Influence of Carbon, Nitrogen and Phosphorous Sources on Glucoamylase Production by *Aspergillus awamori* in Solid State Fermentation

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It was the objective of the present study to increase the production of glucoamylase by *Aspergillus awamori* through solid state fermentation, using wheat bran as the main carbon source and $(NH_4)_2SO_4$, urea, KH_2PO_4, glucose, maltose and starch as additional nitrogen, phosphorus, and carbon sources. The production of glucoamylase is strongly influenced by N and C sources. A 100 % increase was observed when the $(NH_4)_2SO_4$ was replaced by urea, with C/N = 4.8, using maltose as the additional carbon source. C/P ratios in a range of 5.1 to 28.7 did not induce glucoamylase production under the studied conditions.

Key words: *Aspergillus awamori*, Glucoamylase, Solid State Fermentation

Introduction

Solid state fermentation (SSF), a process that occurs in the absence of free water, has advantages over submerged fermentation, especially regarding the economy and simplicity of the process (Costa *et al.*, 1998).

The industrial use of amylases has been increased, and the glucoamylase is the most produced around the world. Glucoamylase (α-1,4-glucan-glucohydrolases), are exoenzymes that catalyses D-glucose production from the non-reducing starch end, is synthesized in the presence of an inductor (Pandey *et al.*, 1996). According to the most accepted mechanism of synthesis (Jacob and Monod, 1961), the regulator gene codifies the production of a repressing protein, which may bind to the operator gene and block the transcription of the structural gene. In the presence of the inductor, the repressing protein binds to it and does not interact with the operator gene, therefore allowing both the transcription process and enzymatic synthesis to occur. Thereby, the induced synthesis of the enzyme is due to its de-repression (Pazur *et al.*, 1980).

Enzyme substrates are typical inductors with different potentials for induction, as in the case of glucoamylase synthesis induced by maltose, when compared with induction mediated by starch (Thomas *et al.*, 1996; Chiquetto *et al.*, 1992; Ramachandran *et al.*, 1979). Additionally, Wang *et al.* (1979) report that the expression of induced enzymes may also be controlled by catabolic repression, or by repression mediated by the product that results from enzyme action (glucose, for instance). In the case of catabolic repression the cell blocks the enzyme synthesis when substrates of easier assimilation are available.

Synthesis of enzymes depends on the type of nutrients available to the organism and besides an adequate carbon source other nutrients may be equally important to the composition of the medium (Thomas *et al.*, 1996). In fact, Chiquetto *et al.* (1992) and Ellaiah *et al.* (2002) indicated that urea was a suitable nitrogen source for glucoamylase synthesis in submerged and solid state fermentation, respectively.

Also, according to Bertolin *et al.* (2001) and Pandey *et al.* (1996), the absence of free water in solid state fermentation always results in very high concentrations of nutrients. In this fermentative process, even minor variations in the C/N or C/P ratios may result in quite distinct responses from the biological system, since the local concentra-

tions are greatly superior to those of the submerged fermentation.

We studied glucoamylase production by *Aspergillus awamori* in solid state fermentations with different C/N and C/P ratios. Wheat bran was used as the main carbon source, supplemented with glucose, maltose, soluble starch or cross-link modified starch as additional carbon sources and ammonium sulfate $(NH_4)_2SO_4$ or urea as additional nitrogen sources.

Material and Methods

Microorganism

The mold *Aspergillus awamori* NRRL 3112 was maintained through successive transfers on potato dextrose agar (PDA). At inoculation, 1 ml of a spore suspension (10^7 spores/ml) prepared in distilled and sterile water, was transferred to each 14 g medium with 63 % moisture.

