 (DESAGA, Heidelberg, Germany), equipped with a 10-μL microsyringe (Hamilton, Switzerland). Best resolution

Table 1. Properties of CELBEADS[14]

Properties	Description
Mean bead size	200 μm (100–350 μm)
Spherecity	0.7–0.9
Nature	Rigid aerogel
Average pore size	~3 μm
Total volume porosity	~57%
Bulk density (water)	1438 kg/m^3

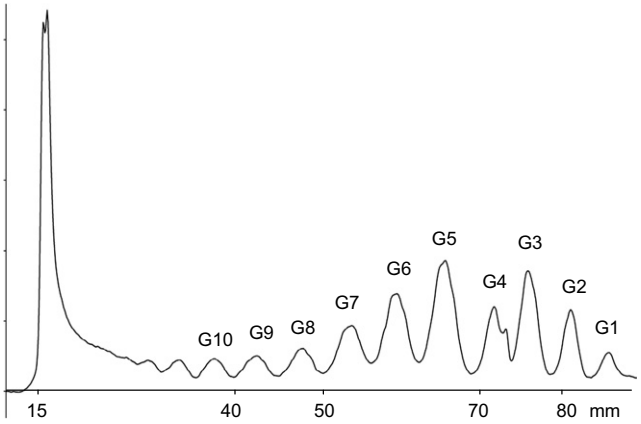

Figure 1. Densitogram of starch hydrolysate of DE 10 produced using immobilized BLA at 55 °C, pH 5.2 and $[S]_0 = 90$ mg/mL. G1 is Glucose and G2–G10 are malto-oligosaccharides with DP 2–10, respectively.

was obtained by triple development. A mobile phase of 70:30 MeCN–0.02 M Na_2HPO_4 was used for the 1st and the 2nd development; and a mobile phase of 80:20 MeCN–0.01 M Na_2HPO_4 was used for the 3rd development. The TLC sheet, after triple development, was stained by dipping for 4 s in a diphenylamine–aniline–phosphoric acid reagent (40 mL acetone, 0.8 g diphenylamine, 0.8 mL aniline, and 6 mL 85% H_3PO_4), followed by heating to 120 °C for 10 min. Densitometry (sample densitogram is shown in Fig. 1) was performed using HPTLC densitometer CD 60 (DESAGA, Heidelberg, Germany) and computer. Samples of starch hydrolysate were quantified by use of external standards of glucose (G1) and maltose (G2) from E. Merck India Ltd and maltotriose (G3), maltotetraose (G4), maltopentaose (G5), maltohexaose (G6), and maltoheptaose (G7) from Sigma Chemical Co. (St Louis, MO, USA). Concentrations of malto-octaose (G8), maltononaose (G9), and maltodecaose (G10) in starch hydrolysate reported later in the text are those that were determined by using the standard curve of G7.

2.2.3. Immobilization of BLA on CELBEADS. Surface hydroxyl groups of the CELBEADS were activated using epichlorohydrin (ECH), while ethylenediamine (EDA) was used as a spacer arm to prevent the possible steric hindrances between the immobilized enzyme and the substrate. Activation and coupling procedures[10,17] were followed for immobilization. CELBEADS (10 mL) were washed with 200 mL of distilled water and suction dried to give a moist cake on a sintered glass funnel. The wet matrix was then added to a conical flask containing 2 M NaOH (34.5 mL), $NaBH_4$ (0.1275 g), and ECH (3.75 mL). To this flask another 2 M NaOH (34.5 mL) and ECH (17 mL) were added in small portions over a period of 2 h under mild stirring. The flask mixture was shaken on an orbital shaker overnight at room temperature. The matrix was then filtered on a sintered glass funnel and washed extensively with 200 mL each of 0.1 M HOAc, 0.2 M $NaHCO_3$ and distilled water sequentially. The washed and suction-dried epoxy-activated matrix (ECH–CELBEADS) was then added to a flask containing a mixture of 0.2 M $NaHCO_3$ (22.5 mL) and EDA (15 mL). The mixture was shaken at 50 °C on an orbital shaker for 24 h. The resulting matrix (ECH–EDA–CELBEADS) was filtered and washed successively with 200 mL each of 0.1 M HOAc, 0.2 M $NaHCO_3$ and distilled water. The matrix (ECH–EDA–CELBEADS) was then further activated overnight for enzyme conjugation using 30 mL of 12.5% w/v aq glutaraldehyde (GA). The activated matrix (ECH–EDA–GA–CELBEADS) was washed well with distilled water to remove traces of glutaraldehyde. The activated matrix was mixed with 20 mL of 100-fold diluted solution (15 mg protein) of BLA in 0.1 M phosphate buffer (pH 7.5) and kept under shaking conditions overnight at 5 °C. After immobilization, $NaBH_4$ (0.08 g) was added and kept under shaking conditions for an additional 30 min. The enzyme-immobilized matrix was filtered on a sintered glass funnel and then sequentially washed with 200 mL each of phosphate buffer, 1 M NaCl solution and distilled water. The protein concentration and free enzyme units in the supernatants and washes were determined. Amount of protein and free enzyme units immobilized on the matrix were calculated by material balance.

2.2.4. Amylolytic activity measurement

2.2.4.1. Free BLA. Soluble starch was added to 0.1 M acetate buffer (pH 5.6) to give a concentration of 9 mg/mL. The mixture was then gelatinized in a stoppered conical flask by heating in boiling water for 6 min. A mixture of 0.5 mL of the gelatinized starch solution (9 mg/mL), 1.4 mL of acetate buffer (0.1 M, pH 5.6), and 0.1 mL of 10,000-fold diluted free BLA was incubated at 55 °C (optimum enzyme activity temperature, found separately) in a water bath for 20 min. The reaction was stopped by adding 1 mL of DNSA reagent. The variation in the concentration of reducing sugar was measured by the DNSA method using dextrose as a standard. One free enzyme unit (FEU) was defined as that required to liberate one micromole of reducing sugar (glucose equiv) per min under conditions of assay.

