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ORIGINAL PAPER

Production of high fructose syrup from *Asparagus* inulin using immobilized exoinulinase from *Kluyveromyces marxianus* YS-1

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Abstract Extracellular exoinulinase from *Kluyveromyces marxianus* YS-1, which hydrolyzes inulin into fructose, was immobilized on Duolite A568 after partial purification by ethanol precipitation and gel exclusion chromatography on Sephadex G-100. Optimum temperature of immobilized enzyme was 55 °C, which was 5 °C higher than the free enzyme and optimal pH was 5.5. Immobilized biocatalyst retained more than 90% of its original activity after incubation at 60 °C for 3 h, whereas in free form its activity was reduced to 10% under same conditions, showing a significant improvement in the thermal stability of the biocatalyst after immobilization. Apparent K_m values for inulin, raffinose and sucrose were found to be 3.75, 28.5 and 30.7 mM, respectively. Activation energy (E_a) of the immobilized biocatalyst was found to be 46.8 kJ/mol. Metal ions like Co^{2+} and Mn^{2+} enhanced the activity, whereas Hg^{2+} and Ag^{2+} were found to be potent inhibitors even at lower concentrations of 1 mM. Immobilized biocatalyst was effectively used in batch preparation of high fructose syrup from *Asparagus racemosus* raw inulin and pure inulin, which yielded 39.2 and 40.2 g/L of fructose in 4 h; it was 85.5 and 92.6% of total reducing sugars produced, respectively.

Keywords *Kluyveromyces marxianus* · *Asparagus racemosus* · Inulinase · Immobilization · High fructose syrup

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Introduction

Inulin, a non-digestible carbohydrate, is a polyfructan that is not only found in many plants as a storage carbohydrate, but has also been part of man's diet for several centuries. This polymer is a recognized source for the production of either ultra high fructose syrup, with D-fructose content over 75% [26], or for the production of oligofructosaccharides [13, 14]. Fructose, having GRAS status, is emerging as a safe alternative sweetener, which is important primarily because of its functional properties rather than sweetness. Its demand is ever increasing due to its beneficial role for diabetics, low cariogenic nature and assistance in iron absorption [11]. It enhances flavor, color and product stability and is thus widely used in many foods and beverages instead of sucrose [12]. Inulin is hydrolyzed to fructose by inulinase (β-D-fructan fructanohydrolase, EC 3.2.1.7), which splits off terminal fructosyl unit and releases fructose monomers with little glucose. It is preferred over the chemical hydrolysis which is associated with drawbacks like production of unwanted by-products (hydroxymethyl furfural, fructose dianhydride) and color forming compounds that lower the product yield and requires a more demanding downstream processing and energy expenditure [20]. These shortcomings can be overcome by more specific enzymatic process. This approach is particularly more effective if an immobilized biocatalyst is used, since it allows either reuse of biocatalyst or a continuous mode of operation and prevents contamination of the processed product. The economic consideration dictates the use of cheap and simple, yet effective immobilization method with carefully characterized system when scale-up is foreseen. Nevertheless, despite of its appreciable stability, inulinase from *K. marxianus* YS-1 is easily inactivated in free form at higher temperatures [24]. Therefore, the improvement in the stability

of inulinase as well as possibility to perform its reuse along with consecutive hydrolysis cycle or continuous mode, are the targets of considerable importance. These findings prompted to investigate the immobilization of inulinase on a suitable support. In this investigation, partially purified inulinase from *Kluyveromyces marxianus* YS-1 has been immobilized on Duolite A568, characterized and evaluated for the preparation of high fructose syrup in batch system. *Asparagus* roots contains 15% inulin [10] and this plant has higher annual world production as compared to chicory and Jerusalem artichoke [1], but there is no report on the utilization of its roots as a source of inulin for the preparation of high fructose syrup. Therefore, *Asparagus* roots were selected as a substrate for the preparation of high fructose syrup.

Materials and methods

Yeast culture

Kluyveromyces marxianus YS-1, an isolate of our laboratory was used in the present work. It was isolated and maintained as discussed earlier [25]. Culture has been identified by Microbial Type Culture Collection (MTCC), Chandigarh, India. It was deposited in the International Depository Authority in the same organization and assigned an accession number MTCC 5201.

