Sucrose Hydrolysis in a Continuous Packed-Bed Reactor with Auto-Immobilised Aspergillus Niger Biocatalyst Obtained by Solid-State Fermentation

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Abstract

Invertase from *Aspergillus niger* C28B25 was produced by solid-state fermentation (SSF), and fermented solids were used directly as a biocatalyst for batch and continuous hydrolysis of sucrose in a packed-bed reactor under different operational conditions with varied temperature, sucrose concentration and feed flow rate. The SSF allowed obtaining a biocatalyst with an invertase activity of 82.2 U/g db. The biocatalyst maintained its activity in the range of 40 to 70ºC for at least 70 h of continuous operation. The highest hydrolysis rate (12.3 mg/U h) was obtained at 70ºC with 2M sucrose under batch conditions. Continuous hydrolysis in 20-mL and 200-mL bioreactors at 60ºC led to sucrose hydrolysis above 60% (8.5 residence times) and above 55% (4.5 residence times), respectively. The auto-immobilised biocatalyst produced by SSF without recovery, purification and immobilisation stages offers an economical alternative for the development of accessible biocatalysts that can be applied in batch or continuous sucrose hydrolysis processes. This study shows the potential of biocatalyst production by SSF for other enzymatic systems.

1. Introduction

Invert sugar syrups are produced by different methods and are mainly used in beverages and in the food industry. Acid hydrolysis at high temperature and pressure has been performed [1]; however, the use of this technique generates undesirable colour products in addition to other drawbacks [2]. Strongly acidic ion exchange resins have been used to decrease colour intensity in products and to improve process costs [1]. Different forms of zeolite have also been used for sucrose hydrolysis [3]. Sulfonic acid membranes and commercial ionic exchange resins [4] have been used for sucrose hydrolysis, showing that the catalytic activity of the sulfonic acid membrane is higher than that of commercial ion exchange resins. Nevertheless, the catalytic activity of the membranes depends on the temperature reaction and the sulfonic acid concentration groups in the membrane [4].

Invert sugar syrups from sucrose are also produced by enzymatic hydrolysis [2, 5]. Invertase (β-D-fructofuranosidase E.C. 3.2.1.26) hydrolyses sucrose into glucose and fructose under mild conditions as compared to chemical hydrolysis, preventing colour formation in products and producing high-quality invert sugar syrups [1].

Invert sugar is not the only important product of invertase. The production and application of fructooligosaccharides obtained from hydrolysis of inulin by invertases and inulinases produced by *A. niger* strains have tremendous commercial importance due to their functional properties as prebiotics [6]. Some of these fructooligosaccharide products improve the intestinal microflora, relieve constipation, reduce cholesterol and lipid concentrations, promote animal growth and are low-calorie sweeteners [6].

Invertase is one of the most used enzymes in the food industry and used to produce lactic acid from molasses [7]. Invertases are also used for the hydrolysis of sucrose for ethanol production, preventing the formation of sorbitol during the fermentation process [8]. Invertase is produced commercially mainly by

Saccharomyces cerevisiae and fungal strains of the genus *Aspergillus*, such as *A. niger*, *A. oryzae* and *A. ficcum* [9]. Although invertase is intracellularly produced by yeast, it is produced both intra- and extracellularly by *Aspergillus*. For instance, multiple forms of invertase have been found in *A. niger* CCT 7415, and 40 to 60% of the total activity was found intracellularly [9].

Invertase is classified within the high-demand soluble enzymes. However, the use of enzymes as catalysts for large-scale industrial processes can be limited by their high production cost and low stability [10]. Different alternatives are being developed to minimize these limitations, such as enzyme immobilisation [10] and the use of different emerging technologies [11, 12, 13]. Immobilisation of cells with intracellular invertase can offer economic advantages compared with soluble invertase. Furthermore, the use of immobilised cells allows the continuous hydrolysis of sucrose [10]. Solid-state fermentation (SSF) and submerged fermentation (SmF) are the main processes for producing enzymes [14]. One of the main advantages of SSF to produce invertase is to directly apply the fermented solids as biocatalysts in hydrolysis reactions under batch or continuous operation conditions [12].

For that, we evaluated the production of invertase in SSF and the direct use of the fermented solids as biocatalysts for the hydrolysis of sucrose in a packed bed reactor operating in batch and continuous conditions. This approach allows avoiding some downstream steps such as extraction and separation, as well as further enzyme immobilisation.

