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ORIGINAL PAPER

Development of a stable continuous flow immobilized enzyme reactor for the hydrolysis of inulin

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Abstract A 23.5-fold purified exoinulinase with a specific activity of 413 IU/mg and covalently immobilized on Duolite A568 has been used for the development of a continuous flow immobilized enzyme reactor for the hydrolysis of inulin. In a packed bed reactor containing 72 IU of exoinulinase from *Kluyveromyces marxianus* YS-1, inulin solution (5%, pH 5.5) with a flow rate of 4 mL/h was completely hydrolyzed at 55 °C. The reactor was run continuously for 75 days and its experimental half-life was 72 days under the optimized operational conditions. The volumetric productivity and fructose yield of the reactor were 44.5 g reducing sugars/L/h and 53.3 g/L, respectively. The hydrolyzed product was a mixture of fructose (95.8%) and glucose (4.2%) having an average fructose/glucose ratio of 24. An attempt has also been made to substitute pure inulin with raw *Asparagus racemosus* inulin to determine the operational stability of the developed reactor. The system remained operational only for 11 days, where 85.9% hydrolysis of raw inulin was achieved.

Keywords *Kluyveromyces marxianus* · Exoinulinase · Immobilized enzyme reactor · Inulin hydrolysis

Introduction

Owing to consumer awareness towards healthy and natural food, attention towards fructose is gaining momentum. Fructose, which is a sweetest natural carbohydrate, is not only sweeter (1.2–2.0 times) than conventional sweetener sucrose, but also has many health benefits mentioned earlier by many workers [3, 6, 12]. One of the most important health benefits of this sweetener is its metabolism which is independent of insulin and it can be considered as diabetic sugar. It is non-cariogenic, non-corpulence, non-atherosclerosis and it helps in the absorption of iron and zinc in the body. Furthermore, its technical superiorities (humectancy, high solubility, low viscosity, low water activity, smooth consistency, high freezing point depression, etc.) over sucrose are attracting many food as well as pharmaceutical industries. Conventionally, starch is used as a substrate for the production of fructose, but now it is losing its hold due to some accompanied drawbacks such as being a multienzymatic process and its low fructose yield. Research is now diverting towards the development of an alternative technology involving hydrolysis of inulin using inulinase (2,1-β-D fructan fructanohydrolase, EC 3.2.1.7). This glycoprotein targets the β-2,1 linkage of the polymer and splits off terminal fructosyl units, releasing fructose. The complete hydrolysis of inulin by a single step using inulinase gives a yield of about 90–95% fructose. Inulin is potential substrate which is present in abundance in plants belonging to the families Gramineae, Compositeae and Liliaceae. A number of studies have been carried out for the development of immobilized microbial and enzyme systems for the hydrolysis of inulin for preparation of high fructose syrup, but they suffer from low stability especially at higher temperature [4, 10, 11, 17].

In our previous studies, the production of inulinase from *Kluyveromyces marxianus* YS-1 using roots of *Asparagus racemosus* [13], its partial purification and characterization [14] and covalent immobilization with a simple low cost method for the hydrolysis of inulin in a batch system [15]

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has been discussed. The improved thermal stability of immobilized inulinase in a batch system prompted us to investigate its application in a continuous system. Here, we report the development of a highly stable continuous flow immobilized enzyme reactor for the preparation of high fructose syrup from inulin.

Materials and methods

Biocatalyst

Exoinulinase from *Kluyveromyces marxianus* YS-1 was used in the present study. Yeast culture has been deposited in the International Depository Authority of Microbial Type Culture Collection (MTCC), Chandigarh, India and assigned an accession number MTCC 5201. It was originally isolated, identified and maintained by the current authors [16]. Enzyme was produced [13] and partially purified using 85% chilled ethanol followed by Sephadex G-100 column chromatography as described earlier [14]. Partially purified exoinulinase (23.5-fold purification, 413 IU/mg specific activity) was used for immobilization.