Biocatalyst and substrate

The production of exoinulinase in the fermentation medium was carried out as mentioned previously [23]. It was partially purified by ethanol precipitation (85%, v/v) followed by chromatography on Sephadex G-100 as described earlier [24] and used for immobilization. *Asparagus racemosus* roots were obtained from Botanical Gardens, Punjabi University, Patiala, India. Raw inulin from roots was extracted in sodium acetate buffer (0.1 M, pH 5.5) as described previously [24].

Enzyme immobilization

Exoinulinase was immobilized on Duolite A568 (Courtesy Rohm and Haas, France) by the method of Peters et al. [22] with modifications. Coupling of enzyme to the phenol formaldehyde resin was done with glutaraldehyde. This weak ionic macroporous resin having amino group (–NH<) was thoroughly and successively washed with 50 mL each of ethanol, water, diluted hydrochloric acid, diluted lye and then equilibrated with sodium acetate buffer (0.1 M, pH 5.5). Subsequently, 5 g of resin was derivatized by gentle stirring in aqueous solution (0.5–2%) of glutaraldehyde at room temperature for 2 h. The derivatized resin was then filtered and washed thoroughly to remove traces of glutaraldehyde. After modification, it was incubated with 20 mL of enzyme (100 IU) at 4 °C, under gentle stirring for 24 h. Immobilized enzyme was recovered by filtration, thoroughly washed and stored at 4 °C in wet state, until its further use. The immobilization yield (%) was defined as: amount of immobilized protein/amount of protein loaded × 100. Activity yield (%) of the immobilized enzyme was defined as $\{C/(A-B)\} \times 100$, where A is the inulinase activity loaded, B is the inulinase activity in the supernatant after immobilization and C is the inulinase activity of the immobilized enzyme.

Characterization of immobilized inulinase

Effect of pH

To find out the optimum pH of the immobilized exoinulinase, assays were carried out at different pH, using sodium acetate buffer (0.1 M, pH 3–6) and phosphate buffer (0.1 M, pH 7–8). pH stability of immobilized enzyme has also been investigated after preincubating it at different pH (4–8) at 55 °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 the optimum pH.

Effect of temperature

To investigate the optimum temperature, assays were carried out at different temperatures (25–80 °C). Thermal stability was examined without substrate and any additives. Immobilized enzyme was kept at 50, 55, 60 and 70 °C in sodium acetate 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 (E_a) has been calculated from the slope ($-E_a/2.3\ R$) of linear representation of log V_{max} versus $1/T$. Temperature was expressed in Kelvin, the gas constant ($R = 8.314$) in J/K mol and the activation energy (E_a) in J/mol.

Effect of different substrates and metal ions

Affinity of immobilized exoinulinase towards inulin, sucrose, raffinose, lactose and maltose was investigated. Apparent Michaelis–Menten constant (K_m) and V_{max} were determined under standard assay conditions using Lineweaver–Burk plot. To analyze the metal ions effect, activity was measured in the presence of various metal ions (Mg^{2+}, Fe^{2+}, Ca^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Ba^{2+}, Hg^{2+}, Ag^{2+}) and EDTA at varying concentrations (1–10 mM).

Inulin hydrolysis

Raw inulin (5%) was incubated with immobilized inulinase (25 IU) under agitation (50–150 rpm) for different time intervals (0.5–6 h) at 55 °C. For comparison, hydrolysis of 5% pure inulin solution was carried out under the same conditions.

Recycling of biocatalyst

Immobilized biocatalyst (25 IU) was added to 10 mL of root extract containing 5% inulin and incubated for 4 h at 55 °C, under shaking (125 rpm). Biocatalyst was recovered and washed thoroughly with the same buffer, and fresh substrate was added for a new bioconversion run. To compare, pure inulin was substituted in place of raw inulin for hydrolysis, under similar conditions. Spent broth from each cycle was analyzed for fructose, glucose and reducing sugars.

