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Glucoamylase Production and Nitrogen Nutrition in *Aspergillus awamori*

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ABSTRACT

Studies related to glucoamylase (GA) production and nitrogen nutrition have been reported, and there has been a general agreement as to its importance, although no conclusions have emerged. The present investigation studies the physiological response of an industrial strain of *Aspergillus awamori* to variable nitrogen nutrition and C/N ratios on GA production. It relates to the use of amino and $NH4^+$ nitrogen or a combination of both. Conditions have been identified that favor the enzyme production. The negative effect of $NH4^+$ concentrations on products, such as antibiotics, pullulan, ligninases, and proteases, is reported in the literature. In some cases, nitrogen metabolite repression has been considered to explain the data obtained. This work discusses a possible role played by nitrogen metabolite repression in glucoamylase production. Batch experiments were carried out in an 8-L working volume fermenter.

Index Entries: Glucoamylase production; *Aspergillus awamori*, nitrogen source; carbon/nitrogen ratio; nitrogen metabolite repression.

INTRODUCTION

Glucoamylase (GA) from *Aspergillus spp* is the second most produced enzyme worldwide, after *Bacillus* protease, and several million tons of starch products are manufactured annually using this enzyme (*1–4*). Strains derived from *Aspergillus awamori* are considered high producers

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(*1,2,5*). Glucoamylase is an inducible extracellular enzyme, and the physicochemical environment, mainly medium composition, affects enzyme induction and repression, the types of glucoamylase and their proportion, and the GA/α-amylase ratio. Medium composition also affects the production of other enzymes, e.g., proteases and glucosidases that act on the enzyme-modification process determining its hydrolytic abilities and stability characteristics (*6–18*). As for carbon source, maltose is an inducer of the enzyme, and glucose has a repressive effect (*19–22*). The influence of the nitrogen source is not clear. Many studies exist related to medium optimization, including the nitrogen source. However, this subject has been managed empirically to a large extent with only few inferences as to the causes of the variability of the results obtained by changing the nitrogen source and/or its concentration and the C/N ratio (*20–25*). The medium composition is a communication code to which the physiology of the microorganism responds in the fermentation process. Its effects must be well understood. This subject has been studied in more detail in relation to other industrial products, such as proteases (*26–29*), antibiotics (*30–43*), ligninases (*44–50*) and the polisaccharide pullulan (*51,52*), where the relationship between control of nitrogen metabolism and product formation has been explored. There is, clearly, a lack of knowledge in this respect concerning GA production. A great deal of information has been accumulated regarding transport and assimilation of organic/inorganic nitrogen sources and the control of nitrogen utilization by fungi (*26–28, 53–64*). The regulation of nitrogen metabolism and control of gene expression in fungi has also been extensively studied (*61,65–68*). All enzymes and permeases in *Aspergillus nidulans*, whose synthesis is subject to nitrogen metabolite repression, are under the control of a positive-acting regulatory gene designated *are A*. This locus is believed to encode a protein that serves as a positive control element. Its role seems to be to monitor the nitrogen status of the cell, and the *are A* protein is presumably inactive in the presence of sufficient levels of nitrogen, reflected in the cellular concentration of a key nitrogen metabolite. Glutamine is, probably, this key nitrogen metabolite, i.e., is the effector for the *are A* gen product (*61,66*). Also, the *ntr* (nitrogen-regulated) operons in *E. coli* and *Salmonella typhimurium* have been well studied. These operons encode 20–25 proteins that are induced at low ammonium concentration and are under the control of the positive-acting regulatory protein NR_1, which is present in cells at all times. When NH_4^+ is plentiful, NR_1 exists in an inactive form. At low ammonia concentrations, it is converted to an active form. When *E. coli* is starved for ammonia, glutamine levels are low, and 2-ketoglutarate levels are high. The relative amounts of these two compounds influence uridylyltransferase and a cycle involving P2 phosphatase and NR_2 kinase, and they determine whether inactive NR_1 or the phosphorylated active NR_1-P predominates (*69*). In addition, the activity of glutamine synthetase (GS) is regulated in a similar fashion (*70*), and GS has been

studied as a regulator of enzyme synthesis in both bacteria and fungi (*61,71*). According to the foregoing, nitrogen metabolite repression is related to a positive-active regulatory protein present in both bacteria (*E. coli*) and fungi (*A. nidulans*), and the glutamine levels, which relates to the NH_4^+ availability, are important in both cases. Experimental data also showed the effect of NH_4^+ in compounds not directly related to nitrogen metabolism, such as antibiotics, so nitrogen metabolite repression would have a wider influence in cell physiology and, as a consequence, on the production of a variety of industrial products other than fungal proteases. Control mechanisms of gene expression involving wide domain regulatory genes or integrator genes could be involved in the repressive mechanisms in fungi (*66*). The present work evaluates the effects of the nitrogen source on GA production in the light of this valuable information.

MATERIALS AND METHODS

Culture Maintenance and Propagation

The fungus, *Aspergillus awamori* 2 B.361 U2/1 is a sequential mutant of NRRL 3112 (The Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, UK). A master culture of the microorganism was made by inoculating a Roux bottle containing potato dextrose agar with spores from a freeze-dried sample that was suspended in sterile physiological solution. The bottle was incubated at 30°C, and a dense sporulation was observed within 1 wk. This young culture was used as a source of conidia to prepare a spore suspension to be added to McCartney bottles containing silica gel (*72–74*). This procedure allowed the use of the same spore source throughout the work. Slopes of potato dextrose agar were used for spore production to be used as inoculum in all fermentations. The slopes were inoculated with a small amount of silica gel containing spores and incubated at 30°C for 1 wk. To obtain a spore suspension, 5.0 cm^3 of sterile water and some glass beads were added aseptically to a slope with a dense sporulation. After a short period of agitation, a black suspension was obtained. Spore concentration was determined using a Coulter Counter, and a standard inoculum of 4×10^3 conidia/cm^3 of growth medium was used in all fermentations.