Enzyme immobilization

Partially purified exoinulinase was immobilized on Duolite A568 (Courtsy Rohm Haas, France) as described earlier [15]. Briefly, the resin was first modified with glutaraldehyde (1.25%) for 2 h at room temperature to generate an activated support containing carbonyl groups and then incubated with exoinulinase for 24 h. Covalent immobilization of enzyme was based on the formation of Schiff's base between the aldehydic group of glutaraldehyde derivatized resin and amino group of the enzyme. By this method, the immobilized biocatalyst contained 6 IU of exoinulinase/g (wet weight) of resin.

Continuous operation of the immobilized enzyme reactor for the hydrolysis of inulin

A jacketed glass column (1 × 20 cm, GE Healthcare Biosciences Ltd., USA) packed with 12 g (wet weight) of resin containing immobilized exoinulinase (72 IU) was used in the continuous flow reactor. The bed height of the bioreactor was 18 cm. The schematic of the continuous reactor system is shown in Fig. 1. Total volume and void volume were 15.7 and 5 mL, respectively. Void volume was calculated as under:

$$V = V_t - V_s$$

where V = void volume, V_s = volume of support, V_t = total reactor volume.

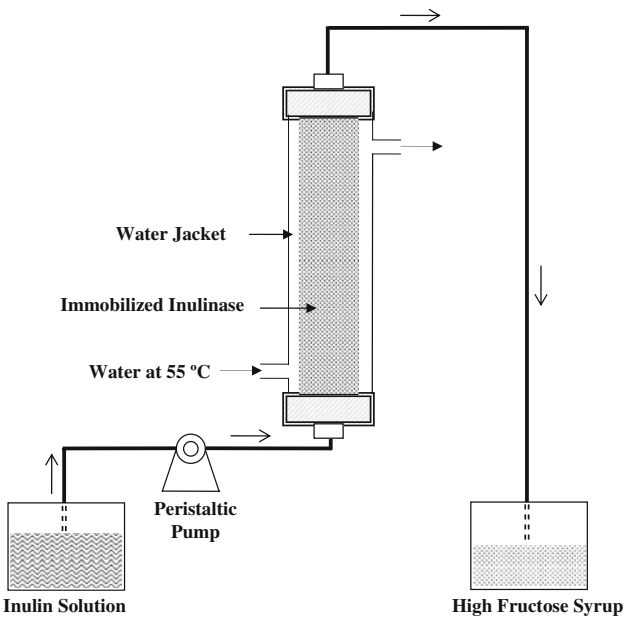

Fig. 1 Schematic layout of the experimental set-up for continuous flow immobilized enzyme reactor

The packed bed reactor was maintained at the desired temperature by circulating water through the outer jacket. The operation of the reactor was facilitated by the upward flow of substrate so that the self compression of the immobilized enzyme causing clogging problems can be minimized. The most important operating parameters affecting the performance of an immobilized enzyme reactor are flow rate, temperature and substrate concentration, which were investigated. To determine the operating conditions necessary for complete hydrolysis, the feeding of inulin solutions (2–12.5%) in the immobilized enzyme reactor was carried out with variable flow rates (2–10 mL/h). Hydrolysis was performed at 45, 50, 55 and 60 °C for each concentration and flow rate of the substrate. The product was analyzed for fructose, glucose and total reducing sugars released. The reactor was equilibrated for 2–10 h according to the residence time at each flow rate to achieve a steady state before the collection of samples for analysis. Residence time and volumetric productivity were calculated as below: Residence time (h)

$$\tau = V/F$$

Volumetric productivity (g/L/h)

$$Q_p = C_p \times F/V$$

where F = flow rate (L/h), V = reactor volume (L), C_p = concentration of reducing sugars released (g/L).

Operational stability

The operational stability of the reactor was assessed by continuously running the immobilized enzyme reactor

under standardized conditions, until enzyme activity of the biocatalyst was reduced to half. For a continuous process, 5% inulin (SD Fine Chemicals, India) in sodium acetate buffer (0.1 M, pH 5.5) was fed continuously into the column using a peristaltic pump (GE Healthcare Biosciences Ltd., USA). The reactor was operated continuously for 75 days by feeding inulin at a flow rate of 4 mL/h. The samples were collected at 6 h intervals and analyzed for the products. An attempt has also been made to study the operational stability of the developed system for the hydrolysis of raw *Asparagus racemosus* inulin. Extraction of raw inulin from the dried roots of *Asparagus racemosus* was carried out as described earlier [14].