2.2.4.2. Immobilized BLA. Gelatinized starch solution (25 mL) of concentration 90 mg/mL (0.1 M acetate buffer, pH 5.2) was incubated with 1 mL immobilized BLA at 55 °C for 1 h, and samples were withdrawn initially and finally. Reducing sugar concentration (glucose equiv) of the samples was measured, and the immobilized enzyme units per mL of CELBEADS were determined. One immobilized enzyme unit (IEU) was defined as that required to liberate one micromole of

reducing sugar (glucose equiv) per min under the conditions of assay. Retained enzyme activity is defined as the ratio of specific activity of immobilized enzyme to specific activity of free enzyme (calculated by the assay procedure of immobilized BLA).

2.2.5. Measurement of kinetic constants of free and immobilized BLA. Gelatinized starch solutions (25 mL each) were prepared in 0.1 M acetate buffer (pH 5.2 for immobilized BLA and pH 5.6 for free BLA) of different initial starch concentrations $[S]_0$ varying in the range of 9–45 mg/mL. These were mixed with the enzyme at suitable concentrations (for immobilized BLA, [IEU] = 0.5 and for free BLA, [FEU] = 0.66 i.e., [IEU equiv] = 0.738) and incubated separately at 55 °C for 2 h. The reactions were carried out on a shaker at 180 rpm. Samples of starch hydrolysate were withdrawn at regular time intervals of 0.5 h and analyzed for reducing sugar concentration. The initial reaction rate (V) was calculated from the slope of the linear part of the reducing sugar concentration versus time plot at all initial starch concentrations for both immobilized and free BLA. Kinetic constants (K_m and V_{max}) were determined for free and immobilized BLA from the Eadie–Hofstee plot of V versus $V/[S]_0$ with $-K_m$ as slope and V_{max} as the y-intercept. Since V_{max} is not a fundamental property of the enzyme and is dependent on the enzyme concentration, it was converted to the turnover number, k_{cat} (i.e., V_{max}/[IEU] for immobilized BLA and V_{max}/[IEU equiv] for free BLA).

2.2.6. Hydrolysis of soluble starch using immobilized BLA in batch mode. Suspension of soluble starch at desired concentration (mg/mL) was prepared with 0.1 M acetate buffer (desired pH) and then gelatinized in a stoppered conical flask by heating in boiling water for 6 min. Immobilized BLA was added to the freshly prepared gelatinized starch solution to have a desired $[IEU]/[S]_0$ and kept at desired temperature for 8 h on the shaker at 180 rpm. A rotational speed of 180 rpm was selected using the following criterion: (1) All beads should always be in the suspended form throughout the batch. (2) The selected speed should be in such a range, at which there is no dependence of the hydrolysis curve on the rotational speed (which is observed to be beyond the speed of 150 rpm). At regular time intervals (0.5 h up to a reaction time of 3 h, and then every 1 h until the end of batch hydrolysis), samples were withdrawn and diluted to a concentration of 9 mg/mL to avoid retrogradation. Samples were then analyzed for reducing sugar concentration and immediately frozen. Samples were thawed, and the saccharide composition of the samples was determined by HPTLC. The effect of reaction conditions on hydrolysis and oligosaccharide composition was studied by varying pH, temperature, $[S]_0$ and [IEU] in the range of 4.4–7, 37–70 °C, 18–180 mg/mL, and 0.2964–1.86, respectively.

2.2.7. Thermostability and reusability of immobilized BLA. Immobilized BLA (5 mL) was resuspended in 50 mL of 0.1 M acetate buffer solution (pH 5.2) in absence of soluble starch and kept under shaking conditions (180 rpm) at 55 °C for 24 h. Similarly free BLA was diluted 10,000-fold with 0.1 M acetate buffer solution (pH 5.6) and kept under shaking conditions (180 rpm) at 55 °C in absence of starch for 24 h. Samples of immobilized BLA and free BLA were taken at various time intervals for the measurement of its activity.

The procedure to carry out the reaction for reusability studies was the same as that described in Section 2.2.6. At end of 8 h, the reaction mixture was separated from immobilized BLA. Then immobilized BLA was first sequentially washed thoroughly with distilled water and acetate buffer solution, and then kept under shaking conditions with acetate buffer (same amount as the reaction mixture, pH 5.2) at 55 °C for 30 min to remove any substrate or product molecules that could have been trapped inside the pores. Then acetate buffer was again separated from immobilized BLA. Fresh acetate buffer solution was added to immobilized BLA and kept at 6 °C, and the same was used for next batch hydrolysis under the same conditions on the next day. For each batch, samples were collected at regular time intervals and analyzed for reducing sugar concentration.

3. Results and discussion

3.1. pH and temperature dependence of the activity of free and immobilized BLA and their catalytic properties

BLA was immobilized onto CELBEADS by covalent binding as described earlier. It was observed that by material balance, 90% of the loaded FEUs (i.e., 300 FEUs per mL of CELBEADS) and 56% of the proteins loaded (i.e., 0.83 mg per mL of CELBEADS) were immobilized. With immobilization of the enzyme on the matrix, the activity of the enzyme may decrease or change due to several reasons. Among these are the orientation of the active site toward the support surface, which decreases the accessibility of the substrate molecule to the active part of the enzyme, the involvement of the active site of the enzyme in binding to the matrix, and denaturation or inactivation of the enzyme due to the reaction conditions used for immobilization. Thus it becomes necessary to determine activity of immobilized BLA separately (reported in Table 2) in addition to FEUs immobilized per mL of CELBEADS from material balance.

After immobilization, the pH–enzyme activity profile shifted toward the acidic side, and the optimum pH

Table 2. Properties of free and immobilized BLA

Parameter	Free BLA	Immobilized BLA
Optimum pH	5.6	5.2
Optimum temperature (°C)	55	55–70
Protein content (mg/mL)	75	0.83[a]
Activity of biocatalyst		
(FEU/mL)	16,500	300[a]
(IEU/mL)	18,450[b]	18.5
Sp activity		
(FEU/mg of protein)	220	361[a]
(IEU/mg of protein)	246.1[b]	22.3
Retained enzyme activity after immobilization	n.a	9.1%
E_a (kcal/mol)	1.63[c]	3.18[d]
K_m (mg/mL)	3.3	15
V_{max} (μmol/(min mL))	1.3	0.46
k_{cat} (min^{-1})	1.76	0.93

[a] Calcd using material balance.
[b] Calcd using assay procedure adapted for immobilized BLA (i.e., IEU equiv).
[c] Calcd using an Arrhenius plot over a temperature range of 32–55 °C.
[d] Calcd using an Arrhenius plot over a temperature range of 37–55 °C.