Fermentations

Eight-liter batch fermentations were carried out in a 9.0-L; stirred-tank instrumented fermenter. The growth media designed to study the influence of the nitrogen (N) source on GA accumulation are presented in Tables 1, 2, and 3. They were organized into three groups according to their N source characteristics. Starch at variable concentrations was the

Table 1
Media Composition Used to Study the Effect of the YE and C/N Ratio on GA Accumulation by *A. awamori*[a]

	Carbon source		Nitrogen source						
Medium	Starch, % w/v	[C], mM	YE, % w/v	$(NH_4)_2SO_4$, % w/v	[N], mM	[C], mM	Total [C], mM	Total [N], mM	C/N
C	3.0	1100	0.5	-	37.7	187.5	1287.5	56.6	22.7
			-	0.125	18.9	-			
D	3.0	1100	1.0	-	71.4	375.0	1475.0	71.4	20.6
E	3.0	1100	1.3	-	92.8	487.5	1587.0	92.8	17.1
F	4.0	1466	1.0	-	71.4	375.0	1841.0	90.3	20.4
			-	0.125	18.9				
G	5.0	1833	1.0	-	71.4	375.0	2208.3	90.3	24.5
			-	0.125	18.9				

[a] Shows carbon (C) and nitrogen (N) sources with millimolar (m*M*) C and N concentrations and C/N ratios.

Table 2
Media Composition Used to Study the Effect of the $(NH_4)_2SO_4$ and C/N Ratio on GA Accumulation by *A. awamori*[a]

	Carbon source		Nitrogen source						
Medium	Starch, % w/v	[C], m*M*	YE, % w/v	$(NH_4)_2SO_4$, % w/v	[N], m*M*	[C], mM	Total [C], m*M*	Total [N], m*M*	C/N
H	3.0	1100	0.1	–	7.1	37.5	1137.0	98	11.60
			–	0.6	90.8	–			
I	4.0	1466	0.1	–	7.1	37.5	1503.5	98	15.34
			–	0.6	90.8	–			
J	5.0	1833	0.1	–	7.1	37.5	1870.5	120.6	15.50
			–	0.75	113.6	–			

[a]Shows carbon (C) and nitrogen (N) sources with millimolar (m*M*) C and N concentrations and C/N ratios.

Table 3
Media Composition Used to Study the Effect of Mixed (Organic and Inorganic) Nitrogen Sources, and C/N Ratio on GA Accumulation by *A. awamori*[a]

	Carbon source		Nitrogen source						
Medium	Starch, % w/v	[C], m*M*	YE, % w/v	$(NH_4)_2SO_4$, % w/v	[N], m*M*	[C], mM	Total [C], m*M*	Total [N], m*M*	C/N
K	3.0	1100	–	0.6	90.8	–			
			0.25	–	17.8	93.8	1194.0	108.6	11.0
L	3.0	1100	–	0.6	90.8	–			
			0.5	–	35.7	187.5	1287.5	126.5	10.0
M	3.0	1100	–	0.6	90.8	–			
			0.75	–	53.6	281.3	1381.3	144.4	9.6
N	3.0	1100	–	0.6	90.8	–			
			1.0	–	71.4	375.0	1475.0	162.2	9.1
P	3.5	1283	–	0.6	90.8	–			
			0.75	–	53.6	281.3	1564.0	144.4	10.8

[a] Shows carbon (C) and nitrogen (N) sources with millimolar (m*M*) C and N concentrations and C/N ratios.

carbon source used in all media, and the salt composition was as follows: $CaCl_2 \cdot 2H_2O$ 0.01%; $FeSO_4 \cdot 7H_2O$ 0.001%; $MgSO_4 \cdot 7H_2O$ 0.05%; KH_2PO_4 0.02% (w/v). Initial pH was 5.5. The C/N ratio of all media was calculated considering its total C and N millimolar concentration regardless of its source. In the first group (media C, D, E, F, and G), organic nitrogen yeast extract (YE) was used as the main nitrogen source. Different YE and starch concentrations were studied, and consequently, the C/N ratio varied. The ammonium sulfate at 0.125%, added to some media, was mainly used as a marker for N consumption (*75*) (Table 1). In the second group of fermentations (media H, I, and J), $(NH_4)_2SO_4$ was used as a main nitrogen source with variable C/N ratios. Yeast extract at 0.1% was added as a source of micronutrients and cofactors (Table 2). The effect of mixed organic (YE) and inorganic (ammonium sulfate) nitrogen was evaluated in the third set of fermentations (media K, L, M, N, P). The strategy adopted was to use in all media a fixed ammonium sulfate concentration (0.6%) and variable YE concentrations, 0.1–1.0%, (Table 3). Another approach to the problem would be to keep the YE concentration constant, i.e., 1%, and to increase the ammonium concentration. Under these circumstances, though, the NH_4^+ would be absorbed as a nitrogen supplement and in similar amounts regardless of its concentration in the medium, since the YE is a preferred nitrogen source (*54,57,63*). Preliminary shaken-flask experiments indicated this to be the case. All fermentations were carried out at 30°C, uncontrolled pH, agitation of 400 rpm, and air flow rate of 0.5 vvm. Citrate buffer (*76*) was used in media containing high ammonium concentration to prevent the pH value from dropping below 2.2.