Analytical techniques

Enzyme activity was determined by measuring the reducing sugars released from inulin after hydrolysis. 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 biocatalyst and keeping resultant mixture at 100 °C for 10 min. After this the resultant mixture was assayed for 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. The hydrolyzed product was collected in fractions of 5 mL. Reducing sugars in the samples were determined by DNSA method [8]. Total sugars were determined as reducing sugars after acid hydrolysis (H_2SO_4, pH 2, 100 °C, 45 min). The extent of hydrolysis (%) was calculated as (amount of reducing sugars released/amount of total sugars) × 100. Glucose in the samples was assayed colorimetrically with the glucose oxidase peroxidase kit (Sigma Aldrich, USA). Fructose was determined as the difference between the amount of total reducing sugars and glucose.

Results

Enzyme immobilization

Three supports, i.e. chitosan beads, Amberlite IRC-50 and Duolite A568 were screened for immobilization of exoinulinase (data not shown). The support chosen for the further study was Duolite A568, a highly porous phenol-formaldehyde ion exchange resin (particle size 150–600 μm; functional group –N–$(R)_2$). The selection was based on its higher capacity, simple immobilization technique and best operational as well as dimensional stability as compared to other two supports. The developed immobilized system contained 6 IU of exoinulinase per gram of wet resin.

Effect of operating conditions on hydrolysis of inulin

The operational parameters like flow rate and substrate concentration were optimized at different column temperatures (45, 50, 55 and 60 °C) to develop an efficient system for the hydrolysis of inulin. Results are graphically given in Fig. 2 and described as below.

Hydrolysis of inulin at 45 °C

Hydrolysis of inulin (2–12.5%) was monitored at 45 °C with varying flow rates (2–10 mL/h). Results showed that at this temperature, the maximum hydrolysis achieved was 90% at the lowest flow rate of 2 mL/h ($\tau = 2.5$ h) and lowest inulin concentration of 2%. The corresponding volumetric productivity and fructose released were 7.96 g/L/h and 18.4 g/L, respectively. Hydrolysis decreased as the flow rate was increased. The maximum volumetric productivity recorded at this temperature was 133.8 g/L/h at flow rate of 10 mL/h ($\tau = 0.5$ h) against the conversion rate of 48.2%.

Hydrolysis of inulin at 50 °C

An increase in column temperature from 45 to 50 °C increased the overall percent hydrolysis, but total hydrolysis was still not attained. Maximum hydrolysis that could be attained was 98% at the flow rate of 2 mL/h and inulin concentration of 2%. Volumetric productivity and fructose concentration after hydrolysis was 8.12 g/L/h and 20.33 g/L, respectively. An increase in flow rate from 2 to 10 mL/h, reduced the percent hydrolysis from 98 to 71.9%, whereas on the other hand, if inulin concentration was increased to 12.5%, the hydrolysis decreased to 80%.

Hydrolysis of inulin at 55 °C

A complete hydrolysis of 10% inulin was achieved at this temperature with a flow rate of 2 mL/h. Volumetric productivity and fructose concentration after complete hydrolysis of 10% inulin was 44.52 g/L/h and 103.59 g/L, respectively. As the flow rate was increased from 2 to 4 mL/h, the same extent of hydrolysis was achieved at lower inulin (5%) concentration. The corresponding volumetric productivity and fructose concentration were 44.32 g/L/h and 51.1 g/L, respectively. The extent of hydrolysis gradually decreased with further increase in either inulin concentration (above 10%) or in flow rate (4 mL/h) at this temperature.