Analytical methods

Enzyme activity was measured by determining the reducing sugars released from inulin. An adequate amount of immobilized enzyme was incubated in 3 mL of 2% inulin in sodium acetate buffer (0.1 M, pH 5.5) at 55 °C in a water bath for 15 min under shaking. Reaction was stopped by filtering out the immobilized enzyme and keeping resultant mixture at 100 °C for 10 min, which was then assayed for released reducing sugars. One unit of enzyme was defined as the amount of enzyme that produces one micromole of reducing sugar per minute under standard assay conditions. Protein content was determined according to Lowry [18] with bovine serum albumin as the standard. Total sugars were determined as reducing sugars after acid hydrolysis (H_2SO_4, pH 2.0, 45 min, 100 °C) by the method of Miller [21]. Glucose was determined by glucose oxidase-peroxidase kit (Sigma, USA) and fructose was measured as a difference between total sugars and glucose. Average degree of polymerization was calculated as the ratio of glucose to fructose in the acid hydrolyzed sample. The average degrees of polymerization were 8.8 in raw inulin (*Asparagus racemosus*) and 13.3 in pure inulin (SD Fine-chem Ltd, Mumbai, India).

Results

Enzyme immobilization

Exoinulinase from *Kluyveromyces marxianus* YS-1 was purified 23.5-fold with a specific activity of 413 IU/mg. It was successfully immobilized on Duolite A 568 via Schiff's base (aldimine) linkage between carbonyl group of the activated support and free amino groups on the protein. Glutaraldehyde was used to activate the support, which resulted in the addition of at least 5-atom spacer arm and a reactive aldehyde end group. Effect of different concentrations (0.5–2.0%) of glutaraldehyde on enzyme activity yield has been examined (data not shown). Results showed 1.25% glutaraldehyde appeared to be optimal for the immobilization of exoinulinase on Duolite A568. By this procedure, the final immobilized system contained 6 IU/g (wet weight) with 86.6% of immobilization yield and 35.6% recovery of enzyme activity.

Characterization of immobilized inulinase

Effect of pH

Immobilized inulinase showed the optimum pH 5.5 and it was the same for the free inulinase. Furthermore, pH stability studies have shown that, the immobilized enzyme retained more than 80% of its initial activity after incubating it in pH 4.5–6.5 for 6 h, whereas activity was only 60% for free enzyme [24]. This indicated better pH stability for immobilized enzyme.

Effect of temperature

The optimum temperature for immobilized inulinase at pH 5.5 was found to be 55 °C, which was 5 °C higher than that of free enzyme (50 °C). After immobilization, exoinulinase has shown a significant increase in thermal stability. Comparing the enzyme in its free and immobilized form there was a remarkable difference. It retained more than 90% of its original activity after incubation at 60 °C for 3 h (Fig. 1), whereas activity of free enzyme reduced to 10% [24]. At 70 °C, it was found to be 56% active till the end of 3 h, but in free form, it was rapidly inactivated. The Arrhenius plot was linear (Fig. 2) and activation energy was calculated to be 46.8 kJ/mol, which was similar to that of free enzyme (45.1 kJ/mol).

Substrate specificity

Immobilized system was active on inulin, sucrose and raffinose solutions (2%, w/v), whereas it failed to hydrolyze starch, maltose and lactose. It showed 145.4 and 56.3% relative activity on sucrose and raffinose with respect to inulin, which was taken as 100%. Effect of different concentrations of inulin, raffinose and sucrose was further investigated and results were plotted using Lineweaver–Burk plot. Minimum apparent Michealis constant (K_m) was observed for inulin (3.57 mM) as compared to raffinose (28.5 mM) and sucrose (30.7 mM) as shown in Table 1.