Analytical Assays

Enzyme and Glucose Concentrations

Samples used for GA and glucose concentrations and pH measurements were first filtered through glass microfiber filters to separate the mycelium. Glucoamylase concentration was determined by measuring the level of catalytic activity of the broth supernatant. For this purpose, 2.0 cm^3 of the supernatant (diluted where necessary) was incubated with 18 cm^3 of 60 m*M* maltose (acetate buffer pH 4.4) at 40°C. The initial rates of glucose production were expressed as micromoles of glucose liberated per minute per cm^3 of broth supernatant (IU/cm^3) (*77*). Glucose concentration was determined using a glucose analyzer (Beckman-2).

Biomass Determinations

Cell dry weight was determined as follows: The samples were filtered through preweighted glass microfiber filters under suction. After being thoroughly washed with distilled water, the filter cake was dried at 100°C for 24 h.

Ammonia Concentration

Berthelot's reaction was used for the colorimetric determination of the ammonia concentration. The reagents and procedure used were as described by SIGMA (Procedure No. 640).

RESULTS

Fermentations Using YE as Main Nitrogen Source: Media C, D, E, F, G

A reproducible sequence of events followed the inoculation of the spores into the growth medium: Within 24 h, germination occurred followed by vegetative growth, enzyme accumulation, and pH drop, concomitant to C and N source consumption. Within the working conditions used to perform the batch fermentations, a pH drop from 5.5 to 2.2 was observed without affecting the enzyme stability. Moreover, according to data previously obtained, the same final enzyme concentration was observed in fermentations using controlled pH 5.0 or uncontrolled pH within the range 5.5–2.2 (*75*). In fermentations using media C, D, and E, the starch concentration was kept constant (3.0%) and the YE content varied. Figure 1 shows GA accumulation and glucose consumption for these media. According to the results using medium C, YE 0.5%, GA concentration increased up to 89 h, and biomass accumulation increased beyond this point (Fig. 2). This additional growth was not, however, true fungal growth, since the time-course of the dissolved oxygen concentration indicates the end of the run at the same time, i.e., 89 h (Fig. 2). This nonreplicatory growth was probably the result of polysaccharide accumulation, since it may be more pronounced under certain conditions, such as excess of carbon source (*20,37*). The halt in real cell growth with glucose still present in the medium indicates nitrogen limitation. Medium D (YE 1.0%) was balanced for the carbon and nitrogen requirements as GA and biomass accumulation, and glucose consumption finished within the same time interval, i.e., 69 h (Fig. 1 and Table 4). The end of growth and glucose depletion also occurred concomitantly with medium E (YE 1.3%), although it was probably carbon limited. Its initial and consumed C/N ratio* of 17 was lower in comparison to the values observed in the fermentations above, i.e., 23.6 and 20.0 (Table 4). The C/N ratio imbalance of medium E was also shown by its lower total specific enzyme production ($Y_{[p/x]}$) of 2.96 in comparison to the value obtained with medium D (Table 4). A steady decrease in fermentation time occurred in media C, D, and E, consequent to the increase in YE concentration and

*The consumed C/N ratios were calculated assuming total consumption of the N from the YE, and the measured concentrations of residual glucose and ammonium sulfate when used.

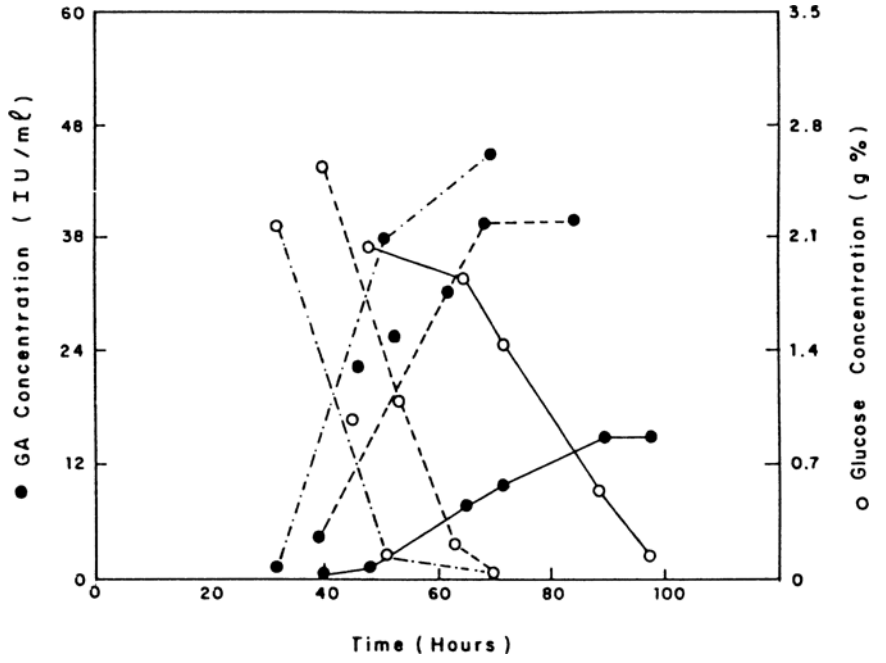

Fig. 1. Glucoamylase (GA) accumulation (●) and glucose consumption (○) by *Aspergillus awamori* in media C (———), D (-----), and E (-·-·-·-). Starch 3.0% plus YE 0.5% (C), 1.0% (D), and 1.3% (E).

