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Research article

Scaling-up batch conditions for efficient sucrose hydrolysis catalyzed by an immobilized recombinant *Pichia pastoris* cells in a stirrer tank reactor

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ABSTRACT

Background: Invert sugar is used greatly in food and pharmaceutical industries. This paper describes scaling-up batch conditions for sucrose inversion catalyzed by the recombinant *Pichia pastoris* BfrA4X whole cells expressing *Thermotoga maritima* invertase entrapped in calcium alginate beads. For the first time, we describe the application of a kinetic model to predict the fractional conversion expected during sucrose hydrolysis reaction in both, a model and a prototype bioreactor with 0.5- and 5-L working volume, respectively.
Results: Different scaled-up criteria used to operate the 0.5-L bioreactor were analyzed to explore the invert sugar large scale production. After model inversion studies, a 5-L scaled-up reaction system was performed in a 7-L stirred reactor. Both scaled-up criteria, immobilized biocatalyst dosage and stirring speed, were analyzed in each type of bioreactors and the collected data were used to ensure an efficient scale-up of this biocatalyst.
Conclusions: To date, there is not enough information to describe the large-scale production of invert sugar using different scaled-up criteria such as dose of immobilized biocatalyst and stirring speed effect on mass transfer. The present study results constitute a valuable tool to successfully carry out this type of high-scale operation for industrial purposes.

1. Introduction

An ideal enzymatic biocatalyst for industrial invert sugar manufactu ring should optimally operate in a highly concentrated sucrose solution (60%, w/v) to minimize microbial contamination and to avoid an initial dilution and final concentration steps. Concentrated sucrose solutions however, can reduce the diffusion of substrate into immobilized systems [1]; thus, the biocatalyst needs to be operated at pasteurization temperatures (60–70°C) and high agitation speed to reduce mass transfer problems.

Stirred tanks reactors are often the system of choice when a bioreactor is designed, and a common approach to enhance mass transfer in these systems is by increasing the impeller speed. However, impeller speed increment also increases the power-volume ratio, which is not economically feasible for large reactors because of high power cost [2]. Therefore, the impeller speed should be chosen such that it is adequate to make all the solid surface areas available for mass transfer [3]. If a satisfactory solid suspension is obtained in a small tank, and this fact becomes evident by visual observations, together with particle velocities or mass transfer rates then the safe scale-up rule is to keep geometrical similarity. In agitated reactors, this type of scale-up criterion is often derived from studies on the minimum rotational speed for complete solid suspension. Scale-up criterion involves selecting mixing variables to achieve the desired performance in both model and pilot scales [4].

Invert sugar has been extensively researched because of its importance in food and pharmaceutical industries [1,5,6,7,8,9,10,11]. Most of the research studies have focused to find the adequate support and immobilization methods to increase invertase thermostability and reuse. Despite the studies on scale-up and/or mass transfer with calcium alginate-immobilized cell-based systems for different processes [12,13,14,15], very few contributions have reported

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stirrer influence in mass transfer, scale-up criteria, and kinetics studies in immobilized cell systems for sucrose hydrolysis.

Thus, the present research focuses on a large-scale reaction system for sucrose hydrolysis catalyzed by the recombinant *Pichia pastoris* BfrA4X whole cells expressing *Thermotoga maritima* invertase entrapped in calcium alginate beads. For the first time, a useful tool for scaling up the sucrose inversion reaction with the PpABfrA biocatalyst is described, together with a new application of a kinetic model [10] to predict the fractional conversion expected during the sucrose hydrolysis reaction in both model and prototype reactors.

2. Material and methods

2.1. Strain, culture conditions, and cell inactivation

P. pastoris GS115 strain BfrA4X was obtained from the culture collection of the Center for Genetic Engineering and Biotechnology, Havana, Cuba [16].