2. Materials And Methods

2.1 Invertase production

2.1.1 Microorganisms

Aspergillus niger C28B25, belonging to the collection of the Department of Biotechnology of the Metropolitan Autonomous University Iztapalapa, was used. This strain has previously been reported as invertase producer [15, 16]. For our study, it was maintained in 20% glycerol at -20ºC.

2.1.2 Inoculum preparation

The strain was first propagated in slants with potato dextrose agar (PDA, Bioxon). After that, 5 mL of a sterile solution of 0.05% (v/v) Tween 80 was used to recover the spores from the slants, and 1 mL of the spore suspension was used to inoculate 50 mL of PDA contained in 250-mL Erlenmeyer flasks. After incubation for 7 days at 30°C, the produced spores were recovered with 20 mL of 0.05% (v/v) Tween 80 and used as inoculum for invertase production by SSF. Spore counts were done with a Neubauer chamber after adequate dilution of the original spore suspension.

2.1.3 Culture medium

The culture medium for invertase production by SSF was the Pontecorvo (ATCC 687) medium with 0.05% yeast extract [17]. The medium was first concentrated 16.66 times and then diluted to the given sucrose

concentration. The composition of the concentrated culture medium was as follows (in g/L): sucrose 500, yeast extract 8.33, $NaNO_3$ 100, KH_2PO_4 25.33, KCl 8.67, $MgSO_4 \cdot 7H_2O$ 8.67, $ZnSO_4 \cdot 7H_2O$ 0.02, $FeCl_3 \cdot 6H_2O$ 0.01, with 1 mL of trace element solution. The composition of the trace element solution was as follows (in g/L): $Na_2B_4O_7 \cdot 10H_2O$ 1.67, $MnCl_2 \cdot 4H_2O$ 0.83, $Na_2MoO_4 \cdot 2H_2O$ 0.83, $CuSO_4 \cdot 5H_2O$ 4.17. To dissolve the reactants of the trace element solution, the pH was adjusted to 3.5 with 10% HCl. All reagents were obtained from J. T. Baker.

2.1.4 Invertase production by SSF

Perlite, used as an inert support for the SSF, was previously sieved to achieve a particle size of 0.8–1.19 mm. After that, it was washed twice with hot tap water and then twice with distilled water; subsequently, it was dried at 60°C for 24 h and maintained in the dark until use.

For SSF, 100 g of dry perlite (g dp) was mixed with 150 to 200 mL of the previously inoculated culture medium (3×10^7 spores/g dp) to attain a moisture content near 60%. The initial sucrose concentration in the culture media was 200 mg/g dp. Twenty grams of inoculated solid fermentation medium was packed in tubular bioreactors (glass columns with 2 cm of internal diameter and 20 cm of height). The tubular bioreactors were placed in a water bath at 30°C with an airflow of 100 mL/min. Samples were taken at 40, 45 and 60 h of cultivation to quantify the enzyme activity by measuring the release of reducing sugars by the Miller assay [18].

2.2 Biocatalyst production

To obtain the dry biocatalyst (db), the fermented solid in the bioreactor was air-dried (atmospheric air at 500 mL/min for 24 h) to a final moisture content below 1.7%. Finally, the biocatalyst was taken out from the bioreactor and stored at room temperature until further activity assay and sucrose hydrolysis processes.

2.3 Continuous hydrolysis

Sucrose hydrolysis was continuously performed in two stainless-steel packed-bed reactors (one with 1 cm in diameter and 25 cm in height and the other with 3 cm in diameter and 30 cm in length). A peristaltic pump (Constametric 3200) was used to feed sucrose solutions to the reactor; the temperature was controlled with a furnace (TC-50 Eppendorf). A sample collector (Bio-Rad 2110) with tubes containing 2 mL of NaOH-$ZnSO_4.7H_2O$ suspension to stop the enzymatic reaction was used. Different reaction temperatures (40 to 80°C) and different sucrose concentrations (0.1 to 2.0 M) at a constant feed flow rate were evaluated. The released reducing sugars were assayed by the method of Miller [18]. The hydrolysis rate was defined as the amount of reducing sugars (mg) released per unit (U) of enzyme activity per hour (mg/Uh).

