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Fungal Invertase Expression in Solid-State Fermentation

Cuitlahuac Aranda[1], *Armando Robledo*[1], *Octavio Loera*[2], *Juan C. Contreras-Esquivel*[1], *Raul Rodríguez*[2] *and Cristóbal Noé Aguilar*[1]*

[1]Food Research Department, School of Chemistry, Universidad Autónoma de Coahuila, Blvd. Venustiano Carranza and Jose Cárdenas, Col. República, PO Box 252, 25000, Saltillo, Coahuila, Mexico

[2]Department of Biotechnology, Universidad Autónoma Metropolitana, Iztapalapa. Av. Michoacán y la Purisma, Col. Vicentina, 09340, México, D.F. Mexico

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Summary

In this study invertase activity expression in *Aspergillus niger* Aa-20 was evaluated under different concentrations of two substrates using solid-state fermentation (SSF) on polyurethane foam. Glucose was used as repressor and sucrose was the inducer. Invertase production increased when glucose was present in the medium (up to 100 g/L); however, higher concentration than this reduced the enzyme production. Induction-repression ratio obtained using any glucose concentration was at least 2.5 times higher than that under basal conditions (without inducer).

Key words: invertase, solid-state fermentation, expression kinetics

Introduction

Invertase (β-D-fructofuranoside-fructohydrolase, E.C. 3.2.1.26) catalyzes the hydrolysis of sucrose to glucose and fructose. Invertase is one of the most widely used enzymes in food industry, especially in the preparation of jams and candies (*1*). The enzyme is a glycoprotein, with some residues of mannose being the major component of the carbohydrate moiety (*2*,*3*). Invertase is mainly used in the food industry, where fructose is preferred over sucrose because it is sweeter and does not crystallize easily. However, the use of invertase is seriously limited because another enzyme, glucose isomerase, can be used to convert glucose to fructose at lower costs. A wide range of microorganisms produce invertase and can, thus, utilize sucrose as the only carbon source and as inducer of such enzyme. It has been extensively studied in yeast and some fungi, mainly *Neurospora* sp. Commercially, invertase is biosynthesized chiefly by yeast strains of *Saccharomyces cerevisiae* or *S. carlsbergensis* in submerged fermentation (SmF) (*4*). However, in SmF, it is well known that invertase is strongly regulated by glucose repression or catabolic repression. This control system regulates expression of sucrose fermentation genes at the transcriptional level. Glucose addition to culture media with sucrose affects transport of sucrose into the cell and thereby affects the function of regulatory mechanisms for induction (*5*).

Several studies have reported high enzymatic activity titers by solid-state fermentation (SSF) over SmF when high glucose concentrations were used. These observations have provoked a great controversy, because several authors considered that SSF was a process where the catabolic repression of inducible enzymes did not take place or was minimized (*6–12*). Several hypotheses have been proposed to explain such differences in the enzyme activity titers, among which are the low water content, the nature of SSF, the diffusion of nutrients on solid matrix and the changes in the ratio between the substrate uptake rate and diffusivity substrate coefficient. Maldonado and Strasser de Saad (*10*) found differences in the fatty acid composition of cell membrane from *Aspergillus niger* grown in SmF and SSF for pectinase production, concluding that those structural differences are directly

*Corresponding author; E-mail: cag13761@mail.uadec.mx

related to enzyme production in SSF. Recently Cerda-Montalvo *et al*. (*13*) demonstrated experimentally the effect of substrate diffusion on enzyme expression. However, today it is still not clear why enzyme titers produced in SSF systems are significantly higher than in SmF systems (*14*); also the effect of addition of high amount of glucose on enzyme production in SSF is still not clear. Hence, the aim of this work was to study the effect of the addition of glucose on invertase production in SSF using *Aspergillus niger* Aa-20 on polyurethane foam.

Materials and Methods

Microorganism and culture medium

Spores of *Aspergillus niger* Aa-20 (UAMI-IRD Collection) used in this work were stored at –20 °C. Inoculum was prepared by transferring the spores to potato dextrose agar medium, incubated at 30 °C for 5 days. Spores were scraped into 0.01 % Tween 80 solution and counted in a Neubauer chamber. Composition of culture medium for invertase production was (in g/L): KH_2PO_4 4.38, $(NH_4)_2SO_4$ 8.76, $MgSO_4{\cdot}7H_2O$ 0.88, $CaCl_2{\cdot}2H_2O$ 0.088, $MnCl_2{\cdot}6H_2O$ 0.018, $Na_2MoO_4{\cdot}2H_2O$ 0.0088, $FeSO_4{\cdot}2H_2O$ 0.012 and sucrose 25. Medium was supplemented with glucose at 6.25, 12.5, 25, 50, 100, 150 and 200 g/L. Basal invertase activity was assayed from cultures with glucose (25 g/L) as the only carbon source.