Fed-batch fermentation was performed in a 7.5-L fermenter (INFORS) as described by Martínez et al. [10]. The culture broth was centrifuged; 150 g of the pellet was resuspended in 300 mL deionized water, and the yeast cells were heat-inactivated at 70°C for 30 min. The heat-killed yeast cells were then pelleted by centrifugation and used for whole cell immobilization or as a free cell source. A total of 150 g (wet weight) of the heat-killed cells was resuspended in distilled water (500 mL) to achieve final biomass concentrations of 300 g L^{-1}.

2.2. Preparation of calcium alginate beads

Sodium alginate solutions were prepared by the stepwise addition of 10 g of alginate powder to 150 g of wet biomass suspended in 500 mL of deionized water and stirred thoroughly to ensure a homogenous distribution of the cells in the alginate solution; the volume of the mixture was adjusted to 500 mL with deionized water before extrusion in the $CaCl_2$ solution. The alginate/cell suspension was added drop-wise through a silicone tube (using a peristaltic pump at a flow rate of 20 mL min^{-1} and a fine needle) to 5 L $CaCl_2$ (0.55% w/v) solution. The $CaCl_2$ solution was stirred at constant speed (100 rpm) using an impeller-type marine propeller to avoid droplet aggregation. Gelation time was restricted to 1 h, after which the $CaCl_2$ solution was discarded. Subsequently, the beads were washed three times and stored in 1.46 M sucrose solution at 4°C. The resulting biocatalyst was named PpABfrA.

2.3. Invertase activity assay

Samples were withdrawn at regular intervals from the stirrer tank reactor to measure the reducing sugars. As described by Miller [17], an equimolar mixture of glucose and fructose was used as standard. Sucrose hydrolysis was determined by measuring the release of reducing sugars using 3′5-dinitrosalicylic acid (DNS).

2.4. Batch process in a 0.5-L model scale reactor

The immobilized cell biocatalyst was tested in a constant-volume batch reactor with a 0.7-L total reactor volume and 0.5-L working volume. The tank was 0.09 m in diameter and the marine impeller was 0.03 m diameter, resulting in an impeller to tank diameter ratio of 0.33. The impeller was placed 0.027 m from the bottom of the tank, resulting in a height to impeller diameter ratio of 0.9. The tank was fabricated of glass to enable the observation of flow patterns in the tank while conducting experiments. Calcium alginate beads of immobilized cells at a concentration of 100 and 200 g L^{-1} were incubated 3 h at a sucrose concentration of 1.75 M at 60°C. To determine the agitation speed needed for satisfactory bead suspension in the model reactor, the speed of 0, 50, 100, and 200 rpm was assayed. To evaluate the sucrose hydrolysis profile, 100 g $L^{-1}$of the biocatalyst was incubated for 12 h at a sucrose concentration of 1.75 M at 60°C at a constant stirring of 100 rpm. Samples were withdrawn at regular intervals to measure the reducing sugars as described above.

2.5. Batch process in a 5-L prototype reactor

The immobilized cell biocatalyst was tested in a constant-volume batch reactor with a jacketed heat transfer fluid and with 7-L total reactor volume and 5-L working volume. The tank was 0.15 m in diameter and the marine impeller was 0.05 m diameter, resulting in an impeller to tank diameter ratio of 0.33. The impeller was placed 0.045 m from the bottom of the tank, resulting in a height to impeller diameter ratio of 0.9. To evaluate the sucrose hydrolysis profile, 100 g L^{-1} of the biocatalyst was incubated for 12 h at a sucrose concentration of 1.75 M at 60°C and a constant stirring of 60 rpm. Samples were withdrawn at regular intervals to measure the reducing sugars as described above.