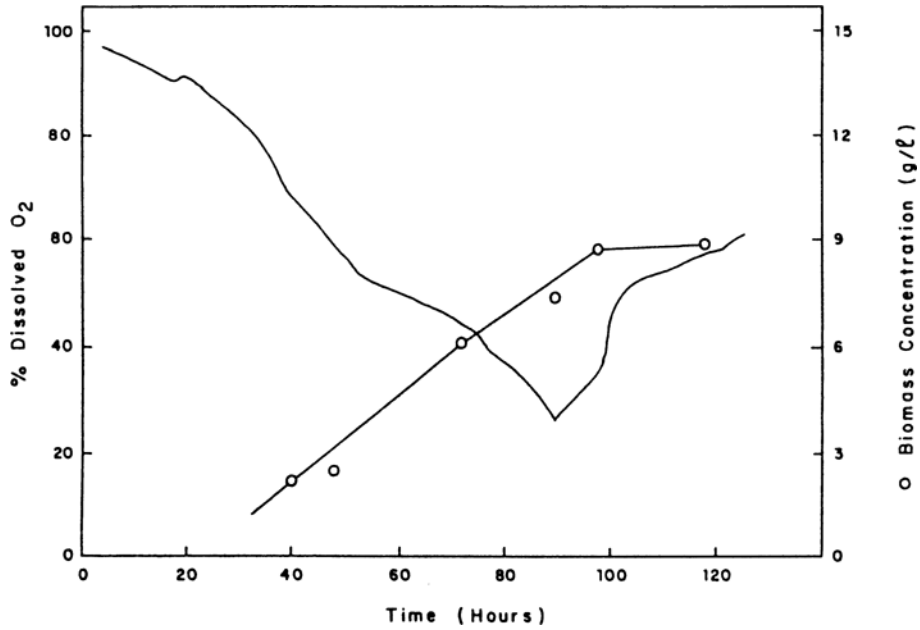

Fig. 2. Biomass accumulation (○—○) and oxygen consumption (———) by *Aspergillus awamori* in medium C (starch 3.0% and YE 0.5%).

Table 4
Effect of Yeast Extract and Starch Concentrations on Glucoamylase (GA) Accumulation[a]

Medium	Time,[b] h	C/N_1	C/N_2[d]	[GA],[e] IU/cm³	[Biomass], mg/cm³	$Y_{(p/x)}$, IU/mg	$Y_{(p/x)}$/h, IU/mg/h
C	89.5[c]	22.7	23.6	15.0	7.4	2.02	0.020
D	69.0	20.6	20.0	37.5	11.6	3.20	0.047
E	52.0	17.1	17.1	45.0	15.2	2.96	0.057
F	72.0	20.4	20.4	41.6	12.0	3.47	0.048
G	72.0[c]	24.5	20.8	48.7	12.5	3.90	0.054

[a] Shows fermentation time, initial (C/N_1) and consumed (C/N_2) carbon-to-nitrogen ratio, final GA and biomass concentration, total specific enzyme production ($Y_{[P/X]}$), and total specific enzyme production per hour ($Y_{[P/X]}$/h).
[b] Glucose depletion.
[c] Nitrogen-limited fermentations, time for maximal GA concentration.
[d] Value at the highest enzyme concentration.
[e] Maximal concentration.

therefore to an increase in cell growth rates. The GA production rates also increased in a proportional fashion. Final biomass and GA concentrations were also correspondingly higher (Table 4). In fermentations using media F and G, the YE concentration was kept constant, 1%, and starch concentrations of 4.0% and 5.0% were used. The results will be discussed in comparison to the data obtained from medium D, which involved the same YE concentration. The time-course of fermentations using media F and G is presented in Fig. 3. Medium F seems to be balanced as both C and N nutrients finished at the same time, i.e., 72 h, and no enzyme and biomass accumulation was observed beyond this point (Table 4). Medium G was shown to be nitrogen limited, similar to medium C. Maximal levels of the enzyme accumulation and nitrogen depletion were observed within 72 h; however, glucose depletion occurred within 97 h. No important differences in final biomass were observed with media F and G in comparison to medium D, showing again the correlation between amino nitrogen, the concentration of which was kept constant, and biomass formation. The enzyme production rates were similarly affected, although final levels increased with the amount of starch present in the growth medium. Starch concentrations of 3.0, 4.0 and 5.0% gave final enzyme concentrations of 37.5, 41.6, and 48.7 IU/cm³. In comparing the data for the consumed C/N ratio obtained for media C, D, E, F, and G, it is apparent that the C/N ratio within the range 20–21 is balanced for amino nitrogen (Table 4). An optimal medium for GA production showed a C/N ratio 19.5 (*7*). It is also apparent from the $Y_{(p/x)}$ datum from medium C, and in agreement with previous reports, that in addition to using a balanced C/N ratio, absolute concentration of the nutrients is significant for the enzyme final yield (*76*). Greater GA activity and total specific enzyme production