Fermentation media

Wheat bran containing 25 % starch and 14 % protein (w/w, dry mass basis) was used as the main carbon source. Each 60 g of this substrate was supplemented with 45 ml of saline solution containing $(NH_4)_2SO_4$ (4 g/l), KH_2PO_4 (2 g/l), $MgSO_4$ (1 g/l), $FeSO_4 \cdot 7H_2O$ (6.3 μg/l), $MnSO_4$ (0.1 μg/l) and $ZnSO_4$ (6.2 μg/l) (Dartora *et al.*, 2002). This broth was set as the standard wheat bran medium (WB). The sterilization was carried out at 110 °C for 30 min. This medium had a C/N ratio of 4.8 and a C/P ratio of 25.7. To study different C/N and C/P ratios, the WB medium was also supplemented with solutions of glucose, maltose, soluble starch (Merck, Germany) or modified cross-link starch, Starch 520 (Lorentz, São Paulo, Brazil). The quantities added were expressed in percentages of the starch amount in the standard medium [(grams of the added substance/grams of starch in the WB medium). 100]. The same procedure was used to substitute ammonium sulfate with urea and to vary the amount of ammonium sulfate, urea, and phosphate in order to study different C/N and C/P ratios. Afterwards, the medium was supplemented with sufficient H_2SO_4 1.5 M and H_2O to adjust the medium pH to 4 and the moisture content to 63 %.

Fermentation occurred in erlenmeyers (250 ml capacity), each with 14 g culture medium, placed in incubators, without agitation, at 30 °C and 98 % relative humidity for up to 40 h. All experiments were carried out in duplicate.

Moisture

Protocol 14004 of the Association of Official Analytical Chemists was used to assess the relative humidity of the media (A. O. A. C., 1997).

Reducing sugars

Total reducing sugars (TRS) were analyzed by hydrolyzing a known amount of sample with 1.5 M H_2SO_4 for 60 min, the resulting sugars, expressed as milligram of glucose per gram of dry matter (mg/gdm), were determined by the glucose-oxidase method (Barham and Trinder, 1972). Free reducing sugars (FRS) were assessed by boiling 500 mg of medium in 20 ml of distilled water for 5 min and determining the quantity of extracted glucose in the same way as for TRS.

Glucoamylase activity

The enzyme was extracted by adding 15 ml of acetate buffer (pH = 4.2) to 1 g of the sample and by shaking the suspension at 200 rpm for 30 min. Once the suspension was filtered, an adequate volume was reacted with 25 ml of a 4 % starch solution at 60 °C for 60 min (Schmidell and Menezes, 1986). After the hydrolysis period, the glucoamylase was heat inactivated and the glucose dosed using the glucose-oxidase method (Barham and Trinder, 1972). One activity unit (U) was defined as the amount of enzyme that in one hour released one gram of glucose under the conditions established for this experiment. The activity of the produced glucoamylase (A) was expressed in units per gram of dry matter (U/gdm).

Statistical analysis

The Tukey honest difference (HSD) test (Box *et al.*, 1978) was used to detect differences in glucoamylase activity between the wheat bran media. All experiments were done in duplicate and the analyses (moisture, reducing sugars and glucoamylase activity) in triplicate.

Results and Discussion

Initially the objective was to verify the effect of supplementing nitrogen and phosphorous sources in the solid state fermentation. The glucoamylase activities produced during 40 h of *A. awamori* growth in the media with different carbon, nitrogen and phosphorous ratios are shown in Table I, 40 h being chosen because after 30 h of fermentation glucoamylase activity was constant.

Although phosphorous is an element of great importance for the cells, the results in Table I show no significant increase, or even a decrease, in glucoamylase synthesis with the addition of phosphorous only, indicating that there was already sufficient phosphorous in the wheat bran. When ammonium sulfate only was added to wheat bran there was also no significant change, or a decrease, in enzyme activity. However, the addition of urea only, even in the small amounts necessary to reduce the C/N ratio from 5 to 4.8, produced a significant rise (p-level = 0.000183) in glucoamylase activity from the 13.9 U/gdm found in the unsupplemented medium to 21.5 U/gdm. The beneficial effects of the addition of urea nitrogen have been reported in submerged fermentations for glucoamylase production by *Aspergillus awamori* and *Aspergillus niger* (Chiquetto *et al.*, 1992; Swift *et al.*, 2002). In our study, the addition of larger amounts of urea resulted in glucoamylase activity dropping to zero. Pandey *et al.* (1996) and Bertolin *et al.* (2001) also found distinct responses by the organisms involved in solid state growth systems associated with elevated local concentrations of some media components.