2.4 Analytical methods

2.4.1 Enzymatic activity

To obtain the enzymatic extract, 0.5 g of biocatalyst was added with 5 mL of distilled water, stirred with a magnetic bar for 5 min and filtered (Whatman 41). Sucrose (0.1 M) in 0.1 M acetate buffer at pH 5 was used as substrate for enzymatic activity assays. Test tubes with 0.9 mL of the substrate and incubated at 50°C were added with 0.1 mL of enzyme extract; after 10 min of incubation, 1.5 mL of DNS reagent was added. All tubes, both for the standard curve and the enzymatic assay, were placed in a boiling water bath for 15 min. Finally, absorbance was read in a Perkin Elmer Lambda 25 UV-Visible spectrophotometer at a wavelength of 640 nm. A standard curve was generated with 250 mg of glucose and 250 mg of fructose in 0.1 M acetate buffer at pH 5. Invertase activity was determined through the release of reducing sugars determined by the Miller method [18]. One unit of enzymatic activity (U) was defined as the amount of enzyme needed to release one micromole of reducing sugars per minute.

2.4.2 Reducing sugars

The reducing sugars were determined to calculate the hydrolysis rate and quantify the continuous hydrolysis of sucrose. A standard curve was generated as described in Sect. 2.4.1. To determine the reducing sugars, a 1:1,000 dilution was made; from these dilutions, 1 mL was taken and 1.5 mL of DNS reagent [18] was added, the tubes were placed in a boiling water bath for 15 min. Absorbance was read at 640 nm on a Perkin Elmer Lambda25 UV-Visible spectrophotometer.

2.4.3 Enzyme deactivation

The enzymatic activity assay was stopped by the addition of 2 mL of a suspension of NaOH-$ZnS0_4.7H_2O$ prepared with 1.2 g of NaOH, and 4.88 g of $ZnS0_4.7H_2O$ was used in 3 mL of enzyme extract.

3. Results And Discussion

This section presents the results obtained to produce the biocatalyst, the effect of the substrate concentration on the hydrolysis of sucrose in batch reactors and the effect of different factors on the hydrolysis of sucrose in packed-bed reactors under continuous operation.

3.1 Invertase production by SSF

Invertase production was performed by SSF in tubular bioreactors. To determine the incubation time for the maximum invertase production, samples were taken at 40, 45 and 60 h of culture. Maximum invertase activity (82.2 U/g db) was reached at 45 h. Invertase can be produced by various organisms, including some animals, plants, bacteria, yeasts and fungi [19]. Due to the importance and widespread application of invertase in various industries, its isolation from natural sources is not affordable. Instead, it is recommended to use yeasts, fungi or bacteria to produce invertase [19].

Table 1 shows some sources of invertase, the enzyme activity and the culture time to reach the maximum enzyme activity. Different studies of invertase production with yeast have shown a range of enzymatic activity of 38–118 U/mL and a culture time of 30–48 h. The results obtained in this work are within this range (82.2 U/g db in 45 h). The enzymatic activity is similar to that reported for *A. niger* ATCC 20611

(100.6 U/mL) cultured in submerged fermentation with sucrose as carbon source [25]; however, this activity was attained within 96 h. We selected 45 h as the culture time to produce the biocatalyst, since at 40 and 60 h, the invertase activity is lower, with 53.7 and 69.3 U/g db, respectively.

3.2 Biocatalyst production

In this work, *A. niger* biocatalyst production was performed in 10 tubular bioreactors incubated for 45 h, as indicated in Section 3.1. At the end of the culture, the fermented solids were air-dried to obtain the biocatalyst with a moisture content lower than 1.7%. After analysis of the extracellular and the total enzymatic activity in the biocatalysts, 50% of the activity was found extracellular. This extracellular fraction was washed during the successive batch and the continuous processes. This extra/intracellular activity was similar to that reported by Vargas et al. [9]. The auto-immobilised (total) invertase activity (40.6 U/g db) was lower than that reported by Prodanovic et al. [28] (5,500 U/g), Mansfeld et al. [1] (1,000 U/g) Szymanskaet et al. [29] (600 U/g) and Cabrera et al. [2] (179 U/g). However, all these values correspond to the enzymatic activity obtained after downstream steps and enzyme immobilisation.