Solid-state fermentation (SSF)

The SSF involved the use of polyurethane foam (PUF) (Expomex, México) as a support to absorb the liquid medium. PUF was washed as reported by Zhu *et al*. (*15*) and then pulverised in a plastic mill. Column reactors (25 x 180 mm) were packed with 10 g of inoculated support ($2{\cdot}10^7$ spores/g of dry inert support). Culture conditions were: 30 °C, aeration rate of 20 mL of air per gram of support per min, initial pH=5.5, initial moisture content of 65 % and incubation time of 32 h. For enzyme extraction, the content of each reactor was mixed with distilled water (1:10, mass per volume) and vortexed for 1 min. Solids were filtered (Whatman 41) and the clear filtrate was assayed for extracellular invertase activity. The remaining solids were washed three times with 50 mL of distilled water. Intracellular enzyme was recovered by freezing the cells in liquid nitrogen and by macerating in a chilled mortar and mixed with acetate buffer (200 mM, pH=5.5). The fungal debris was removed by centrifugation and the resulting supernatant was assayed for invertase activity. SSF system was conducted in three sets and the values were averaged.

Analytical methods

Fungal biomass production

Considering that fungal biomass could not be measured accurately by gravimetry, it was determined by measuring the protein concentration with a Bradford microassay (Bio-Rad®) according to the technique reported by Córdova *et al*. (*16*), which evaluates the lag and exponential growth phases only.

Enzyme assay

Invertase activity was assayed by the dinitrosalicylic acid technique (*17*) to estimate the release of reducing sugars using the following reaction mixture: 1.9 mL of substrate (100 mM sucrose in 100 mM acetate buffer, pH=5.0) and 0.1 mL of enzyme extract incubated for 30 min at 30 °C. One international unit (IU) of enzyme was defined as the amount of catalyst able to release 1 μmol of reducing sugar per minute per millilitre. All data points correspond to triplicates of independent experiments.

Sugar determinations

Total sugar quantification was carried out using the phenol-sulphuric acid method (*18*). Sucrose, glucose and fructose concentrations were evaluated by HPLC chromatography using a Shodex SC-LG column.

Presence of other metabolites

Production of other metabolites (trehalose, citric acid and polyols) was evaluated by HPLC chromatography using a C-18 column.

Calculation of metabolic parameters

The major metabolic parameters including specific growth rate (μ), biomass yield ($Y_{X/S}$), enzyme yield (Y_P), specific sugar uptake rate (q_s) and induction-repression ratio ($R_{I/R}$) were calculated. Specific growth rate, μ, was obtained from growth curves fitted by a Marquardt computer program (Solver-Excel, Microsoft) using the logistic equation as follows:

$$\frac{dX}{dt} = \mu\left(1 - \frac{X}{X_{max}}\right)X \qquad /1/$$

where μ (1/h) was the maximal growth rate and X and X_{max} were the biomass concentrations at times t and $t \to \infty$, respectively. The algorithm minimized the sum of the least square errors, comparing experimental data with the theoretical values given by the next equation:

$$X(t) = \frac{X_{max}}{1 + \frac{X_{max} - X_0}{X_0} e^{-\mu t}} \qquad /2/$$

X_0 being the biomass concentration at time t=0.

The overall biomass yield, $Y_{X/S}$, defined as the amount of biomass produced per gram of sugar utilised, was approximated from:

$$Y_{X/S} = \frac{\Delta X}{\Delta S} = \frac{X_{max} - X_0}{S_0 - S} \qquad /3/$$

where S and S_0 are substrate concentrations in the fermentor at times t=0 and $t \to \infty$, respectively.

Enzyme yield, Y_P, the invertase activity produced per gram of biomass of fungal cells was approximated from:

$$Y_P = \frac{\Delta E}{\Delta X} = \frac{E_{max} - E_0}{X_0 - X} \qquad /4/$$

where E_{max} and E_0 are the international units of invertase activity in the extract at times t=0 and $t \to \infty$, respectively.