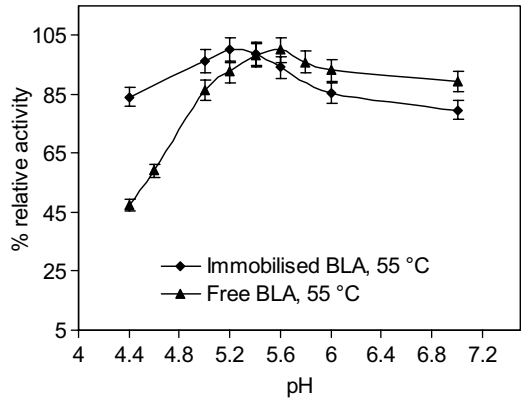

Figure 2. pH–% relative activity profile of free and immobilized BLA.

slightly decreased from 5.6 to 5.2 (Fig. 2). Shift in the optimum pH toward the acidic side[6,11,18] might be due to the difference in the hydronium-ion concentration in the bulk solution and the microenvironment in the vicinity of the immobilized enzyme molecule.[11] Figure 3 shows that the relative activity (%) above 55 °C was marginally better and approximately the same over the temperature range of 55–70 °C after the immobilization, indicating that optimum temperature changes from 55 °C to 55–70 °C (Fig. 3) upon immobilization. Figure 3 also shows that the relative activity (%) at a temperature less than 55 °C was reduced after immobilization. This could be because at low temperature the starch solution is more viscous, and hence the diffusional resistance for the migration of the starch molecules through the macropores is likely to be more at low temperature (<55 °C) as compared to higher temperature. The increase[11] or no change[13,18] in the optimum temperature

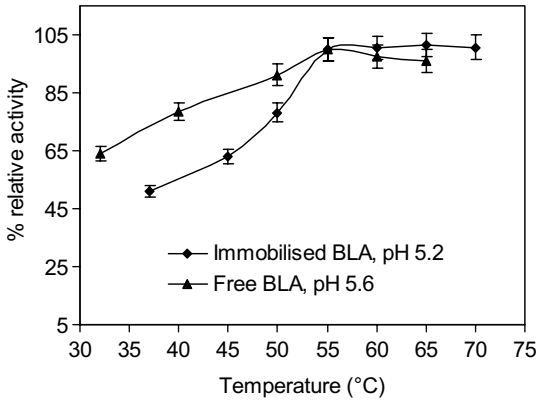

Figure 3. Temperature–% relative activity profile of free and immobilized BLA.

may be because of the improvement in the enzyme rigidity upon immobilization by covalent binding.

Properties of free and immobilized BLA are summarized in the Table 2. Activation energy (E_a) of immobilized BLA (3.18) was higher than that of free BLA (1.63). Similar increase in the E_a after immobilization is reported and attributed to the change in enzyme structure upon immobilization.[18] K_m^{app} of immobilized BLA (15 mg/mL) was 4.5 times of the K_m^{free} (3.3 mg/mL). Higher value of K_m for immobilized BLA indicates less affinity between immobilized BLA and substrate molecules, which could be because of either the similar nature of the charges carried by the support and the substrate or structural changes in the enzyme occurring upon immobilization or lower accessibility of substrate to the active enzyme site of the immobilized BLA due to steric hindrances and still persisting diffusion. The apparent value of K_m is reported to increase up to 2.6 times and 9 times for α-amylase (from porcine pancreas) immobilized on HEMA and styrene–HEMA microspheres,[11] respectively, and up to 10 times for α-amylase (*B. licheniformis*) immobilized on different types of matrices.[18] k_{cat}^{app} of immobilized BLA (0.93) was about half of the k_{cat}^{free} (1.76). The lower value of k_{cat} of immobilized BLA is due to lower accessibility of substrate to the active enzyme site of the immobilized BLA, which subsequently results in a lower reaction rate. Apparent V_{max} is reported to decrease marginally[11] for nonporous support, as well as significantly up to 20 times[18] for porous support.

3.2. Effect of reaction conditions on hydrolysis of soluble starch using immobilized BLA and saccharide composition

3.2.1. Effect of pH. Hydrolysis performed at pH 5.2 showed the maximum initial hydrolysis rate as well as maximum hydrolysis rates in the later stages of hydrolysis (see Supplementary data), indicating that the stability

of the immobilized BLA is relatively high at pH 5.2. Hence pH 5.2 was taken as an optimum operating pH, and experiments to take into account the effect of temperature, $[S]_0$ and $[IEU]/[S]_0$ were all performed at pH 5.2. Initial hydrolysis rates with unbuffered solution (i.e., soluble starch gelatinized in distilled water) are comparable to those at pH 5.6, but in the later stages of hydrolysis (i.e., beyond the reducing sugar concentration of 20 mg/mL), the hydrolysis rate decreases significantly as compared to that at pH 5.6 (Supplementary data). This indicates that, although the initial hydrolysis rate with unbuffered solution is high, the stability of the immobilized BLA in unbuffered solution is relatively low due to continued exposure to varying pH conditions.

It was observed that at low DE (8.5 and 12.5), there are no significant changes in the composition of maltodextrins (data not shown). However, Figure 4a shows that at DE of 20.5, the wt % of G5 and G3 significantly increases from 7.7 to 13 and from 4.7 to 6.7, respectively, with an increase in the pH from 4.4 to 7, but there are only marginal increases in the wt % of G1, G2, G4, G6, and G7. It can be also seen in Figure 4a that the wt % of oligosaccharides with DP > 7 (which mainly constitute branched dextrins) decreases from 76.8 to 70.5. It also indicates that an increase in the pH of the reaction mixture up to pH 7 favors the binding of higher dextrins to immobilized BLA and the subsequent hydrolysis of the same.