3.3 Batch hydrolysis

Batch studies were performed to determine the effect of temperature (from 20 to 80ºC) on the sucrose hydrolysis rate. Experiments were conducted in test tubes containing 50 mg of biocatalyst and 1 mL of substrate (0.1 M sucrose in 0.1 M acetate buffer at pH 5). After 60 min of the enzymatic reaction, we added 9 mL of 0.04 N H_2SO_4 to stop the reaction. The reducing sugars release increased with temperature, with a maximum value at 70ºC and a strong reduction at 80ºC (Fig. 1). The reducing sugar release rate increased 3.3 times from 20 to 70ºC (Table 2). Based on the velocity data from 20 to 50°C, the activation energy (E_a) was calculated. The Q_{10} coefficient was calculated from 20 to 30°C, and the obtained value (1.49) indicates that the reaction rate increases by 49% when the temperature is changed from 20 to 30°C. The E_a value (29.7 kJ/mol) corresponds to the typical values of activation energy for diffusive processes (25.1–30.1 kJ/mol) in which mass transfer limitations are suspected. This profile was similar to that obtained with the invertase from *C. utilis* [30]. In our study, invertase showed activity in the interval of 30–90°C and reached its maximum activity at 70°C. The invertase produced by *Saccharomyces cerevisiae* SAA-612 was stable between 30 and 60°C, with a half-life between 65 h and 30 min [20]. The invertase produced by *Azotobacter chroococcum* had an optimal sucrose hydrolysis temperature of 45°C; however, at 30°C the enzyme had 90% of its maximum hydrolysis rate [31].

In the case of invertase produced by *Aspergillus* strains, the temperature at which they present their highest activity is lower than that reported in this work; for example, the temperature for the highest invertase activity for *A. terreus* is 60°C [24], similar to that reported for the invertase produced by *A. niger* IBK1 [26]. A temperature above 60ºC is preferable for industrial invert sugar production to reduce the viscosity of the solutions and decrease the risk of contamination [5]. Based on our results, we determined the temperature range where invertase activity was observed; in addition, the naturally immobilised invertase produced by *A. niger* C28B25 was capable of hydrolysing sucrose solutions.

3.4 Continuous hydrolysis with different sucrose concentrations

Once the impacts of the reaction temperature on the enzyme stability and the sucrose hydrolysis rate were evaluated under batch conditions, the effect of the sucrose concentration and the feed flow rate on the hydrolysis rate and sucrose conversion efficiency were evaluated under the continuous feed of a 5-mL stainless-steel bioreactor. Sucrose (from 0.1 to 2 M) was fed at 6 mL/h for 40 h at 40ºC to reduce enzyme denaturation. The reducing sugar release increased from 0.1 to 1.8 M sucrose (Fig. 2), and the release rate increased 5.6 times from 0.1 to 2 M sucrose (Table 3). These results suggest that the hydrolytic reaction is not limited by any enzyme inhibition mechanism (Table 3). Concentrated sucrose solutions [near 2 M] are recommended for industrial purposes [28]. It has been observed that the sucrose concentration has a marked effect on free invertase; a sucrose concentration greater than 50 g/L decreases the activity of the free enzyme [32]. The biocatalyst produced by SSF could hydrolyse concentrated sucrose solutions (2 M) at 40°C; in Section 3.3, the temperature interval for the batch hydrolysis of sucrose was established. However, it is necessary to know the effect of temperature for the continuous hydrolysis of sucrose.

3.5 Continuous hydrolysis at different temperatures

To determine the effect of temperature (from 40 to 80ºC) on the enzymatic hydrolysis under a continuous operation regime, a 20-mL stainless-steel bioreactor was used. The substrate solution (2 M sucrose) was fed at a flow rate of 6 mL/h for 40 h. The release of reducing sugars increased when the temperature increased, reaching the highest production at 70°C in a bioreactor continuously for 40 h (Fig. 3). This result is important if we consider the origin of the catalyst used in this work. For example, Soares et al. (2019) [13] used a stirred tank reactor operating under batch conditions with ultrasound to hydrolyse sucrose with commercial invertase produced by *Saccharomyces cerevisiae*. The authors report a production similar to that found in our study (Fig. 3), albeit with a lower production temperature (55°C). Further, the period for which it remained stable was 8 times shorter than that reported in this work (40 h). In all cases, the initial hydrolysis was close to 100%. Subsequently, a decrease was observed in the conversion for all conditions studied, most likely because of the extracellular invertase present in the biocatalyst, representing nearly 50% of the total activity (see Section 3.2). These temperatures are similar to those used for the continuous hydrolysis of sucrose with yeast cells immobilised on wool, where temperatures between 40 and 70°C have been used, finding the highest conversion of sucrose at 70°C in a reactor with 1.2 cm in diameter and 220 cm in length, with a flow of 204 mL/h and a constant hydrolysis not below 50% for 60 days [5].

The invertase immobilised in polystyrene for the continuous hydrolysis of sucrose showed a range similar to that used in this work (40 to 70°C), although the reactors used with invertase immobilised in polystyrene are of greater volume (0.3 and 50 L) [1]. For the immobilised enzyme of *Cladosporium cladosporioides* [33], the temperature higher conversion of sucrose is similar to that reported for this work (70°C); however, the sucrose concentration and the period with stable enzyme (5% and 3 h, respectively) were lower than those found for the naturally immobilised enzyme from *A. niger* by SSF.