Costa *et al.* (1998) have reported that the best moisture content in a solid state fermentation depends on the nature of the substrate, the type of microorganism and the final product. Singh and Soni (2002) showed that nutrient concentration is inversely related to the amount of water in the medium. Seemingly an increase in osmotic potential can negatively affect growth and enzyme production. The C/N ratio of 4.8 (with urea) and a C/P ratio of 25.7 apparently provided the best osmotic balance for highest enzymatic activity among the studied conditions in our system.

The previous results also indicate that during the growth and enzyme synthesis period, the presence of free sugars was not observed, in spite of the decrease in TRS concentration. This suggests that an additional carbon source would prolong the fermentative process, thus allowing for a greater production of glucoamylase.

As mentioned before, polysaccharides may induce glucoamylase synthesis and glucose may repress it, so a series of experiments was conducted to investigate the performance of *A. awamori* in the presence of other additional carbon sources. Fig. 1 shows the relative enzyme activity after 40 h of fermentation and after the addition of different carbohydrates, with a constant amount of nitrogen added (equal to that of the standard medium), or maintaining constant the C/N ratio. The relative

Table I. Glucoamylase activity (A) produced by *Aspergillus awamori* after 40 h of solid state fermentation in media with different carbon, nitrogen and phosphorous ratios.

Wheat bran media	C/N ratio	C/P ratio	A* [U/gdm]
Without supplement (N, P)	5.0	28.3	$13.9^{B,C,D} \pm 0.8$
Standard wheat bran medium	4.8	25.7	$13.7^{B,C,D} \pm 1.1$
Supplemented with $(NH_4)_2SO_4$	3.3	25.7	$10.5^{D} \pm 1.4$
	3.1	25.7	$12.5^{C,D} \pm 1.1$
	2.9	25.7	$12.2^{C,D} \pm 1.0$
Supplemented with urea	4.8	25.7	$21.5^{A} \pm 1.5$
	3.1	25.7	$16.9^{B} \pm 1.8$
	1.0	25.7	$0.0^{E} \pm 0.0$
Supplemented with KH_2PO_4**	4.8	20.1	$15.5^{B,C} \pm 1.1$
	4.8	14.3	$14.3^{B,C} \pm 2.3$
	4.8	5.1	$13.3^{C,D} \pm 1.8$

* Mean ± standard deviation.
** Using $(NH_4)_2SO_4$ as nitrogen source.
[A,B,C,D,E] Enzymatic activities with the same exponent do not differ significantly (p < 0.05) in agreement with the Tukey test.
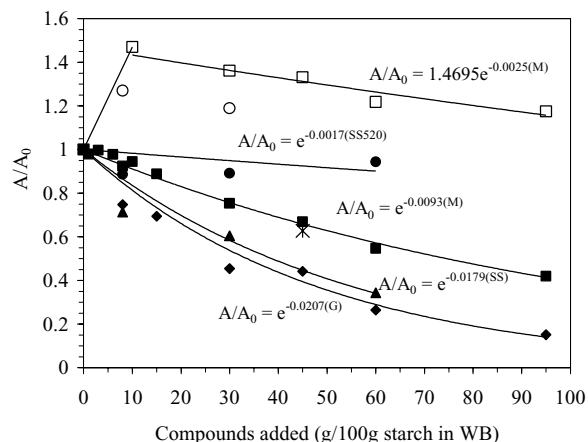
Fig. 1. Relative enzymatic activity (A/Ao = glucoamylase activity in the studied medium/glucoamylase activity of the standard medium) associated with the addition of various carbon sources, after 40 h of fermentation, maintaining fixed the amount of $(NH_4)_2SO_4$ added, or maintaining fixed the ratio C/N = 4.8 by the addition of $(NH_4)_2SO_4$ (indicated by "suf") or urea (indicated by "urea"): (◆) glucose-G, (▲) soluble starch-SS, (■) maltose-M, (●) starch 520-SS520, (✕) maltose (sulf), (○) starch 520 (urea), (□) maltose-M (urea). Curves plotted according to the indicated equations.

enzymatic activity (A/A_0) was defined as the relationship between the glucoamylase activity in the studied medium (A) and that of the standard medium (A_0).