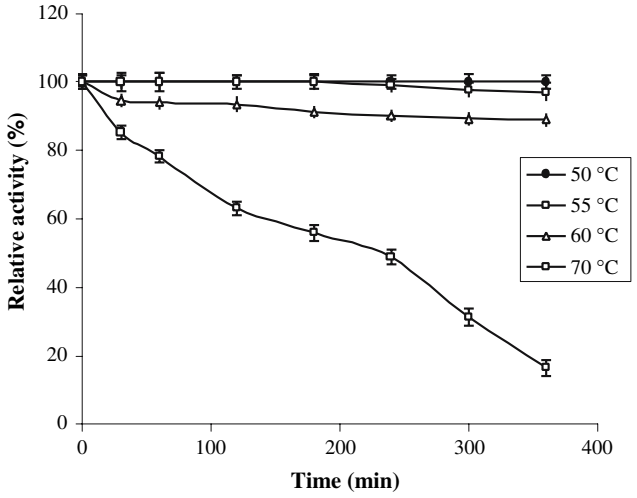

Fig. 1 Thermal stability of immobilized exoinulinase at different temperatures

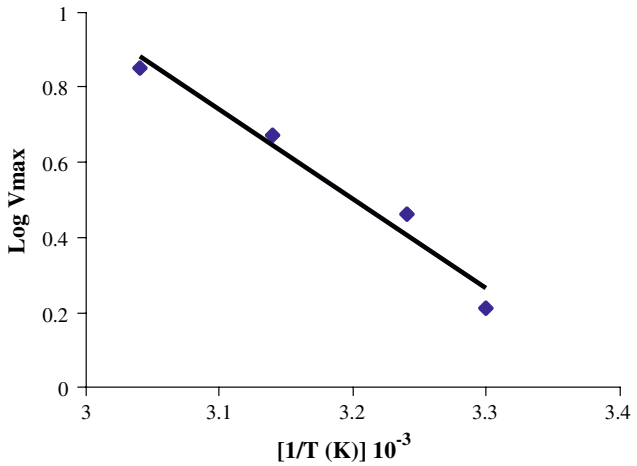

Fig. 2 Arrhenius plot for the determination of activation energy of immobilized exoinulinase

Table 1 Michaelis-Menten constant (K_m) and V_{max} of immobilized exoinulinase

Substrate	K_m (mM)	V_{max} (μM/min)
Inulin	3.57	7.14
Raffinose	28.5	1.53
Sucrose	30.7	16.1

Effect of metal ions

The effect of metal ions on immobilized enzyme system was studied using different ions at varying concentrations (data not shown). Maximum increase in enzyme activity (1.6 times each) was observed on the addition of Mn^{2+} (5.0 mM) and Co^{2+} (2.5 mM) as compared to control where no metal ions were added. These metal ions were followed by Ca^{2+} (10 mM), which increased the activity by 1.3 times. Hg^{2+} and Ag^{2+} were found to be potent inhibitors of the immobilized inulinase even at the lowest concentration of 1 mM used. Zn^{2+}, Fe^{2+} and EDTA also inhibited immobilized enzyme to 35, 20 and 49% of its initial activity, respectively.

Inulin hydrolysis

Hydrolysis of raw inulin from *Asparagus racemosus* and pure inulin was carried out with the immobilized inulinase (25 IU) under agitation (50–150 rpm) at 55 °C. In both the substrates, shaking at 125 rpm was found to be optimal for hydrolysis (Table 2). At this mode of agitation, hydrolysis was studied as a function of time. Initially the rate of hydrolysis of raw *Asparagus racemosus* inulin was very high but after 4 h a distinct slackening in the hydrolysis was observed. After 4 h of hydrolysis, 82.6% of raw inulin was hydrolyzed, whereas after 6 h extent of hydrolysis was 85.0% only. Since the objective of the work was the production of high fructose syrup, it is important to evaluate not only the degree of saccharification but also the proportion of fructose released after hydrolysis. Hydrolysis of raw inulin yielded 39.2 g/L of fructose in 4 h, which was 85.5% of the total reducing sugars (Fig. 3a). Similar hydrolysis pattern was observed with pure inulin (Fig. 3c). Pure inulin was hydrolyzed 78.3% in 4 h and, thereafter, hydrolysis was very slow with only 83.3% hydrolysis reached at 6 h. The amount of fructose produced after hydrolysis of pure inulin was 40.25 g/L in 4 h, which constituted 92.6% of total reducing sugars released under the optimal conditions.

Recycling of biocatalyst

The reutilization of the immobilized enzyme has been investigated using both (raw and pure inulin) types of substrates. There was 10% loss of immobilized inulinase activity at 4th cycle in both cases and 20% loss at 7th cycle of hydrolysis (Fig. 4). After this the system has shown a good stability and was repeatedly used for 55 batches till it lost half of its original activity.