The specific sugar uptake rate, q_S, defined as the amount of sugar consumed per gram of biomass per hour, was calculated from:

$$q_S = \frac{\mu}{Y_{X/S}} \qquad /5/$$

Induction-repression ratio, $R_{I/R}$, defined as invertase activity induced in a medium with glucose and sucrose per basal invertase activity in a medium without sucrose, was calculated from:

$$R_{I/R} = \frac{IAI}{BIA} \qquad /6/$$

where *IAI* is the invertase activity produced in a medium with inducer (sucrose) and repressor (glucose), and *BIA* is the basal invertase activity produced in a medium without inducer.

Statistical analysis

All results were evaluated using the analysis of variance and Tukey's test for comparison of means in the statistical program InStat for Macintosh.

Results and Discussion

In this study the strain *A. niger* Aa-20 was adapted on media with sucrose, or mixtures of glucose-sucrose, although it has generally been characterized as producer of tannase (*19*). Fig. 1 shows the values of maximum biomass production (X_{max}) and the specific growth rate values (μ). Evidently, under conditions used, the X_{max} was not significantly affected by the addition of glucose in a range from 6.25 to 150 g/L, and increased to 200 g/L. The mould cultured in SSF grew faster (0.2 to 0.53 h^{-1}) when initial glucose concentration was increased from zero to 25 g/L.

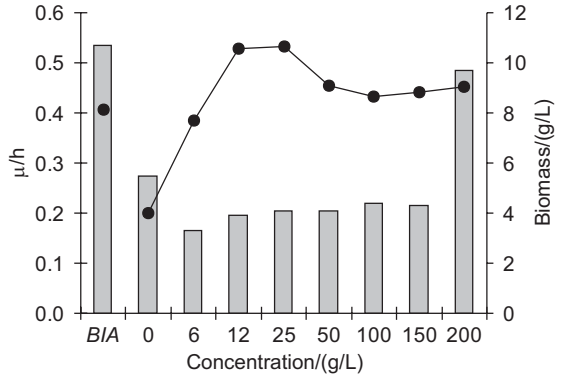

Fig 1. Effect of glucose concentration on biomass production and specific growth rate of *Aspergillus niger* Aa-20. Bars represent the X_{max} values and line is μ.

Maximal invertase production values are shown in Fig. 2a. *BIA* (or constitutive level of invertase activity) produced by *A. niger* Aa-20 in SSF with glucose as the only carbon source was always lower than in any other experiment, which was due to the presence of sucrose in the medium. Intracellular invertase activity was seriously affected by the addition of glucose, but extracellular invertase activity was not affected. Fig. 2b shows

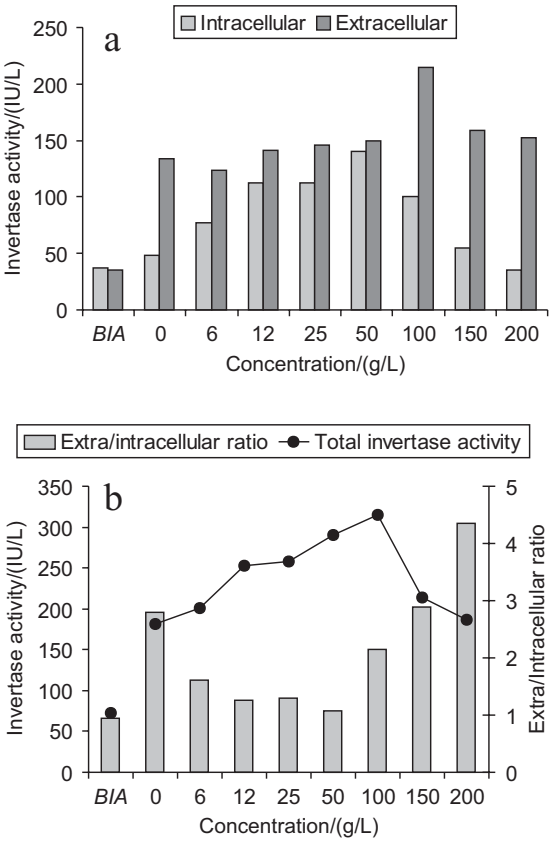

Fig 2. Effect of glucose concentration on (a) intracellular and extracellular invertase production, and (b) intra/extracellular ratio and total invertase activity

that the extra/intracellular ratio was decreased by glucose addition in a range of 0 to 50 g/L, but it increased with higher initial glucose concentration. Glucose addition in a range of 6.25 up to 100 g/L increased the total invertase activity, but it decreased 32 and 41 % when glucose concentrations of 150 and 200 g/L were used, respectively. However, total invertase activity obtained for 200 g/L (187 IU/L) was higher than *BIA*. This result appeared important, as it demonstrated that at high glucose concentration the phenomenon of catabolic repression was not present in SSF process under the conditions used. Fig. 3 showed that $R_{I/R}$ value increased when the initial glucose concentration was in a range from 0