3.6 Continuous hydrolysis in a 20- and 200-mL reactor

When the residence time increases, the contact period between the enzymes and the substrates is longer, resulting in higher conversions. First, the effect of the feeding rate (1.2 and 3.6 mL/h) on continuous hydrolysis was evaluated. The 20-mL stainless-steel bioreactor with 4.48 g of dry biocatalyst was used at 60°C, with 2 M sucrose and a flow rate of 1.2 mL/h (hydraulic residence time 16.67 h). A baseline near 100% hydrolysis (Fig. 4a) was achieved. After 20 h, hydrolysis remained constant at 58.9 ± 5.2% after 140 h operation (representing more than 8 residence times). At 70 h of operation, the feed flow was increased to 3.6 mL/h (hydraulic residence time 5.56 h). A decrease in conversion was observed and recovered from 120 h of continuous operation (Fig. 4a). This behaviour is similar to that reported by Mukherjee et al. [34], namely that invertase immobilised on silica monoliths with hierarchical structure pores for hydrolysis continuous sucrose. The authors reported that when decreasing the residence time of 3.5 s to 0.4 s, conversion decreased from 88 to 44%.

In our study, hydrolysis was sensitive to small changes in the flow rate. To reduce fluctuations in flow rate, it was decided to use a larger column (200 mL) packed with 51.1 g of biocatalyst with a flow of 12 mL/h (hydraulic residence time 16.67 h). A similar behaviour was observed (Fig. 4b), with initial hydrolysis of 100% and a rapid decrease, reaching 55.4 ± 4.8% of sucrose hydrolysis for a period of 75 h of continuous operation (approximately 5 residence times). By increasing the reactor volume, hydrolysis was not affected.

Cell systems with immobilised invertase to hydrolyse sucrose can provide economic advantages over systems with free enzymes, also achieving hydrolysis percentages as high as those reported for the auto-immobilised enzyme *Cladosporium cladosporioides*, reaching 94.2% of hydrolysis at 70°C [33], greater than that reported for the enzyme used in this study. However, the sucrose concentration used and the time that the enzyme remained stable were considerably decreased (5% sucrose and 3 h, respectively). In systems with commercial enzymes, sucrose hydrolysis was studied in continuous experiments with yeast-immobilised wool [5], achieving a hydrolysis of 2.0 M sucrose not less than 50% at 70°C, with flows of 180–240 mL/min and a retention time of approximately 9 min. These systems remained stable for a period of 30 days, after which this percentage decreased. With immobilised invertase in polystyrene [1], hydrolysing sucrose solutions up to 2.5 M, 80% hydrolysis and a stability period of 360 days could be achieved. Recently, Szymanska et al. [29] immobilised invertase in silica monoliths with hierarchical structure pores and an activity of 600 U/mL for continuous hydrolysis of sucrose; the system remained stable for 2 weeks.

When directly applying enzymes produced by SSF naturally immobilised, the results were superior to those reported by Buenrostro-Figueroa et al. [35], who found the greatest conversion (32%) early in the process, with a decrease to 19% for 4 h of continuous operation at a concentration of 0.1% substrate w/v. In our case, the initial conversion was greater than 90% in most cases, and more than 50% retained activity for 70 h continuous operation with a substrate concentration of 68% w/v. Both studies were conducted in a packed-bed reactor. It should be mentioned that although the method of producing the

enzymes was similar, the produced enzymes and substrates used were not the same, which might explain the large differences between the two studies.

4. Conclusions

A high-sucrose solution was enzymatically hydrolysed under batch and continuous processes with a naturally immobilised invertase produced by solid-state fermentation with *Aspergillus niger*. This naturally immobilised biocatalyst allowed to continuously hydrolyse concentrated sucrose solutions for prolonged periods at high temperatures. The production of this robust and stable biocatalysts does not require additional recovery, purification, and immobilisation steps. These characteristics are outstanding, and the obtained biocatalysts can be applied in industrial processes since they are stable, active and can be applied directly in batch or continuous operating systems.