Discussion

In continuation of our previous studies [23, 24], the present investigation has been carried out on characterization of exoinulinase immobilized on Duolite A568 and production of high fructose syrup from pure and raw asparagus inulin in a batch system. Inulinase purified to 23.5-fold (413 IU/mg protein) was immobilized on the resin via glutaraldehyde. The capacity of the support for immobilization was demonstrated by the immobilization yield of 86.6%, however the activity recovery was 35.6%. pH activity profiles

Table 2 Hydrolysis of (A) raw *Asparagus racemosus* inulin and (B) pure inulin at 55 °C with time and agitation as function

Time (min)	Hydrolysis (%) under agitation (rpm)									
	50		75		100		125		150	
	A	B	A	B	A	B	A	B	A	B
30	7.3	2.9	16.1	14.6	23.8	18.1	27.1	22.4	20.3	19.4
60	18.6	15.3	41.2	31.3	49.6	45.6	53.2	47.0	35.7	30.6
120	29.0	23.1	59.7	44.3	63.7	58.1	68.4	60.8	55.0	48.5
180	35.0	33.1	66.2	52.4	73.7	70.3	75.6	72.4	64.2	60.5
240	48.3	45.3	70.3	60.7	79.6	75.2	82.6	78.3	70.3	67.4
300	56.0	52.1	72.0	65.7	81.4	80.3	83.8	82.6	72.1	70.1
360	60.3	56.1	73.9	69.8	82.3	81.6	85.0	83.3	75.4	73.4

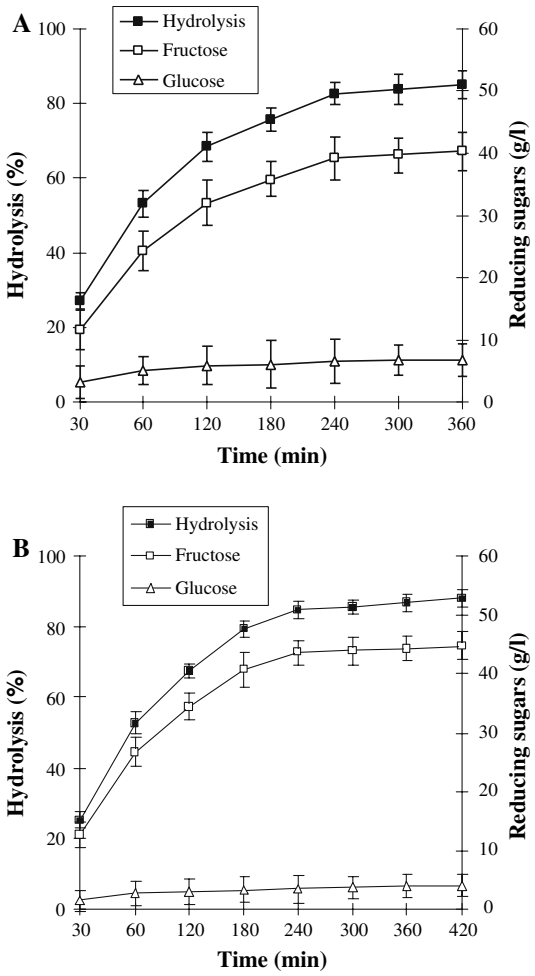

Fig. 3 Time course of sugars released from: **a** raw *Asparagus racemosus* inulin, **b** pure inulin under agitation

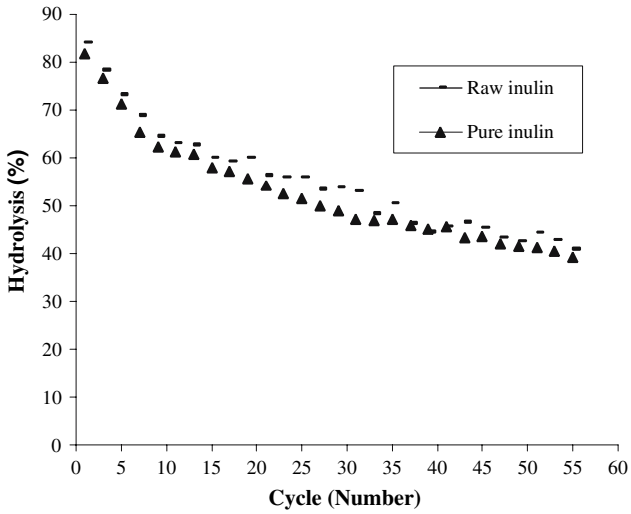

Fig. 4 Recycling of immobilized biocatalyst for hydrolysis of raw *Asparagus racemosus* inulin and pure inulin