3.2.2. Effect of temperature. The initial rate of reaction (V) increases significantly with an increase in the temperature from 37 °C to 55 °C, but a further increase in the temperature results in a lesser increase in V, and initial rates at temperatures above 60 °C are approximately the same (see Fig. 3 and Supplementary data). At a DE of 20, the wt %s of G5 and G6 remain constant with an increase in the temperature from 36 to 50 °C, at 12–13 and 6–7, respectively, but further increases in the temperature up to 70 °C significantly decreases the wt % of G5 and G3 from 12.5 to 8 and from 7 to 4.8, respectively (Fig. 4b). At DE 20, the wt % of G1, G2, G4, G6, G7, and oligosaccharides higher than G7 increases from 0.9 to 1.35, from 2.4 to 3.1, from 2.3 to 2.6, from 3.9 to 5.7, from 0.7 to 1 and from 69 to 73.5, respectively, with an increase in the temperature from 36 °C to 70 °C (Fig. 4b). This indicates that an increase in the temperature decreases the product specific-

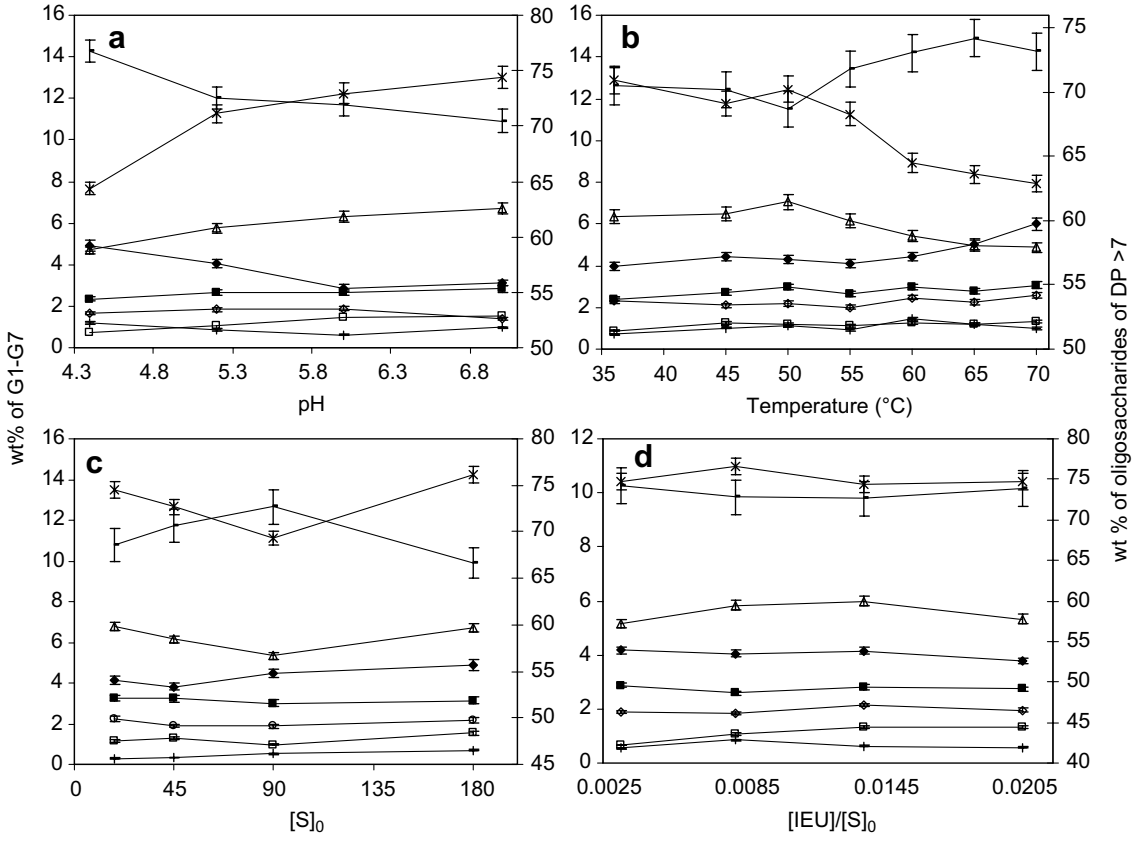

Figure 4. Effect of (a) pH ($[S]_0 = 90$ mg/mL, 55 °C and $[IEU]/[S]_0 = 8.27 \times 10^{-3}$), (b) temperature ($[S]_0 = 90$ mg/mL, pH 5.2 and $[IEU]/[S]_0 = 6.22 \times 10^{-3}$), (c) $[S]_0$ (55 °C, pH 5.2 and $[IEU]/[S]_0 = 3.3 \times 10^{-3}$) and (d) $[IEU]/[S]_0$ (pH 5.2, 55 °C and $[S]_0 = 90$ mg/mL) on the saccharide composition at DE 20–21. □ G1, ■ G2, △ G3, ◇ G4, ✳ G5, ◆ G6, + G7—oligosaccharides with DP > 7.

ity of immobilized BLA toward G3 and G5, whereas specificity toward G1, G2, G4, G6, and G7 increases. This results in more homogeneous molecular weight distribution with an increase in the operating temperature. Similar decrease in wt % of G5 and G3 and increase in wt % of G2 and G4 with an increase in the temperature from 50 to 90 °C, for free α-amylase (*B. licheniformis*, Maxamyl), are reported and is attributed to the combination of the following aspects: (1) A decrease in product specificity of α-amylase with increasing temperature; (2) an increase in amount of transglycosylation products with increasing temperature; (3) a change in the ratio of rate of hydrolysis of different linear oligosaccharides (of different DP) with increasing temperature.[13]

3.2.3. Effect of initial starch concentration, $[S]_0$ and $[IEU]/[S]_0$. In order to compare hydrolysis curves (Supplementary data) at different values of $[S]_0$ (varying from 18 to 180 mg/mL), DE versus time is plotted in Figure 5. Since the ratio $[IEU]/[S]_0$ was the same, it was expected that all curves would lie on the same line. But at low value of $[S]_0$ (18 mg/mL), [IEU] was also kept low, that is, 0.0593 in order to maintain $[IEU]/[S]_0$ constant, that is, 3.3×10^{-3}. Hence, although the viscosity of the starch solution was less, due to lesser concentrations of IEU and starch, the probability of contact of an enzyme active site on the beads with a starch molecule becomes less, which results into lesser increase in DE (5.7) in 3 h of reaction time (Fig. 5). But as $[S]_0$ increases, the probability of contact of the enzyme active site on the beads with a starch molecule increases, however, due to an increase in the viscosity, the diffusion resistance for a starch molecule to reach the active enzyme site inside the pore increases. This could be the reason for initial increase and then the maxima in the increase in DE in 3 h of reaction time (for $[S]_0$ of 45 and 90 mg/mL, the increase in DE was 9.2 and 10, respectively; Fig. 5) with an increase in $[S]_0$. Further increase in the $[S]_0$ results in an increase in the viscosity and the diffusion resistance for a starch molecule to reach the active site inside the pore. This could be the reason for the observed lower increase in DE (8.9) in 3 h of reaction at high starch concentration, that is, 180 mg/mL (Fig. 5). A decrease in the rate of hydrolysis at high starch concentration is reported for starch hydrolysis using free α-amylase (*B. licheniformis*, Termamyl), which is attributed to the imposed restriction on the free movements of both the starch and enzyme molecules due to viscosity effects and/or reduced water activity.[19]