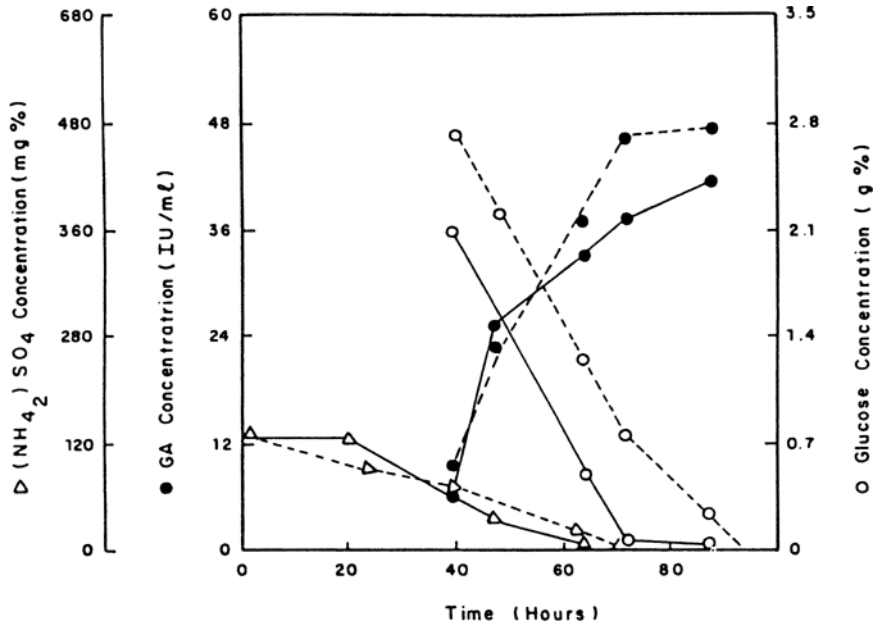

Fig. 3. Glucoamylase (GA) accumulation (●), glucose (○), and NH_4^+ (△) consumption by *Aspergillus awamori* in media F (—) and G (--). Yeast extract 1.0% plus starch 4.0% (F) and 5.0% (G).

per hour ($Y_{[p/x]}/h$) were obtained with media E and G. It is possible though that a higher carbon supply in medium E would increase the enzyme production (Table 4).

Fermentations Using $(NH_4)_2SO_4$ as the Main Nitrogen Source

Media H, I, J

The results presented in Table 5 and Fig. 4 relate to the effect of ammonium sulfate and media C/N ratio on glucoamylase accumulation. Medium H was shown to be carbon limited, since residual NH_4^+ was observed after glucose depletion. Media I and J were balanced in terms of carbon and nitrogen requirements, since both nutrients finished at the same time. The consumed C/N ratio in the later case was 15.3 (Table 5). Reported values from the literature, using inorganic N are 14 (*7*) and 16 (*9,20*). The differences in media composition had little influence on final biomass concentration (Table 5). The different media composition, though, affected GA production and, consequently, the yield ratio $Y_{(p/x)}$. The final data for these parameters were 23.8 IU/cm^3 and 2.3 for medium H in comparison to 51.7 IU/cm^3 and 5.2 (medium I), and 58 IU/cm^3 and 5.3 (medium J). A lower consumed C/N ratio of 14.2 was also observed in medium H in comparison to 15.3 and 15.5 in media I and J, respectively (Table 5). This result indicates a higher relative NH_4^+ intake by the fungus

Table 5
Effect of Ammonium Sulfate and Starch Concentrations on Glucoamylase (GA) Accumulation[a]

Medium	Time,[b] h	C/N_1	C/N_2[c]	[GA],[d] IU/cm³	[Biomass], mg/cm³	$Y_{(p/x)}$, IU/mg	$Y_{(p/x)}/h$, IU/mg/h
H	72.0	11.6	14.2	23.8	9.96	2.3	0.030
I	92.0	15.3	15.3	51.8	9.80	5.2	0.056
J	103.0	15.5	15.5	58.0	10.80	5.3	0.051

[a]Shows fermentation time, initial (C/N_1) and consumed (C/N_2) carbon-to-nitrogen ratio, final GA and biomass concentration, total specific enzyme production ($Y_{[P/X]}$), and total specific enzyme production per hour ($Y_{[P/X]}/h$).
[b]Glucose depletion.
[c]Value at the highest enzyme concentration.
[d]Maximal concentration.

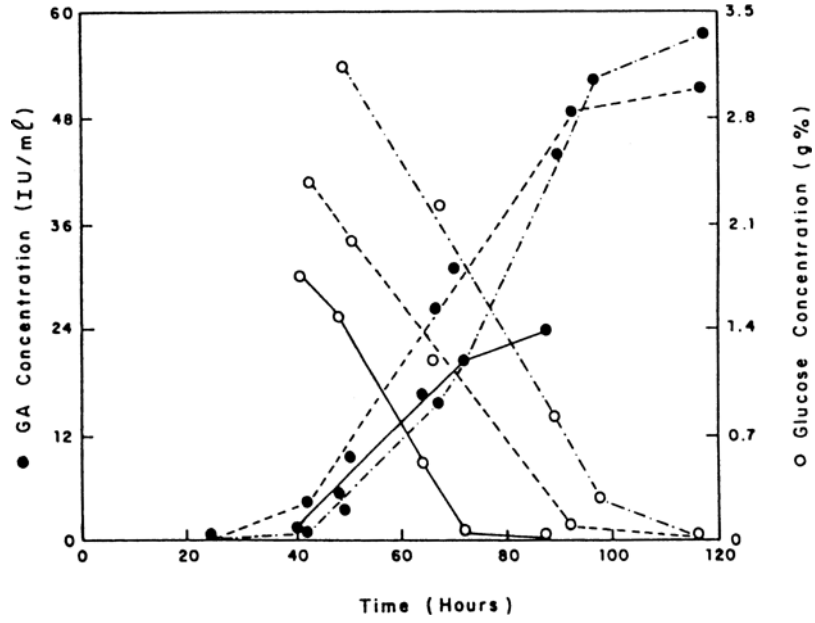

Fig. 4. Glucoamylase (GA) accumulation (●) and glucose consumption (○) by *Aspergillus awamori* in media H (——), I (----), and J (-·-·-·-).
Medium H: Starch 3.0%, $(NH_4)_2SO_4$ 0.60%.
Medium I: Starch 4.0%, $(NH_4)_2SO_4$ 0.60%.
Medium J: Starch 5.0%, $(NH_4)_2SO_4$ 0.75%.

in medium H and, consequently, a higher NH_4^+ availability in the metabolic pool that could have a repressive effect on GA production. The higher enzyme concentration observed in media I and J could be explained by the provision of a balanced C/N ratio translated into a balanced metabolic pool in terms of the relative amounts of 2-ketoglutarate and glutamine,

and consequently, derepressed conditions for GA biosynthesis. It is also important to consider that enzyme production may be affected not only by repression, but also by depletion of a common carbon/energy supply in conditions where cell biosynthesis is favored. Most of the substrates in a living cell are used in branched and crosslinked reaction pathways. In such reactions, enzymes often compete for a common substrate, its availability being vital.