Declarations

- **Ethical Approval**. Not applicable.
- **Consent to Participate.** All authors gave their consent to participate in this research.
- **Consent to Publish.** All authors gave their consent to submit this research for publication.
- **Authors Contributions.** The study was conceived, done, and written by AMR and EFT. All authors: AMR, LTC, CNA, GSC, and EFT participated in the preparation of the study, discussion, critical review of the draft of the manuscript, and revision of the final version of the manuscript. All authors read and approved the final manuscript.
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- **Competing Interests.** All authors have read and approved this manuscript and have declared that they have no conflicts of interest.
- **Availability of data and materials.** Not applicable

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Tables

Table 1. Different carbon sources for microbial invertase production.

Source	Genus	Carbon source	Enzymatic activity (U/mL)	Cultivation time (h)	References
Yeast	*Saccharomyces cereviciae* SAA-612	Sucrose	118	30	[20]
	Pichia sp BCCS M1	Sucrose	38.71	48	[21]
	Saccharomyces cerevisiae, *Hanseniaspora uvarum*	White sugar	115.5	14 (days)	[22]
	Cryptococcus laurentii MT-61	Sucrose	39.1	30	[23]
Fungus	*Aspergillus terreus*	Sucrose	1	24	[24]
	Aspergillus niger ATCC 20611	Sucrose	100.65	96	[25]
	Aspergillus niger IBK1	Pineapple peels	24.20	120	[26]
	Cladosprium herbarum ER-25	Molasses	36.4	96	[27]
	***Aspergillus niger* C28B25**	**Sucrose**	**82.22**	**45**	**This work**
Bacteria	*Komagataeibacter spp, Lactobacillus spp*	White sugar	115.5	14 (days)	[22]

Table 2. Initial enzymatic sucrose hydrolysis rate at different temperature in a 20 mL packed bed reactor.

Temperature (°C)	Sucrose hydrolysis initial rate (mg/g db min)
20	3.31
30	4.71
40	6.03
50	10.78
60	11.76
70	**11.85**
80	0.174

Table 3. Initial enzymatic sucrose hydrolysis rate at different sucrose concentrations in a 20 mL packed bed reactor.

Concentration (M)	Sucrose hydrolysis initial rate (mg/g db min)
0.1	1.85
0.5	5.42
1.0	8.33
1.5	11.21
2.0	**12.13**

Figures

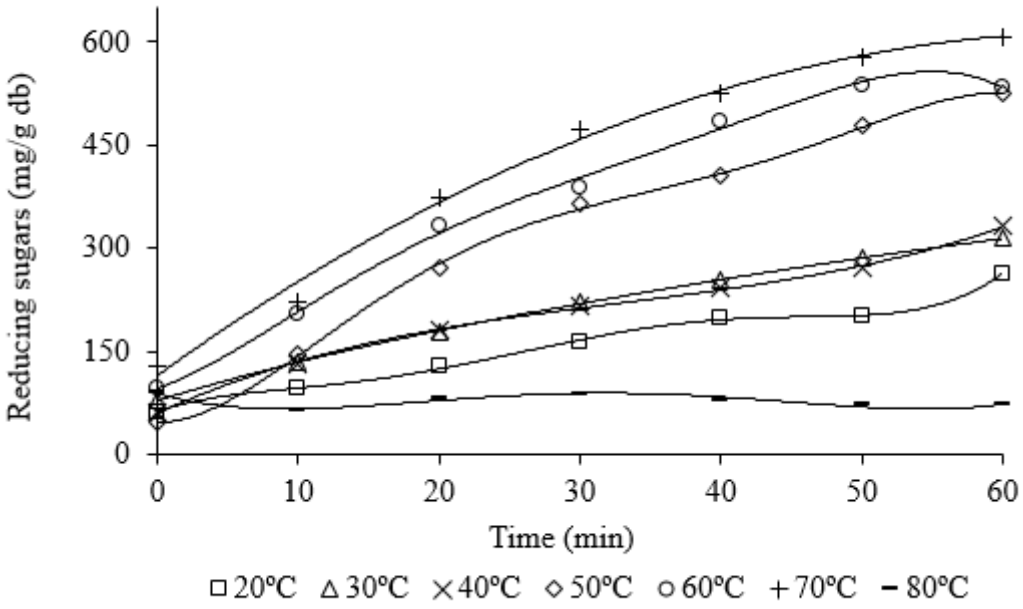

Figure 1

Effect of temperature on enzymatic sucrose hydrolysis (□ 20, Δ30, × 40, ◊ 50, ● 60, + 70, and − 80 ºC).