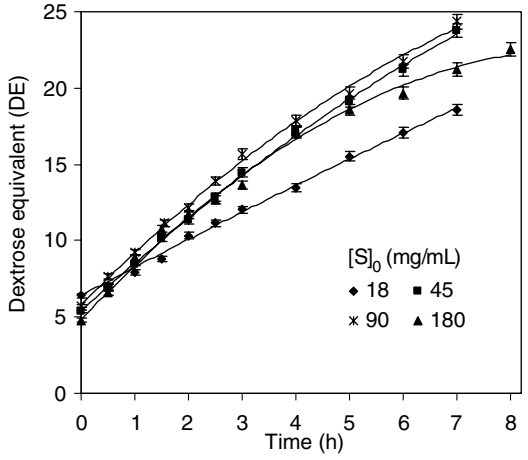

Figure 5. Comparison of DE versus time curves with $[S]_0$ as a parameter at 55 °C, pH 5.2 and $[IEU]/[S]_0 = 3.3 \times 10^{-3}$.

It can be seen from Figure 4c that the wt % of G1, G2, and G4 remains approximately the same with an increase in the $[S]_0$ at any value of DE; whereas the wt % of G3 and G5 decreases (from 6.8 to 5.3 and from 13.5 to 11.1, respectively, at a DE of 20) with an increase in $[S]_0$ from 18 to 90 mg/mL, and further increases in $[S]_0$ from 90 to 180 mg/mL result in an increase in the wt % of G3 and G5 (from 5.3 to 6.7 and from 11.1 to 14.2, respectively, at a DE of 20). It can be also seen from Figure 4c that the wt % of G6 and G7 marginally increases (from 4.2 to 4.9 and from 0.3 to 0.7, respectively) with an increase in $[S]_0$ from 18 to 180 mg/mL, whereas the wt % of higher oligosaccharides increases (from 68.6 to 72.7 at a DE of 20) with an increase in $[S]_0$ from 18 to 90 mg/mL and then decreases (from 72.7 to 66.7 at a DE of 20) with further increase in $[S]_0$ from 90 to 180 mg/mL.

With an increase in $[IEU]/[S]_0$ hydrolysis obviously takes place fast (Supplementary data). It can be seen from Figure 4d that there are marginal changes in saccharide composition with an increase in the ratio of $[IEU]/[S]_0$.

3.3. Comparison of saccharide composition of starch hydrolysate using free and immobilized BLA

The DE of gelatinized starch solution was ~5–6. For free BLA, the hydrolysis ceased at a DE of around 42–43 because BLA could not hydrolyze more α-(1→4) linkages due to the presence of branched dextrins, whereas for immobilized BLA, the DE of the starch hydrolysate at hydrolysis equilibrium was marginally low (around 36–37). The reason could be attributed to steric hindrances for branched dextrins in the vicinity of active enzyme site. The DE of starch hydrolysate can be correlated with reaction time (t) by the following exponential equation,

$$DE = DE_0 + A(1 - \exp(-Bt)) \quad (2)$$

in which DE_0 is initial DE of the gelatinized starch solution (~5–6), $A + DE_0$ is the maximum attainable DE by hydrolysis (which is around 36–37 for immobilized BLA) and B is the pseudo first-order hydrolysis constant (h^{-1}). The hydrolysis constant (B) was correlated with

operating parameters for immobilized BLA by the following empirical equation:

$$B = 5.67 \times 10^4([\mathrm{IEU}]/[\mathrm{S}]_0)^{0.892}(-0.002[\mathrm{S}]_0^2 + 0.521[\mathrm{S}]_0 + 45.18)\exp(-33.65/(0.008314T)) \quad (3)$$

In the case of free BLA, it can be seen from Figure 6 that the concentration of G1, G2, G3, G4, and G5 increases with the reaction time. The concentration of G6 and G7 increases with an increase in DE and attains maxima at a DE of about 37, and then shows a minor decrease with a further increase in DE. G8, G9, and G10 also show similar behavior.

For immobilized BLA, it can be seen from Figure 7 that the concentration of G1, G2, G3, G4, and G5 increases with an increase in the hydrolysis time, whereas the concentration of G6 and G7 increases up to DEs of 25 and 20, respectively, and then decreases (due to their further hydrolysis) with a further increase in the DE. The data of G8, G9, and G10 are not shown in Figure 7 because at any DE their wt % was always lower than 0.3. For immobilized BLA at any DE, G5 and G3 are the principal products (Figs. 7 and 8). Similar high production of G5 and G3 from soluble starch has been reported[9] with immobilized BLA, but variation in the concentration of oligosaccharides with DE or time has not been reported. This information will be useful in deciding the appropriate reaction quenching time to get the final product of desired saccharide composition.

Figure 8 shows that at the same DE, wt %s of G1, G2, G3, and G5 produced by immobilized BLA were much higher than those produced by free BLA, and there was no significant change in the wt % of G4, whereas the wt %s of G6, G7, G8, G9, and G10 produced by immobilized BLA were significantly lower than those produced by free BLA. The composition of starch hydrolysate produced by free BLA (reported here) is different from that reported[9,13] (produced by α-amylase, *B. licheniformis*), which could be due to a different type of strain and different botanical source of starch (i.e., corn, tapioca, potato, wheat, etc), which mainly differ in the amylose:amylopectin ratio, and average molecular weight.