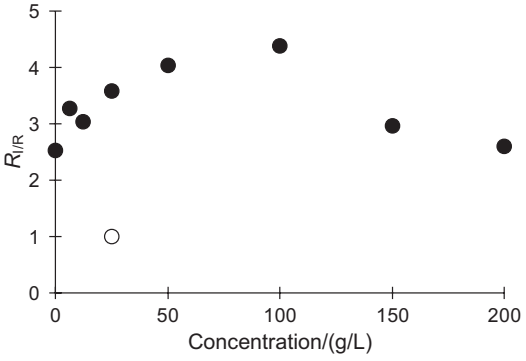

Fig 3. Effect of glucose concentration on induction-repression ratio, $R_{I/R}$

Table 1. Kinetic parameters of invertase production by *Aspergillus niger* Aa-20 in SSF

γ(glucose)	γ(sucrose)	$Y_{x/s}$	Y_p	q_s	q_p	*IE*
g/L	g/L	g/g	IU/g	g/(g·h)	IU/(g·h)	IU/g
25	0	0.781	6.729	0.521	2.739	–0.192
0	25	0.233	33.091	0.858	6.618	0.222
6.25	25	0.107	60.909	3.584	23.389	0.165
12.5	25	0.111	64.872	4.747	34.317	0.235
25	25	0.103	62.927	5.151	33.477	0.443
50	25	0.075	70.732	6.081	32.183	0.530
100	25	0.064	71.591	6.721	30.999	0.775
150	25	0.042	49.767	10.536	21.997	0.312
200	25	0.070	19.278	6.471	8.733	0.094

to 100 g/L, but it decreased at higher concentrations. It is important to note that $R_{I/R}$ value obtained in the presence of any glucose concentration was at least 2.5 times higher than the value obtained for *BIA*.

Table 1 shows data obtained for kinetic parameters during the evaluation of invertase production on solid-state culture at different substrate conditions. *A. niger* Aa-20 presented the highest value of $Y_{X/S}$ using glucose as the sole carbon source, however, when the initial glucose concentration was incremented in the presence of sucrose, a dramatic reduction of its value was observed. In contrast, increments in Y_P, q_S and q_P values were registered. Under solid-state fermentation of the culture at 32 h, the increment in the initial glucose concentration favoured the invertase production and seriously affected the biomass formation. Invertase expression (*IE*), defined as the enzyme produced per gram of biomass generated, demonstrated that it was maximum when 100 g/L of glucose and 25 g/L of sucrose were used, and when substrate concentration was increased, a serious reduction of its value, mainly due to strong effect of inhibition of substrate on fungal growth, was observed.

The production of invertase from *Penicillium* sp. and *Aspergillus* sp. has been reported (*20–24*). Production of invertase by SSF has potential advantages over the SmF with respect to simplicity in operation, high productivity fermentation, it is less favourable for growth of contaminants and concentrated product formation. The kinetics of invertase production by filamentous fungi in SmF has been studied extensively (*25–27*). However, there is not enough information about the comparison of the invertase expressed by SSF and SmF conditions. Generally the fermentation media and their components and strains that are used in SmF have been used in SSF too (*28*). However, certain investigations clearly indicated that the strains used in SmF are less efficient in SSF. Ashokkumar *et al.* (*28*) described the comparison of invertase production by *A. niger* under two different fermentation conditions and optimization of media using a fractional factorial design, to improve the invertase production under SmF and SSF conditions.

Romero-Gómez *et al.* (*23*) published data showing that three strains of *A. niger* produced higher titers of invertase and had higher observed productivity when cultured by SSF as compared to SmF. The trend for SmF and SSF systems was followed up by the Monod equation with substrate inhibition. Nevertheless, it was worth noticing that inhibition constant value for SSF was significantly higher than for SmF. Altogether, results showed that *A. niger* grew more efficiently in SSF than in SmF when the initial level of sucrose was high. They indicated that excess sucrose helped to prevent the breakdown of invertase in SSF system (*12*). It was interesting to note that there was a consistent behaviour in the enzyme induction patterns and the results in this study were well correlated with those obtained for tannase production reported previously (*11*).

Conclusions

These results seemed important as they demonstrated that a high glucose concentration, the phenomenon of catabolic repression, was not present in SSF process under conditions used for invertase production.

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