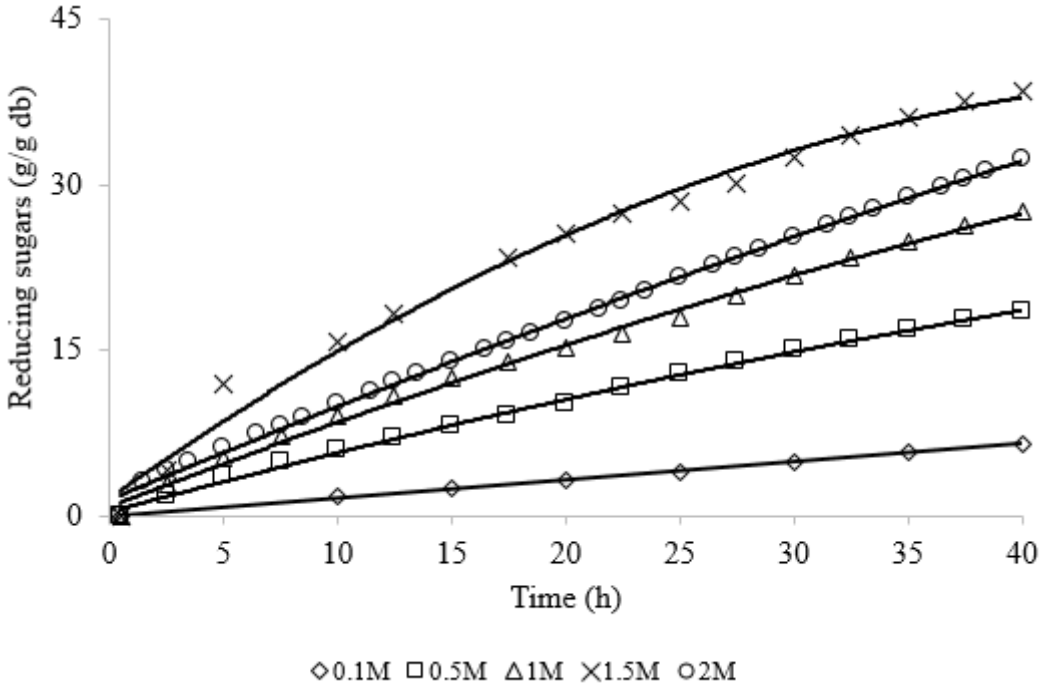

Figure 2

Effect of sucrose concentration (◊ 0.1, □ 0.5, Δ 1.0, × 1.5, and ● 2.0 M) on the initial rate of the enzymatic sucrose hydrolysis in a 20 mL packed bed reactor.

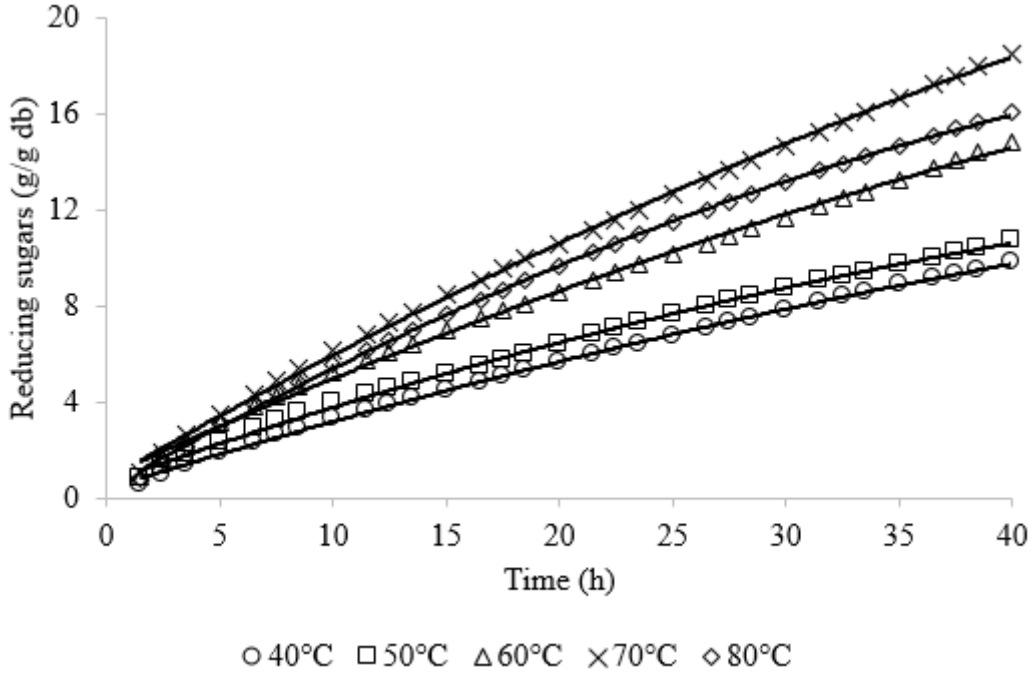

Figure 3

Effect of temperature (● 40, □ 50, Δ 60 × 70, and ◊ and 80 °C) on the enzymatic hydrolysis of 2 M sucrose under continuous conditions.