of an enzyme 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 optimum pH of the enzyme was not changed after immobilization, whereas pH stability was improved by 20% in the pH range of 4.5–6.5. Lower pH optimum is advantageous for the preparation of fructose syrup, because it prevents undesired color formation. Immobilized exoinulinase showed higher optimum temperature (55 °C) and considerably high thermal stability. Higher hydrolysis temperature is advantageous to increase the solubility of inulin and accelerate the reaction velocity. The increase in thermal stability may be a result of the fact that immobilization limits the thermal movement of the enzyme at higher temperature, hence decreases denaturation. Enhancement of thermal stability, due to immobilization, has also been reported earlier [2, 17, 28]. Activation energies for free (45.1 kJ/mol) and immobilized (46.8 kJ/mol) exoinulinase were approximately similar, indicating that the reaction is kinetically controlled. This has also been observed earlier [6, 7]. Whereas, activation energies for free and Amberlite immobilized inulinases were reported as 30.0 and 26.6 kJ/mol [5]. Immobilized enzyme has greater affinity towards inulin as shown by lower K_m values as compared to raffinose and sucrose. This K_m value for inulin was lower as compared to values 7.7–10 mM and 13.3 mM, reported earlier for immobilized inulinase from *K. fragilis* [17] and *K. marxianus* [3], respectively. Immobilization of exoinulinase had no effect on its affinity towards inulin. As the crude substrate and partially purified exoinulinase has been used for the preparation of high fructose syrup, it is important to characterize the immobilized system for the metal ions effect. The increase in enzyme activity by the addition of Mn^{2+} and Co^{2+} indicates that these metal ions are required for better catalytic action.

Several types of raw materials have been used as a source of inulin, but in the present investigation *Asparagus*

has been selected owing to its appreciable inulin content and higher worldwide production. Raw *Asparagus* inulin (5%) was hydrolyzed to 82.6% in 4 h and released 39.2 g/L of fructose. The production of glucose and fructose from raw *Asparagus racemosus* inulin was faster than that of pure inulin during initial stages of hydrolysis, which shows polymers having low degree of polymerization were hydrolyzed more rapidly than those with higher degree of polymerization. Similar results have been reported earlier [2, 4, 9]. Most of the earlier studies have been carried out with Jerusalem artichoke. In a batch system, 90% conversion of Jerusalem artichoke extract yielding 34 g/L [16] and 77.5 g/L [15] of D-fructose have been reported. In other studies, it has been reported that hydrolysis of dried topinamber extract produced free fructose which was 85.7% of total reducing sugars [19]. In a continuous system, hydrolysis of Jerusalem artichoke yielding a mixture of 85% D-fructose and 15% D-glucose has been reported [28]. Hydrolysis of chicory extract produced a mixture of fructose and glucose in the ratio of 3:1 [8]. Kuth roots yielded 70% of fructose after hydrolysis with inulinase [27]. Amount of fructose obtained from hydrolysis of raw inulin from *Asparagus racemosus* was comparable with the fructose produced from Jerusalem artichoke, chicory and kuth roots as reported in the earlier studies.

The ease of separation and increased stability are the advantages of using an immobilized biocatalyst. The immobilized biocatalyst was continuously recycled for 55 batches. The decrease in hydrolysis in the initial batches may be due to the loss of loosely bound exoinulinase during washings of the biocatalyst. The immobilized system showed a good stability in batch system suggesting that it can be successfully used for the continuous preparation of high fructose syrup.

Conclusions

Partially purified exoinulinase was successfully immobilized on Duolite A 568 by simple glutaraldehyde treatment, with 36.5% activity yield and good thermal stability. The recycling of immobilized biocatalyst has shown a good operational and mechanical stability making it an effective system. The easy immobilization technique and stability of this enzyme system is very promising. Further, substituting pure inulin with raw inulin from *Asparagus racemosus* with the results at par as compared to earlier studies is a successful approach towards high fructose syrup preparation from a cheap and easily available source. These promising results prompted the further use of this immobilized system for the continuous preparation of high fructose syrup.

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