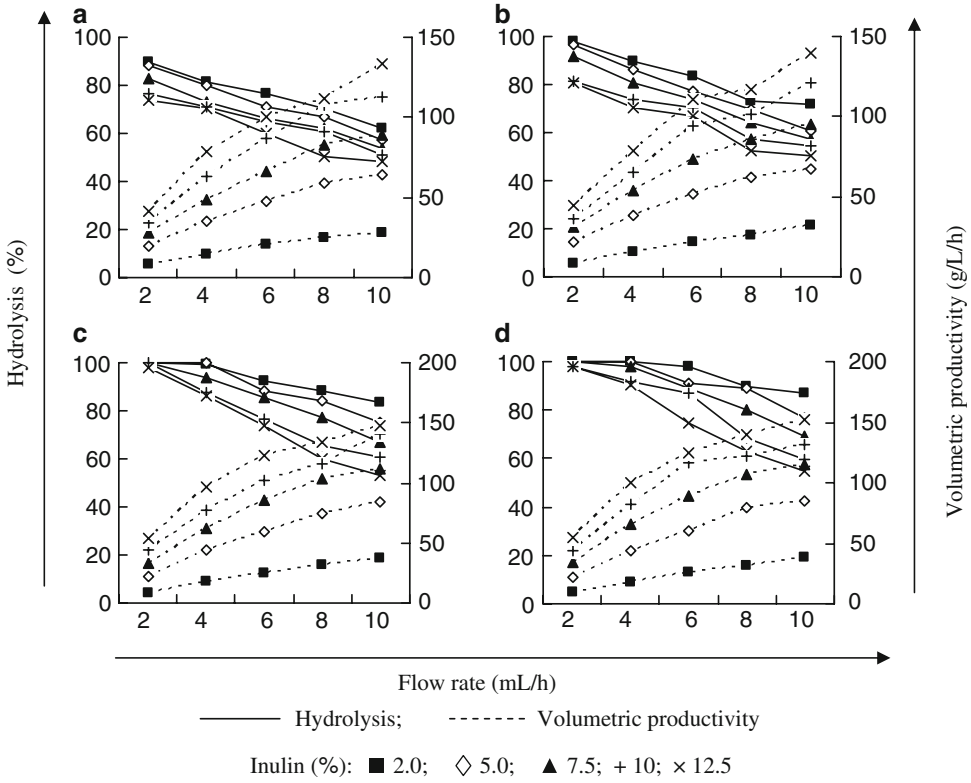

Fig. 2 Inulin hydrolysis as a function of flow rate in an immobilized enzyme reactor. Inulin solutions (pH 5) of different concentrations were hydrolyzed continuously at (**a**) 45 °C, (**b**) 50 °C, (**c**) 55 °C, (**d**) 60 °C

Hydrolysis of inulin at 60 °C

Hydrolysis of inulin (2–12.5%) was also carried out as a function of flow rate above the temperature optima of immobilized exoinulinase. As the temperature was increased, percent hydrolysis also increased and complete hydrolysis of 7.5% inulin was observed at 2 mL/h of flow rate. More than 98% hydrolysis was achieved at inulin concentration of 12.5%. Volumetric productivity and fructose concentration after complete hydrolysis of 7.5% inulin were 33.64 g/L/h and 77.9 g/L, respectively.

Operational stability

The success of any developed bioreactor is dependent largely upon the maintenance of the biocatalytic activity over the operational time. The operational stability of the reaction was studied under the optimized conditions of complete hydrolysis. The operational stability of the immobilized enzyme reactor was assessed by monitoring continuous hydrolysis of 5% inulin solution at a flow rate of 4 mL/h and column temperature of 55 °C. The reducing sugars released in the hydrolyzate were also measured. The results of this protocol are presented in Fig. 3 and the product profile is given in Table 1. The flow rate was corresponding to residence time (τ) of 1.25 h. A complete hydrolysis of inulin to reducing sugars was continued for 5 days and after that it decreased gradually. Even after 14 days of continuous operation, more than 90% hydrolysis was recorded. A slow reduction in inulolytic activity of immobilized enzyme reactor was observed and after 63 days of operation almost 60% hydrolysis of the inulin was still maintained. As the continuous operation proceeded, the inulolytic activity was further decreased. The reactor was operated continuously with a flow rate of 4 mL/h at 55 °C for 75 days and half-life of the reactor