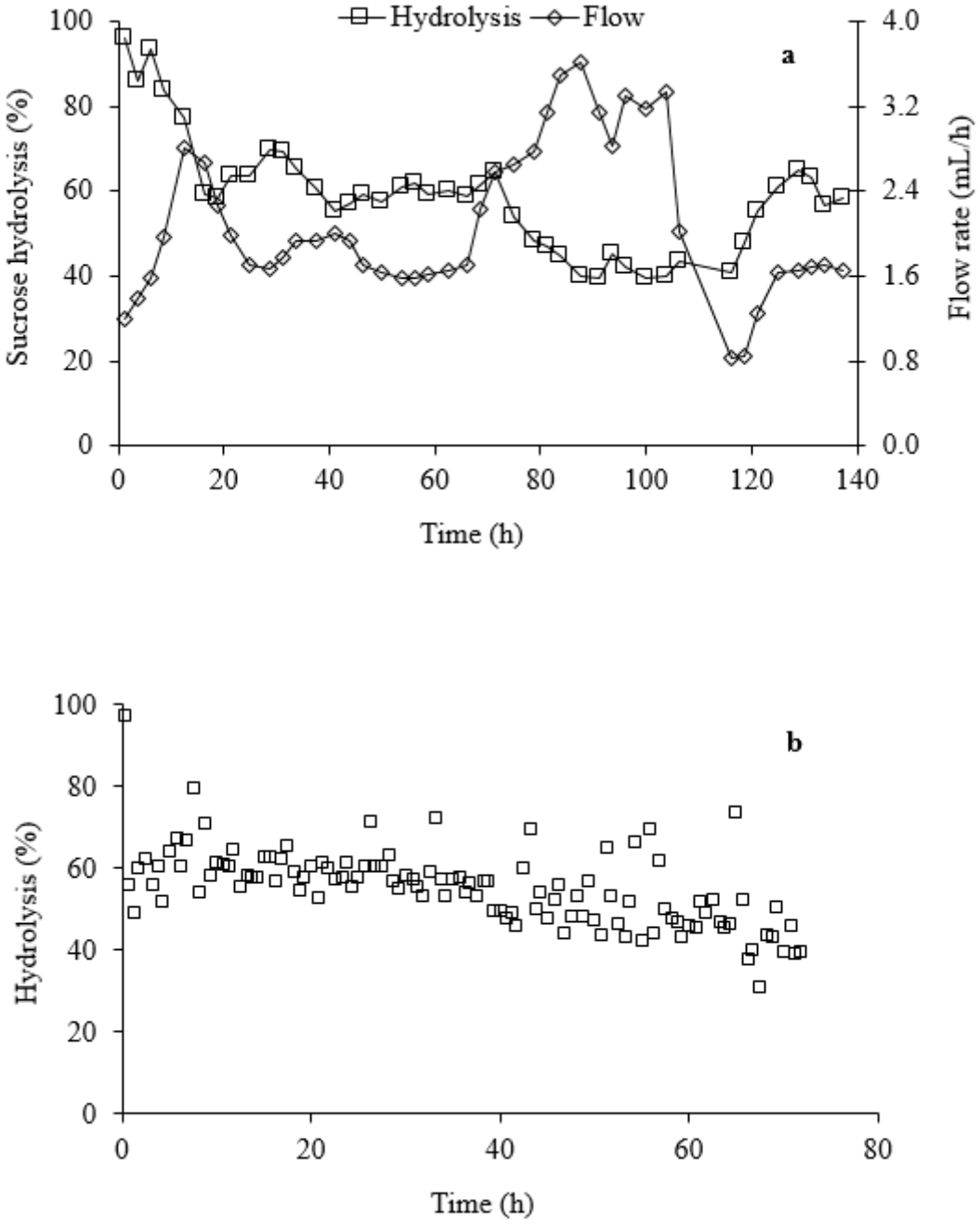

Figure 4

Enzymatic hydrolysis of 2 M sucrose in (a) 20 mL and (b) 200 mL packed bed bioreactors operating at 1.2 and 12 mL/h respectively at 60 °C.