Comparative Evaluation of Amino and Ammonium N on GA Production

Fermentations using organic and inorganic nitrogen with starch at 4.0 and 5.0%, therefore without carbon limitation, were compared. According to data in Tables 4 and 5, higher enzyme and lower biomass levels were obtained using medium I (starch 4.0%, $[NH_4]_2SO_4$ 0.6%) in comparison to medium F (starch 4.0%, YE 1.0%). The same response was given by medium J in comparison to medium G. The figures for the $Y_{(p/x)}$ and $Y_{(p/x)}/h$ data for these same media also favor the use of inorganic nitrogen, i.e., average values of 5.25 and 0.056 (media I and J) compared to 3.7 and 0.051 (media F and G) for glucoamylase production. It is important to consider, however, that the fermentation using medium E (starch 3.0%, YE 1.3%) gave an equivalent specific productivity figure, i.e., 0.057 (Table 4). It could, thus, be expected that a higher starch availability would increase the enzyme production. As to optimized C/N ratios using amino or ammonium nitrogen, lower values were obtained for ammonium (15.3) in comparison to amino (20.8) nitrogen. The dissimilar effects of amino and ammonium nitrogen on cell growth and product formation are the result of differences in the basic biochemical steps related to their use by the cell. As far as amino acids are concerned, they are assimilated and directly incorporated into proteins, and are not first degraded into ammonia. This process favors biomass accumulation, and the available carbon source is highly allocated into it (*78*). Abundant nitrogen is incorporated into cell constituents with a consequent increase in the rate of respiration and glucose consumption. With respect to ammonium, its use is limited by the rate at which it is incorporated into its organic counterparts. The metabolite 2-ketoglutarate fed from the Krebs cycle and thus derived mainly from the carbohydrate metabolism must be provided for the assimilatory reaction to take place. Biomass formation is, comparatively, a more limited process, and more carbon/energy and N sources may be channeled toward maintenance of cell metabolism and product biosynthesis. These general considerations would explain to a certain extent the results that were obtained for optimized C/N ratios for GA production using either amino or ammonium nitrogen.

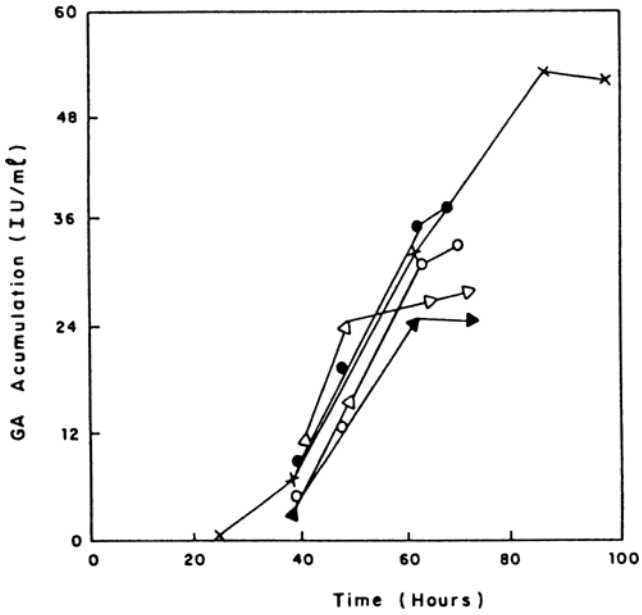

Fig. 5. Glucoamylase (GA) accumulation by *Aspergillus awamori* in media K (○-○-○), L (●—●—●), M (△—△—△), N (▲—▲—▲), and P (XXX). Starch 3.0%, $(NH_4)_2SO_4$ 0.6% plus YE at 0.25% (K), 0.5% (L), 0.75% (M), 1.0% N. Medium P: Starch 3.5%, $(NH_4)_2SO_4$ 0.6% plus YE 0.75%.

Fermentations Using Combined (Amino/Ammonium) Nitrogen Sources

Media H, K, L, M, N

The effect of combined nitrogen sources on GA production was studied in carbon limited fermentations, where the starch and ammonium concentrations were kept constant, i.e., 3.0 and 0.6%, respectively, and the YE concentrations increased within the range 0.1–1.0% (Table 3). The time-course of the relevant fermentations is presented in Fig. 5, and Table 6 gives a summary of the final data concerning enzyme and biomass accumulation, and fermentation time. Except for YE concentrations of 0.1 and 0.25%, which showed similar values for final biomass concentration, the biomass increased steadily with the amount of YE. Final enzyme accumulation, though, showed a different pattern: Although a steady increase was observed within the range 0.1–0.5% YE, a decrease occurred at higher concentrations. The negative effect of using organic and inorganic N as a supplement to each other has been reported for complementing wheat bran with $(NH_4)_2SO_4$ (*7*) and sodium nitrate with corn steep liquor (*18,79*). The use of inorganic salts as a nitrogen supplement is a current industrial practice (*1*). Table 6 also compares the initial and consumed C/N ratios, total specific enzyme production, and total