2.6. Kinetic equation evaluation for sucrose hydrolysis

The experimental fractional conversions (X_A) in the model and pilot-scale reactors were compared with the theoretical X_A expected according to the kinetic equation $-\ln(1 - X_A) = kt$ previously reported by Martínez et al. [10]. From the equation, the theoretical X_A expected at different time intervals was calculated as $X_A = 1 - e^{-kt}$ where t is the time (h); S_{A0} is the initial sucrose concentration (mol L^{-1}), W is the biocatalyst weight (gL^{-1}), and k is the reaction kinetic coefficient: $k = (-0.0432\ g^{-1}\ h^{-1}\ M^{-1} S_{Ao} + 0.1054) W\ (h^{-1})$ according to Martínez et al. [10].

2.7. Statistical analysis

The statistical package for social sciences (SPSS) 15.0 was used for the data analyses. The data are presented as means ± S.D. The level of significance used in this study was $P < 0.05$.

3. Results and discussion

3.1. Effect of agitation and biocatalyst loading on sucrose hydrolysis in a model bioreactor

The effect of agitation and biocatalyst loading on mixtures and sucrose hydrolysis was determined in a 0.5-L reactor through a bifactorial design for the agitation factor, with four speed levels of 0, 50, 100, and 200 rpm and two load levels of 100 and 200 g $L^{-1}$of the biocatalyst. Operational conditions of 60°C, sucrose concentration of 1.75 M, and reaction time of 3 h remained constant.

The percentages of sucrose hydrolysis under the assayed conditions described above are shown in Table 1. The biocatalyst loading affects the percentage of sucrose hydrolysis, regardless of agitation speed. With the increase in the biocatalyst load, a significantly increased percentage of sucrose hydrolysis was achieved ($F = 13.0$; $p = 0.005$).

Table 1
Influence of agitation and biocatalyst loading on the percentage of sucrose hydrolysis in the model bioreactor.

Biocatalyst loading (g L^{-1})	Sucrose hydrolysis (%)			
	0 rpm	50 rpm	100 rpm	200 rpm
200	14.9 ± 1.4[a]	24.7 ± 1.2[a]	51.4 ± 17.7[b]	45.2 ± 16.4[b]
100	8.0 ± 2.1[a]	18.7 ± 0.9[a]	36.7 ± 5.5[b]	32.0 ± 4.6[b]

Tabulated data are the means of triplicate measurements ± standard deviation. Different letters imply significant differences between sucrose hydrolysis results for the same biocatalyst loading (Tukey DHS test: $F = 28.4$; $p \leq 0.000$).

The agitation speed also significantly affected sucrose hydrolysis ($F = 28.4$; $p \leq 0{,}000$). The multiple means comparison obtained from the four agitation speed levels showed no significant differences in sucrose hydrolysis between 100 and 200 rpm, but these were different from the lowest agitation speed levels. In this experiment, no interaction was observed between agitation and biocatalyst load ($F = 0.600$; $p = 0.620$). In several studies, immobilized invertase-based biocatalysts are stirred at 100 rpm or higher to maintain homogeneity of the beads in the sucrose solution [18,19,20,21], but magnetic stirrer or orbital shaker [22] is not recommended for higher volumes. Other systems such as a rotating basket reactor showed that the rotation speed should be higher than 200 rpm to consider this reactor as homogeneous [23] wish could cause a high power consumption during scale up.

The beads are surrounded by an unstirred layer that causes external diffusion. The thickness of this layer depends on the relative velocity of the particle to the bulk solution. For rapid stirring, this external diffusion can be neglected [24]. External mass transfer resistance can be easily reduced or eliminated, mainly by improving the mixing characteristics of the reactor system. However, internal mass transfer resistance is often unavoidable [12]. When the hydrolyzed sucrose produced by the immobilized cells increases with the increase in the agitation speed to 100 rpm, it suggests that the external diffusion resistance could be minimized in this condition, but once the reaction product approaches a constant value independent of the increase in agitation to 200 rpm, then the intraparticle diffusion resistance could determine the biochemical reaction rate and become independent of external agitation.