Fig. 1 indicates that the addition of distinct carbon sources in the presence of ammonium sulfate does not stimulate the synthesis of glucoamylase by *A. awamori.* In contrast, there was a reduction in enzyme synthesis as the additions were increased. Enzyme activity without carbon addition (A_0) reached 13.7 U/gdm, as observed in Table I.

Apparently the addition of glucose appeared to strongly repress the enzyme synthesis, and the exponential model fit to the experimental data had a negative exponent (− 0.0207). A similar response was obtained when soluble starch was added to the medium (exponent = − 0.0179). The addition of maltose caused a minor repressive effect (exponent = − 0.0093) and the use of Starch 520 had little influence (exponent = − 0.0017) on glucoamylase synthesis.

Fig. 1 also shows the data obtained using urea as a nitrogen source and varying the amount of maltose, while maintaining the C/N (4.8) and the C/P (25.7) ratios fixed. The experiments without the addition of maltose, but using urea, increased

the activity to above 20 U/gdm (Table I). Small additions of maltose (about 10 % of the starch present in the WB medium) allowed significantly higher activities to occur (A/A_0 = 1.48). However, higher values of maltose reduced the activity of glucoamylase, although the exponent of the adjusted model was smaller than that obtained with maltose and ammonium sulfate (similar observation was obtained in the case of Starch 520). This additional fact emphasizes the importance of having suitable sources of distinct nutrients to obtain good performance in such fermentations.

Fig. 2 presents the time course of the free glucose concentration for the experiments in which distinct saccharides were added up to 60 % of the starch present in the WB medium. In all the experiments the amount of free glucose was greater in the supplemented media, which may affect enzyme synthesis. Free glucose was not so high in the run with addition of Starch 520 whose chemical structure may limit its hydrolysis. In contrast, maltose had less negative effect in enzyme production than soluble starch, probably because maltose is a better inducer than soluble starch, as already reported (Chiquetto *et al.*, 1992; Ramachandran *et al.*, 1979). Furthermore, the accumulation of glucose tended to be lower in the experiments with addition of maltose and urea, which may be an additional factor for better performance in glucoamylase production of this me-
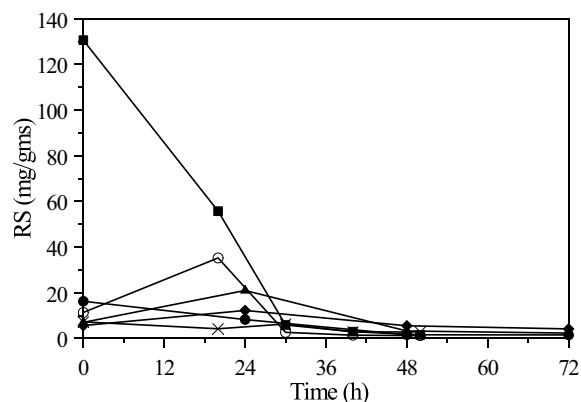

Fig. 2. Time course of free reducing sugars (RS), represented as glucose, for experiments supplemented with carbohydrates (addition of 60 % relative to the starch in the WB medium): (■) glucose, (○) maltose and $(NH_4)_2SO_4$, (●) maltose and urea, (▲) soluble starch, (◆) starch 520, (×) standard wheat bran medium.

dia. It must be emphasized that this better performance was not due to a different cellular growth, since the growth curves obtained for the assays with additional 60 % of maltose and adding sulfate or urea were practically coincident (data not shown).

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