At hydrolysis equilibrium with immobilized BLA, there were only traces of G6 and G7 (Fig. 7). Since BLA can hydrolyze linear G6 and G7, and not the branched one, we can say that immobilized BLA produces linear G6 and G7 from higher linear or branched dextrins in the early stages of hydrolysis (DE < 25), which gets further hydrolyzed, leaving only traces of G6 and G7 at hydrolysis equilibrium. However, the presence of significant quantities of G6–G10 at hydrolysis equilibrium with free BLA (Fig. 6) indicates that free

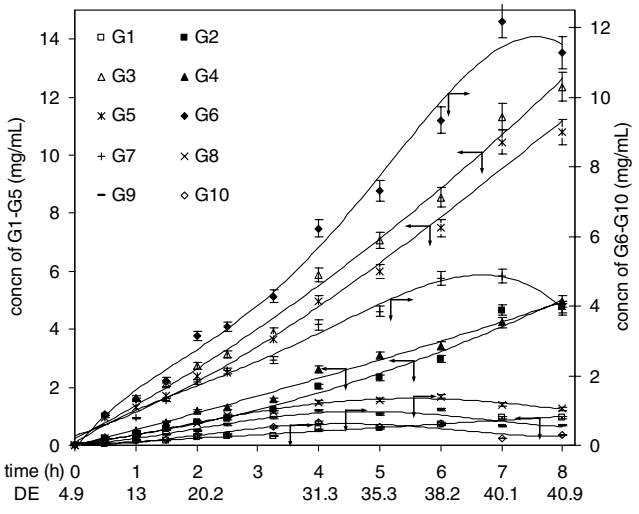

Figure 6. Change in concentration of oligosaccharides with time and DE, with free BLA. $[S]_0 = 90$ mg/mL, [IEU equiv]/$[S]_0 = 8.3 \times 10^{-3}$, pH 5.6, 55 °C.

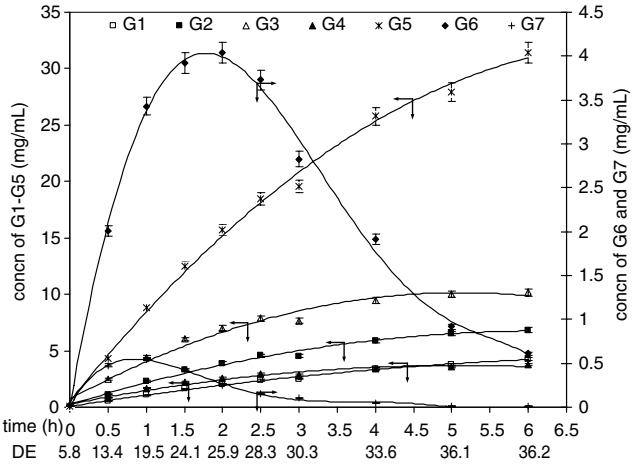

Figure 7. Change in concentration of oligosaccharides with time and DE, with immobilized BLA. $[S]_0 = 90$ mg/mL, [IEU]/$[S]_0 = 20.7 \times 10^{-3}$, pH 5.2, 55 °C.

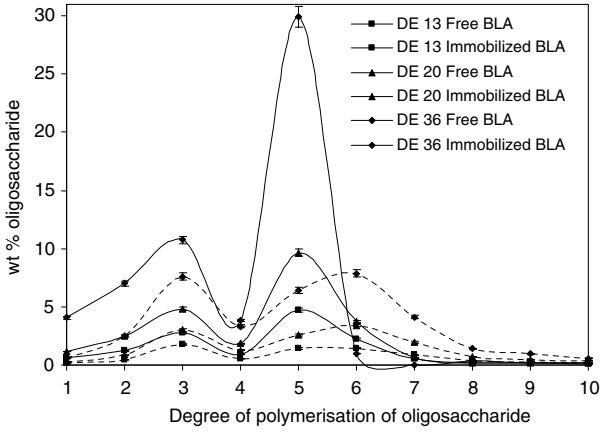

Figure 8. Comparison of saccharide profile produced by free and immobilized BLA.

BLA produces a significant amount of branched G6–G10 as opposed to the linear one. One can speculate that this could be the case of immobilized BLA. The linear part of higher dextrins must form a productive complex with the active enzyme site rather than with the branched part. The reason for this could be attributed to steric hindrance, which is a property of both the porous nature of the support and the extent of branching of the starch.[13] However, there is no such steric hindrance in the case of free BLA.

A few experiments were also performed on the hydrolysis of G4, G5, G6, and G7 separately using immobilized BLA at 55 °C. It was observed that immobilized BLA could not hydrolyze G4 and G5, but it completely hydrolyses G6 and principally produces G5 and G1 (this is in agreement with the literature report[9]). It also completely hydrolyses G7 and mainly produces G5, G2 along with a small quantity of G1. This must be because the immobilized BLA hydrolyses a major fraction of G7 to G5 and G2, and the rest to G6 and G1. The G6 thus produced, further hydrolyses to G5 and G1.

The action pattern and subsite mapping of free BLA with modified malto-oligosaccharide substrates have been reported.[20] The binding region or active site of BLA is composed of five glycone- and three aglycone-binding subsites and a barrier subsite, all of which have different binding energies.[20] Free BLA cleaves G5 as the main product from the nonreducing end of G6, G7, and G8 with yield or bond-cleavage frequencies of 68%, 84%, and 88%, respectively.[20] However, as the DP of the substrate increases, attack shifts toward the reducing end, and BLA cleaves G8, G9, and G10 into a main product G3 with yields of 88%, 83%, and 83%, respectively.[20] Our results on the hydrolysis of G6 and G7 using immobilized BLA are very similar to these results, which are reported for the hydrolysis of G6 and G7 using free BLA. However, the yields of G5 were 80% and 88%, respectively, for the hydrolysis of G6 and G7, which are higher than those reported[20] with free BLA.

Major production of G5 by the hydrolysis of G6 and G7 and the higher production of G5 and G3 from soluble starch, suggest that the action pattern of immobilized BLA is more like an exoamylase with dual product specificity mainly toward G5 and G3. Free BLA is also reported[20] to have dual product specificity mainly to G5 and G3 (due to the existence of the barrier subsite) using results of hydrolysis of linear G6–G10; however, the effect of the branching characteristics of starch was not considered. Our results of the hydrolysis of soluble starch using free BLA do not show high specificities toward G5 and G3 (discussed earlier), and this must be because of the absence of steric hindrance for the formation of a productive complex between free BLA and branched dextrins.