Data obtained from different experimental design (Table 1) describing the ratio of sucrose hydrolysis percentage at 100 rpm using 100 g of biocatalyst L^{-1} revealed that 2.16 g of sucrose could be hydrolyzed per gram of beads. This sucrose hydrolysis value is higher than that noted when 200 g biocatalyst L^{-1} was used, corresponding to 1.53 g; hence, on the basis of the experimental data, 100 g biocatalyst L^{-1} was used for scale-up studies.

This result also indicates that if the immobilized cells are uniformly distributed in the stirred tank, the data obtained from experiments conducted in a small bioreactor can be reproduced when the same process is scaled-up.

3.2. Consequences of the application of different scale-up criteria to operate the prototype bioreactor (5 L).

Before the scale-up of the hydrolysis reaction, the consequences of the application of different scale-up criteria based on the principle of similarity were analyzed. For marine impellers, a ratio of impeller diameter (D)/tank diameter (d) between 0.2 and 0.5 and a ratio of stirrer blade height (h)/impeller diameter (D) between 0.5 and 1.0 are recommended. To ensure the geometrical similarity between the model and prototype reactors, constant ratios $D/d = 0.3$ and $h/D = 0.9$ were fixed according to the design range established by Kasatkin [25].

The followed scale-up criteria were analyzed:

Constant impeller tip speed $(ND)_M = (ND)_P$.

Constant impeller speed $(N_M) = (N_P)$.

Constant power input $(P_M) = (P_P)\ \alpha\ N^3D^5$.

Constant Reynolds $Re = ND^2\rho/\mu$.

Constant power per unit volume $(P/V)_M = (P/V)_P \alpha\ N^3D^2$.

where N is rotational speed (rps); P is power input (W); D is impeller diameter (m); Re is Reynolds number (dimensionless); ρ is density (kg/m^3); μ is viscosity (Pa s); $_M$ and $_P$ refer to the model and prototype, respectively.

The selection of a scaling-up criterion depends mainly on the analysis of the consequences of applying each one in our reaction system.

Table 2 shows the values of the parameters analyzed under different scale-up criteria. The criterion based on equal impeller tip speed increases the Reynolds number by 1.6 times and the power input by 1.5 times and decreases the power per unit volume by 0.2 times for the pilot-scale tank in comparison with the model tank. Despite the mixing increment, the Reynolds number did not change the turbulent flow. Therefore, by applying this criterion, a uniform distribution of the immobilized cells can also be achieved in a large-scale reactor. The application of other scale-up criteria such as constant rotation speed or power per unit volume causes large differences in terms of power requirement between the small- and large-scale reactors, which is not feasible for industrial operations. It is also evident that at a constant Reynolds number or power input, the power per volume unit and the rotational speed decrease greatly with the used scale; thus, it can affect the bead suspension in a large-scale vessel. This fact explains why these criteria should not be used for the present scale-up.

3.3. Sucrose hydrolysis in the model and prototype bioreactors

To evaluate the sucrose hydrolysis profile, 100 g L^{-1} of the PpABfrA biocatalyst was incubated for 12 h at 60°C with a sucrose concentration of 1.75 M. By applying the constant impeller tip speed criterion, stirring of 100 and 60 rpm was used for the model and prototype, respectively.

Fig. 1 shows a similar fractional conversion (X_A) profile obtained in both bioreactors. The experimental fractional conversion (X_A) in the model and prototype reactors was compared with the theoretical X_A expected according to the equation $X_A = 1 - e^{-kt}$ reported by Martínez et al. [10]. A reasonable agreement was found between the experimental data and the X_A values predicted by the theoretical model for sucrose hydrolysis in the stirred tank bioreactors under the studied operational conditions for both model and prototype reactors. The expected final X_A was 0.83, and the experimental X_A was 0.90 and 0.84 for the model and prototype stirrer tanks, respectively.