Table 6
Effect of Combined Nitrogen Sources, YE, and Ammonium Sulfate on Glucoamylase (GA) Accumulation[a]

Medium	Time,[b] h	C/N_1	C/N_2[c]	[GA],[d] IU/cm³	[Biomass], mg/cm³	$Y_{(p/x)}$, IU/mg	$Y_{(p/x)}$/h, IU/mg/h
H	73.0	11.6	14.2	23.8	9.9	2.3	0.030
K	65.0	11.0	13.9	33.3	9.4	3.5	0.050
L	55.0	10.0	13.7	37.5	10.6	3.5	0.052
M	58.0	9.6	13.6	28.0	11.8	2.3	0.034
N	65.0	9.1	12.3	27.0	14.0	1.9	0.020
P	78.0	10.5	12.2	51.0	12.3	4.1	0.057

[a]Shows fermentation time, initial (C/N_1) and consumed (C/N_2) carbon-to-nitrogen ratio, final GA and biomass concentration, total specific enzyme production ($Y_{[P/X]}$), and total specific enzyme production per hour ($Y_{[P/X]}$/h).
[b]Glucose depletion.
[c]Value at the highest enzyme concentration.
[d]Maximal concentration.

specific enzyme production per hour. The absorbed C/N ratios were higher in comparison to the media C/N ratios, confirming carbon limitation. The increase in the YE concentration keeping constant starch concentration in media H, K, L, M, and N resulted in a steady decrease in the media C/N ratios. The same decreasing tendency was observed in the consumed C/N ratio, indicating a proportional consumption of amino nitrogen by the fungus. The data for the $Y_{(p/x)}$ and $Y_{(p/x)}$/h parameters showed the same pattern observed for enzyme production above, i.e., a diminishing tendency by the use of YE at 0.5% onward (Table 6).

Ammonium/Amino Nitrogen Consumption

Table 7 shows the data for the specific ammonium and amino nitrogen consumption, total specific glucoamylase production, and final biomass concentration in media H, K, L, M, and N. The relative percentage of these values is depicted in Fig. 6. These results indicate a gradual decrease in ammonium nitrogen consumption as the amino nitrogen concentration increased in the growth medium. Curves from Fig. 6 representing these phenomena show an apparent correlation indicating the consumption of the amino N by the fungus and, therefore, the metabolic preference toward this source by the cell. Consequent to the use of amino nitrogen is the gradual increase in final biomass concentration, although glucoamylase accumulation was negatively affected by YE concentrations beyond 0.5%. Figure 6 also shows normalized data for $Y_{(p/x)}$ and final biomass concentration from medium D (YE 1.0%, starch 3.0%). The comparison between these two parameters from media D and N shows medium N with higher biomass and lower $Y_{(p/x)}$. The higher nitrogen availability in

Table 7
Specific Amino and Ammonium Nitrogen Consumption, Specific GA Production ($Y_{[P/X]}$), and Final Biomass Accumulation in Media Using Combined Nitrogen Source

Medium	Specific N consumption, m*M*/g		$Y_{(p/x)}$, IU/mg	Biomass, mg/cm³
	Amino	Ammonium		
H	0.71	7.30	2.4	9.96
K	1.89	7.72	3.5	9.40
L	3.36	5.48	3.5	10.6
M	4.50	4.03	2.3	11.8
N	5.10	3.49	1.92	14.0
P	4.30	6.04	4.1	12.3

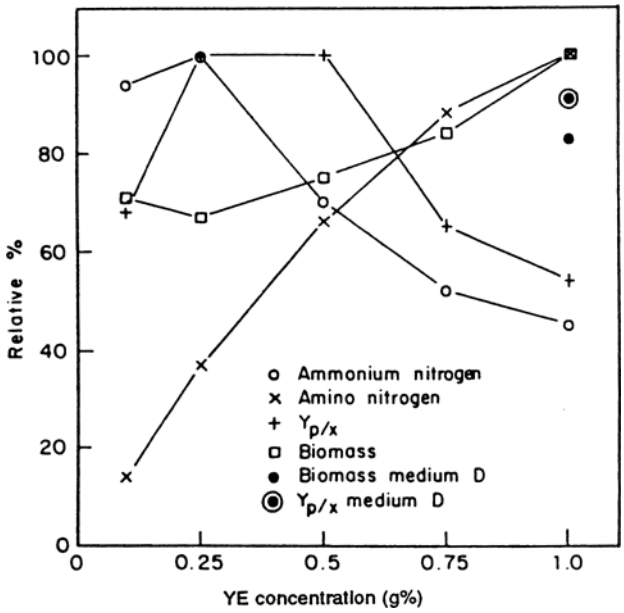

Fig. 6. Normalized data for final amino and ammonium nitrogen consumption, product yield ($Y_{[p/x]}$) and final biomass concentration in media H, K, L, M, and N. Media composition: starch 3.0%, ammonium sulfate 0.6%, and YE 0.1% (medium H), 0.25% (medium K), 0.5% (medium L), 0.75% (medium) M, and 1.0% (medium N). Normalized data for biomass concentration and $Y_{[p/x]}$ from medium D (starch 3.0% and YE 1.0%) are also shown. Percent data originated from the results presented in Table 7 and are shown relative to the largest value in each category.

medium N (YE plus $[NH_4]_2SO_4$) stimulated biomass accumulation with a decrease in GA biosynthesis. According to data presented in Table 8, although it was not possible to identify a relationship between total specific nitrogen consumption and enzyme yields, a better correlation was observed between the specific consumed C/N ratio and total specific GA