3.4. Thermostability and reusability of immobilized BLA

Figure 9 shows that the thermostability BLA improved after immobilization. Relative activity (%) of free BLA decreases from 100 to 75, whereas it remains nearly the same for immobilized BLA. It is well established that the immobilization of an enzyme by covalent binding often improves thermostability.[21] The reason is attributed to improvement in enzyme rigidity after immobilization.

Unlike the free enzyme, the immobilized enzyme can easily be separated from the reaction mixture and reused. Hence reusability or operational stability is an important criterion for the industrial use of immobilized enzymes. A reusability study for immobilized BLA shows that 100% activity of immobilized BLA was retained even after eight batches of hydrolysis, which indicates good reusability. For comparison, the hydrolysis curves of the 1st and 8th batch are shown in Figure 10.

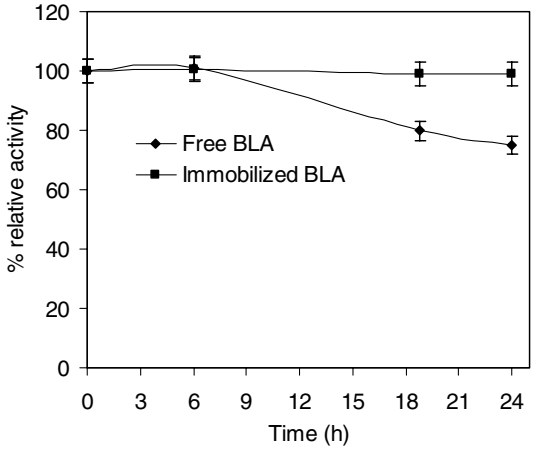

Figure 9. Thermostability of free and immobilized BLA.

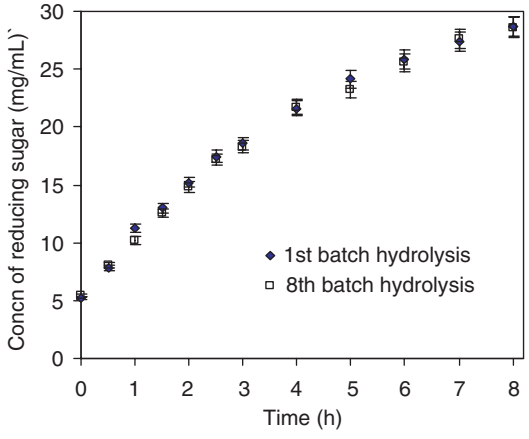

Figure 10. Reusability of immobilized BLA at pH 5.2, 55 °C, $[IEU]/[S]_0 = 6.13\ e{-}3$, $[S]_0 = 90$ mg/mL.

3.5. Semiempirical model for prediction of saccharide composition

Molecular weight distribution of soluble starch and starch hydrolysate were not calculated in this work. Also, the kinetic mechanism of starch hydrolysis is quite complex due to the presence of multisubstrates. Therefore, semiempirical equations for the concentrations of oligosaccharides (G1–G7) versus time were used, which are analogous to reported[22] semiempirical equations. It was observed that plotting the rate of formation of oligosaccharides (G1–G5) versus the concentration of oligosaccharides higher than G5 yields a straight line. This indicates that rate of formation of oligosaccharides is first-order with the concentration of oligosaccharides higher than G5. Since, as stated earlier oligosaccharides with DP lower than 6 can not be hydrolyzed by immobilized BLA, there is no need for the depletion term in the differential equations expressing the time-dependent variation of G1–G5, which are as follows:

For $i = 1$–5,

$$\frac{d[G_i']}{dt} = k_i[\mathrm{IEU}]\left(\mathrm{TDW} - \sum_{n=1}^{5}[G_n']\right) \tag{4}$$

in which $[G_i']$ is the concentration (mg/mL) of oligosaccharide with a DP of i, TDW is the dry weight concentration of starch hydrolysate (mg/mL) and k_i is kinetic constant ($h^{-1}\,[\mathrm{IEU}]^{-1}$) of the formation of oligosaccharide with a DP of i. Concentration terms in Eq. 4 were made dimensionless by dividing Eq. 4 by TDW on both sides. Eq. 4 thus takes the following form:

For $i = 1$–5,

$$\frac{d[G_i]}{dt} = k_i[\mathrm{IEU}]\left(1 - \sum_{n=1}^{5}[G_n]\right) \tag{5}$$

in which $[G_i]$ is the dimensionless concentration or weight fraction of the oligosaccharide with a DP of i.

As stated earlier, immobilized BLA hydrolyses G6 and G7, so it becomes essential to add a depletion term ($k_i'[\mathrm{IEU}][G_i]$) while constructing a differential equation expressing the time-dependent variation for G6 and G7. The differential equations for G6 and G7 are as follows,

For $i = 6$–7,

$$\frac{d[G_i]}{dt} = k_i[\mathrm{IEU}]\left(1 - \sum_{n=1}^{i}[G_n]\right) - k_i'[\mathrm{IEU}][G_i] \tag{6}$$

where k_i' is kinetic rate constant ($h^{-1}\,[\mathrm{IEU}]^{-1}$) for hydrolysis of oligosaccharide with DP of i. Differential equations of G8–G10 are not considered because the wt %s of G8–G10 were always lower than 0.3. Values of kinetic constants k_1–k_7, k_6' and k_7' were determined by minimizing the sum of square of the error between predicted (obtained by simultaneously solving Eq. 5 and Eq. 6 from $t = 0$ h to $t =$ time required to attain the equilibrium DE of 36.5, which was calculated using Eq. 2) and the experimental wt %s of the oligosaccharides (G1–G7). This was done by developing a code in MATLAB. Kinetic constants for reaction conditions ($[\mathrm{IEU}] = 1.86$, 55 °C, pH 5.2 and $[S]_0 = 90$ mg/mL) are $k_1 = 0.0069$, $k_2 = 0.0119$, $k_3 = 0.0191$, $k_4 = 0.0072$, $k_5 = 0.0508$, $k_6 = 0.0489$, $k_7 = 0.0148$, $k_6' = 0.9725$ and $k_7' = 3.2536$ (the unit of all the kinetic constants is $h^{-1}\,[\mathrm{IEU}]^{-1}$). A comparison of the experimental and predicted wt %s of oligosaccharides (G1–G7) under the above-mentioned reaction conditions is shown in Figure 11. It can be seen from Figure 11 that the predicted wt % fits well with the experimental values of wt %s for G1–G5, whereas for G6–G7 the predicted wt % lies slightly below the experimental values of the wt % up to a DE of 20. Beyond DE 20 the model overpredicts the wt %s of G6–G7. These kinetic constants were empirically correlated with [IEU], $[S]_0$ and temperature using the following type of correlation:

$$k = A[\mathrm{IEU}]^B(T/273)^C[S]_0^D \tag{7}$$

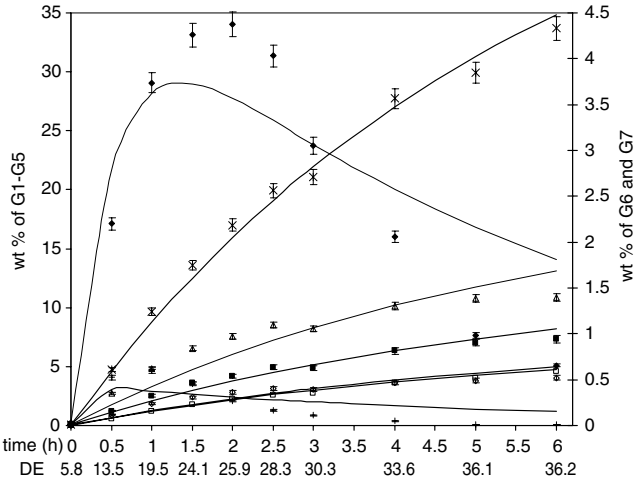

Figure 11. Comparison of experimental and predicted saccharide composition; $[\mathrm{IEU}] = 1.86$, 55 °C, pH 5.2, $[S]_0 = 90$ mg/mL. Symbols represent experimental data; □ G1, ■ G2, △ G3, ◇ G4, ✳ G5, ◆ G6 and + G7; continuous lines represent predicted data.

Table 3. Values of A, B, C, and D for kinetic constants

Kinetic constant	A	B	C	D	Correlation coefficient
k_1	0.0083	−0.0807	14.58	−0.6237	0.97
k_2	0.0086	−0.3975	13.28	−0.4374	0.99
k_3	0.0415	−0.4727	7.30	−0.4145	0.99
k_4	0.0060	−0.3673	12.42	−0.4160	0.99
k_5	0.0899	−0.3777	7.26	−0.4033	0.98
k_6	0.3580	0.1086	12.01	−0.9275	0.99
k_7	0.0022	−0.0633	14.62	−0.0905	0.80
k_6'	102	0.5063	8.84	−1.439	0.93
k_7'	7.8800	0.1496	9.36	−0.6200	0.89

where A, B, C, and D are correlation constants and T is temperature in K.

Values of A, B, C, and D for kinetic constants k_1–k_7, k'_6 and k'_7 calculated by nonlinear regression (using POLYMATH) are given in Table 3.

4. Conclusions

B. licheniformis α-amylase (BLA) was immobilized on superporous CELBEADS. After immobilization, the optimum pH marginally decreases and the optimum temperature remains the same. The pH, temperature and initial starch concentration all have significant effects on the saccharide composition at the same value of DE. The saccharide composition of the starch hydrolysate produced by immobilized BLA is different than that produced by free BLA at any value of DE. At any DE, the free BLA principally produces maltotriose, maltopentaose and maltohexaose, whereas the immobilized BLA principally produces maltotriose and maltopentaose. Immobilized BLA has better thermostability than free BLA and is found to retain 100% activity even after eight batches of hydrolysis. Immobilized BLA can be used as an additional tool for production of maltodextrins solely or in combination with variation in pH, temperature and starch concentration. A semiempirical model was used to predict the wt %s of oligosaccharides (G1–G7) that satisfactorily fit with experimental data of G1–G5, but the model over predicts the wt %s of G6 and G7. Such a model can be used for selecting temperature and starch concentration as design parameters to obtain the desired saccharide composition in maltodextrins. However, care should be taken because the values of the kinetic constants are likely to vary with changes in the source of the substrates.

5. Abbreviations

A, B, C, D	see Eq. 7
$[G'_i]$	concentration of oligosaccharide with DP of i (mg/mL)
$[G_i]$	dimensionless concentration or wt fraction of oligosaccharide with DP of i
[FEU]	number of free enzyme units per mL of starch solution
[IEU]	number of immobilized enzyme units per mL of starch solution
[IEU equiv]	number of equivalents of immobilized enzyme units per mL of starch solution
$[S]_0$	initial starch concentration (mg/mL)
DE	dextrose equivalent of starch hydrolysate
k_1–k_7	kinetic constant for formation of G1–G7 (h^{-1} $[IEU]^{-1}$)
k'_6	kinetic constant for depletion of G6 (h^{-1} $[IEU]^{-1}$)
k'_7	kinetic constant for depletion of G7 (h^{-1} $[IEU]^{-1}$)
k_{cat}^{free}	turnover number of free enzyme (min^{-1}), $V_{max}^{free}/[IEU]$
k_{cat}^{app}	turnover number of immobilized enzyme (min^{-1}), $V_{max}^{app}/[IEU\ equiv]$
K_m	intrinsic michaelis constant (mg/mL)
K_m^{free}	K_m of free enzyme (mg/mL)
K_m^{app}	apparent K_m of immobilized enzyme (mg/mL)
n	average degree of polymerization (DP) of starch hydrolysate
T	temperature (K)
t	reaction time (h)
TDW	dry weight concentration of starch hydrolysate (mg/mL)
V	initial reaction rate
V_{max}	intrinsic maximum reaction rate
V_{max}^{free}	V_{max} of free enzyme
V_{max}^{app}	apparent V_{max} of immobilized enzyme

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Supplementary data

Supplementary data contains Figure S1. Comparison of hydrolysis curves: (A) pH as a parameter, (B) temperature as a parameter, (C) $[S]_0$ as a parameter, and (D) $[IEU]/[S]_0$ as a parameter. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carres.2007.02.027.

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