A simple linear regression showed that the X_A variability explains more than 99% of the theoretical and experimental fractional conversion profiles. The regression model coefficients were evaluated by a t test, and the resulted slope was significantly different to zero ($t = 36{,}3$; $p \leq 0{,}000$). Despite the model constant was statistically different to zero ($t = -4{,}0$; $p = 0{,}002$), the absolute value was 0.082, indicating a linear relationship between these variables. These results demonstrate that by using both variables, that is, geometrical

Table 2
Values of different parameters according to the applied scale-up criteria.

Scale-up criteria	Model	Values in the prototype				
		N (rpm)	ND (rpm·m)	Re	P (W)	P/V (W/L)
$N_{constant}$	100	$1.0N_M = 100$	$1.7ND_M = 5.0$	$2.7Re_M = 918$	$6.6P_M = 1.0 \times 10^{-3}$	$0.7(P/V)_M = 2.0 \times 10^{-4}$
$ND_{constant}$	3	$0.6N_M = 60$	$1.0NDM = 3.0$	$1.6Re_M = 547$	$1.5P_M = 2.3 \times 10^{-4}$	$0.2(P/V)_M = 4.6 \times 10^{-5}$
$Re_{constant}$	330	$0.4N_M = 36$	$0.59ND_M = 1.8$	$1.0Re_M = 330$	$0.4P_M = 6.6 \times 10^{-5}$	$4.3(P/V)_M = 1.3 \times 10^{-5}$
$P_{constant}$	1.5×10^{-4}	$0.4N_M = 42$	$0.71ND_M = 2.1$	$1.2Re_M = 392$	$1.0P_M = 1.5 \times 10^{-4}$	$10.0(P/V)_M = 3.0 \times 10^{-5}$
$P/V_{constant}$	0.0003	$0.7N_M = 70$	$1.18ND_M = 3.5$	$1.9Re_M = 647$	$5.0P_M = 1.5 \times 10^{-3}$	$1.0(P/V)_M = 0.0003$

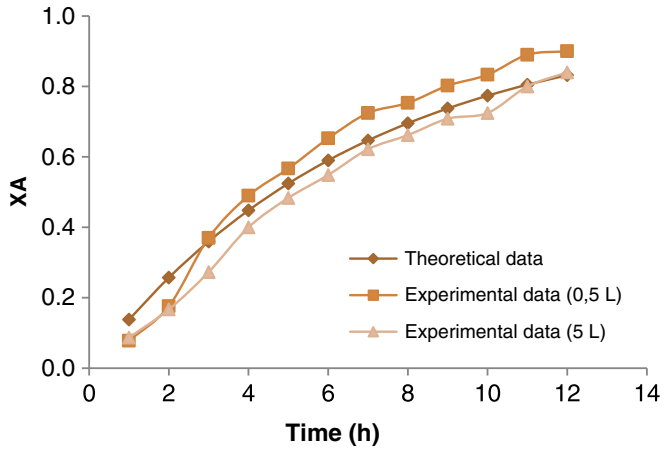

Fig. 1. Fractional conversion profile in the 0.5-L model and 5-L prototype bioreactors and theoretical fractional conversion. Samples were withdrawn at every hour during the reaction with 100 g $L^{-1}$of the PpABfrA biocatalyst in a sucrose solution at a concentration of 1.75 M at 60°C.

similarity and constant impeller tip speed, it is possible to successfully scale up the sucrose inversion reaction with the PpABfrA biocatalyst.

4. Conclusions

To date, there is not enough information to describe the large-scale production of invert sugar using different scale-up criteria such as dose of immobilized biocatalyst and stirring speed effect on mass transfer. The agreement between the theoretical prediction and the data obtained from the experiments conducted in the prototype bioreactor indicates that both the kinetic model and these experimental conditions can be successfully reproduced when the process is scaled up; thus, this information constitutes a valuable tool to successfully perform this type of high-scale operation for industrial purposes.

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