Table 8
Total Specific Nitrogen Consumed (m*M* N/biomass [mg/cm³], the Specific Consumed C/N Ratio (C/N/biomass [mg/cm³]), and the Specific Enzyme Production ($Y_{[P/X]}$) in Fermentations Using Combined Nitrogen Sources

Medium	Total specific N consumed	Specific consumed C/N ratio	$Y_{(p/x)}$
H	8.00	1.43	2.3
K	9.61	1.47	3.5
L	8.84	1.29	3.5
M	8.53	1.15	2.3
N	8.59	0.87	1.9

production, since both values show the same decreasing pattern. Considering that the balance of 2-ketoglutarate and glutamine in the intracellular pool is related to the specific consumed C/N ratio, the correlation above could be the result of repression of GA biosynthesis. Aiming to confirm the negative effect of carbon-source deficiency in the above conditions, a richer carbon environment was offered to a "repressive" condition to GA production, i.e., medium M. Accordingly, a fermentation using medium P was performed. It showed the same nitrogen composition as medium M with a higher starch content of 3.5%. The results are presented in Table 6 and Fig. 5. Final biomass accumulation was quite similar in both media. Enzyme production rates were also similar, although the final enzyme concentration in medium P was considerably higher. A comparable phenomenon had already been observed in fermentations using media H and I (Table 5 and Fig. 4), where glucoamylase concentration increased in a similar fashion, without increase in cell growth, on a higher carbohydrate availability and using the ammonium nitrogen surplus. Therefore, the cause for a lower enzyme production in medium M was carbon shortage and its effects on cell metabolism or/and on the control of gene expression.

DISCUSSION

The reported general effects of the nitrogen source on antibiotics, ligninase, and pullulan production were also identified in this work concerning glucoamylase production. A stepwise investigation of the separate effects of amino, NH_4^+, and combined nitrogen sources allowed a better understanding of the phenomenon. Biomass synthesis was a preferred process within cell metabolism, and under carbon-limited conditions, GA biosynthesis was not a favored process. The reason for this metabolic shift could be depletion of metabolic pools or cofactors supplying the relevant metabolic pathways in conditions where cell growth or/and control of gene expression, i.e., repression of GA biosynthesis, is

favored. In that case, nitrogen metabolite repression could be involved. The *are A* protein, which controls the nitrogen status of the cell, could act on the regulatory sequence of the glucoamylase operon. The activity of the *are A* protein could be regulated in a similar fashion as the NR_1 protein studied in bacteria. The possibility of the expression of an enzyme, such as glucoamylase, mainly related to carbon metabolism, and a target for carbon catabolite repression being affected by nitrogen metabolite repression is likely. There is a relationship between carbon catabolite repression and nitrogen catabolite repression. In *N. crassa*, the production of protease induced by bovine serum albumine is produced in response either to N, S, or C limitation (*61*) and is repressed by high concentrations of fructose (*28*). The structural gene that encodes the relevant enzymes may be served by a complex regulatory region that contains recognition sequences for signals arising independently from the nitrogen and carbon circuits. The role of nitrogen metabolite repression on glucoamylase production, as discussed in the present work, has a reasonable degree of theoretical basis and could provide a starting point for a better understanding of the effects of the nitrogen source not only on glucoamylase production, but also on other industrial products equally affected.

CONCLUSIONS

The results discussed in the previous sections indicate carbon-source limitation to be the cause for the lower glucoamylase yields; therefore, the provision of a balanced C/N ratio is vital to avoid the enzyme production to be hindered by carbon-source depletion. Optimized growth media for GA production using YE or ammonium sulfate as main nitrogen source were, respectively starch 4.7%, YE 1.0%, ammonium sulfate 0.125% and starch 5.0%, ammonium sulfate 0.75%, YE 0.1%. These media compositions showed a C/N ratio of 20.8 and 15.5, respectively, and the following data were obtained for final enzyme accumulation, total-specific enzyme production ($Y_{[p/x]}$), and total specific enzyme production per hour ($Y_{[p/x]}/h$), respectively: 48.7 IU/cm^3, 3.9 IU/mg and 0.054 IU/mg/h for YE as N source and 58.0 IU/cm^3, and 5.3 IU/mg and 0.051 IU/mg/h for ammonium sulfate as N source. The yeast extract proved to be more favorable to cell growth and also a preferred nitrogen source. The data from fermentations where ammonium sulfate and YE were used concomitantly indicate a gradual decrease in the ammonium consumption by the microorganism as the YE concentration increased in the growth medium. Consequent to the use of amino nitrogen by the fungus was a gradual increase in final biomass concentration, although glucoamylase accumulation was negatively affected by YE concentrations beyond 0.5%, i.e., 0.75% and 1.0%, using starch at 3.0% and in the presence of ammonium sulfate at 0.6%. Accordingly, the figures for final enzyme accumulation

and the $Y_{[p/x]}$ and $Y_{[p/x]}/h$ parameters were, respectively, 37.5 IU/mL, 28.0 IU/mL, 27.0 IU/mL; 3.5 IU/mg, 2.3 IU/mg, 1.9 IU/mg, and 0.052 IU/mg/h, 0.034 IU/mg/h, 0.02 IU/mg/h. This negative effect on the enzyme production was owing to the starch deficiency in conditions of high nitrogen availability, which favored biomass accumulation. Two cellular mechanisms were discussed in connection with this metabolic shift: depletion of metabolic pools and/or control of gene expression involving nitrogen metabolic repression.

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