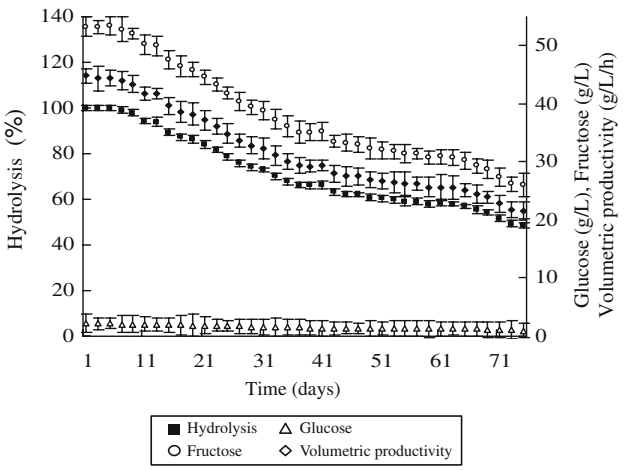

Fig. 3 Operational stability of immobilized enzyme reactor containing exoinulinase from *Kluyveromyces marxianus* YS-1 with pure inulin as substrate. Inulin solution (5%, pH 5.5) was hydrolyzed continuously with a flow rate of 4 mL/h at 55 °C

Table 1 Volumetric productivity and sugars released from pure inulin solution (5%, 4 mL/h) during the operation of immobilized enzyme reactor at 55°C

Operational days	Hydrolysis (%)	Volumetric productivity (g/L/h)	Fructose (g/L)	Glucose (g/L)	*F/G*
1	100	44.5	53.3	2.22	24.0
11	94.4	41.8	50.2	2.09	24.0
21	83.9	37.2	44.6	1.88	23.7
31	73.0	32.4	38.9	1.59	24.4
41	66.3	29.4	35.3	1.4	25.2
51	60.3	26.7	32.0	1.31	24.4
61	58.1	25.7	30.9	1.28	24.1
71	51.6	22.8	27.4	1.14	24.0

calculated was 72 days. Further, the operational stability of the system was investigated using the raw inulin from *Asparagus racemosus* roots (Fig. 4). Pure inulin was substituted with raw inulin and hydrolysis was carried out under similar operational conditions. A complete hydrolysis of raw inulin was continued for 3 days of operation and thereafter it decreased. The system was continuously operated for 11 days where 85.9% hydrolysis of raw inulin was recorded. But after this, a decrease in flow rate of the substrate was observed which may be due to pore blockage of the carrier. Ultimately, the bioreactor became non-operational, because of complete blockage. Volumetric productivity and fructose yield in the initial batches were 44.16 g/L/h and 49.7 g/L, respectively from raw inulin.

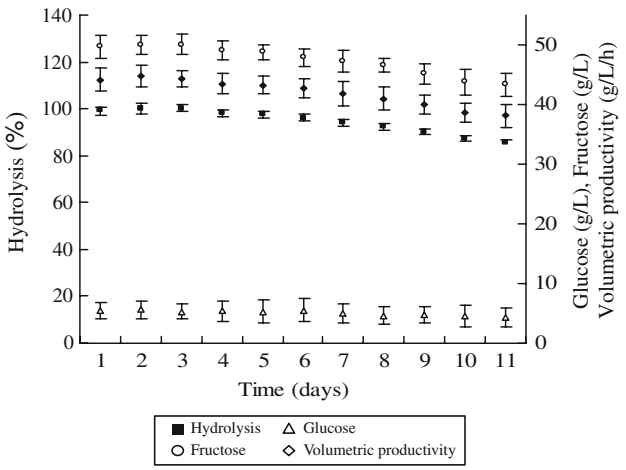

Fig. 4 Operational stability of immobilized enzyme reactor containing exoinulinase from *Kluyveromyces marxianus* YS-1 with raw *Asparagus racemosus* inulin as substrate. Inulin solution (5%, pH 5.5) was hydrolyzed continuously with a flow rate of 4 mL/h at 55 °C

Discussion

The results obtained were very encouraging and supports the feasibility of inulin hydrolysis in a continuous flow immobilized enzyme reactor for the preparation of high fructose syrup. The covalent immobilization of exoinulinase on Duolite A568 has been found very effective in terms of enzyme stability. The repeated use of immobilized enzyme in successive batches discussed earlier [15] has formed the basis of continuous mode of operation of immobilized biocatalyst.

Since packed bed reactors are continuing to dominate the large scale industrial applications of immobilized enzymes, it was selected for the hydrolysis of inulin. The column temperature is an important factor in a reactor to maintain initial enzyme activity for a longer operational time. Although, the optimum temperature of immobilized enzyme activity was 55 °C [14], low column temperatures were also studied to prevent loss of activity during prolonged operational period. However, at lower working temperatures, the conversion yields were also lower. At 55 °C, a complete hydrolysis of 10 and 5% inulin solutions was achieved at flow rates of 2 and 4 mL/h, respectively with the similar volumetric productivity (44 g/L/h approx.). However, higher flow rate (4 mL/h) was preferred to prevent column contamination as well as product inhibition. A complete hydrolysis and better volumetric productivity were also obtained at a column temperature of 60 °C, but characterization of immobilized exoinulinase has proved that enzyme is more prone to deactivation at this temperature [15] and it was avoided. Hence, hydrolysis of 5% inulin with a flow rate of 4 mL/h at a column temperature of 55 °C were chosen as best conditions for the complete hydrolysis of inulin in a packed bed reactor.

Half-life of the immobilized enzyme reactor was also determined. Activity loss can be resulted from enzyme denaturation, pore blockage and physical loss of enzyme from the carrier due to erosion or from the fracture of the bonds between enzyme and carrier. Since, factors other than thermal deactivation may also be responsible for the low operational stability especially in case of packed bed reactors, the half-life was not calculated theoretically. The reactor was run continuously for 75 days under the standardized operational parameters and half-life of the reactor calculated was 72 days. There was no microbial contamination observed in the column over the operational time. To the best of our knowledge, this is the longest half-life of the immobilized enzyme packed bed reactor for the hydrolysis of inulin reported so far. The literature survey reveals several continuous systems for the production of HFS using whole cells or enzyme immobilization. The reported performances of the reactors were compared with our results with respect to the efficiency and stability of the

reactor, especially at higher temperature. An immobilized yeast cell reactor was operated at a dilution rate of 1.65/h for 240 h at 50 °C with 90% conversion of inulin [1]. The operational half-life of 30 days has been reported for the molecular sieve bound inulinase reactor operated at 55 °C [2]. Inulinase from *Aspergillus ficuum* immobilized on chitin was used for the continuous production of HFS. The column was operated only for 2 weeks at a residence time of 2.6 h at 40°C for the hydrolysis of Jerusalem artichoke inulin [7]. Inulinase from *Aspergillus niger* immobilized on Amino-Cellulofine was used for the continuous hydrolysis of 5% inulin and 16 days half-life of the reactor at 60 °C has been reported [9]. The half-life of the packed bed column reactor for the hydrolysis of 4.5% Jerusalem artichoke fructans at 50 °C has been reported 32 days [17]. Recently, a half-life of 42 days for the ConA-linked amino activated silica bead immobilized inulinase reactor operated at 60 °C has been reported, but the productivity of the reactor was 3.4 g/L/h [5]. The experimental data gathered and its comparison with the earlier studies highlights the operational and mechanical stability of the developed continuous flow immobilized enzyme reactor for the hydrolysis of inulin.

Conclusions

The immobilized biocatalyst was successfully used in a continuous flow reactor for the hydrolysis of inulin. The half-life of the developed system was 72 days at 55 °C, which is very encouraging. A literature survey reveals no reports of such a long period for the hydrolysis of inulin in a continuous system. The remarkable operational and mechanical stability of the biocatalyst suggests that it is a promising candidate for the development of a highly effective set-up on higher scale for the preparation of high fructose syrup in a continuous system.

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