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Xylanase Production by *Aspergillus awamori* in Solid-State Fermentation and Influence of Different Nitrogen Sources

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Abstract

The use of purified xylan as a substrate for bioconversion into xylanases increases the cost of enzyme production. Consequently, there have been attempts to develop a bioprocess to produce such enzymes using different lignocellulosic residues. Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications, including xylanases, whose levels in fungi are generally much higher than those in yeast and bacteria. Considering the industrial importance of xylanases, the present study evaluated the use of milled sugarcane bagasse, without any pretreatment, as a carbon source. Also, the effect of different nitrogen sources and the C:N ratio on xylanase production by *Aspergillus awamori* were investigated, in experiments carried out in solid-state fermentation. High extracellular xylanolytic activity was observed on cultivation of *A. awamori* on milled sugarcane bagasse and organic nitrogen sources (45 IU/mL for endoxylanase and 3.5 IU/mL for β-xylosidase). Endoxylanase and β-xylosidase activities were higher when sodium nitrate was used as the nitrogen source, when compared with peptone, urea, and ammonium sulfate at the optimized C:N ratio of 10:1. The use of yeast extract as a supplement to the these nitrogen sources resulted in considerable improvement in the production of xylanases, showing the importance of this organic nitrogen source on *A. awamori* metabolism.

Index Entries: *Aspergillus awamori*; xylanases; nitrogen nutrition; solid-state fermentation; sugarcane bagasse.

Introduction

Filamentous fungi have been widely used to produce hydrolytic enzymes for industrial applications including xylanases. The genera

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Aspergillus and *Trichoderma* have shown to be efficient producers of these enzymes on an industrial scale *(1)*. *Aspergillus awamori* has been used industrially for the production of several enzymes such as glucoamylase, α-amylase, and protease. Another important advantage of *A. awamori* is that it has a long history of safe use for the manufacture of food products destined for human consumption and is regarded as a nontoxigenic and nonpathogenic fungus *(2)*.

Xylanases have been considered for clarifying fruit juices and wines, food processing in combination with cellulases, and improving the nutritional properties of agricultural silage and grain feed. Its main potential application, though, relates to cellulosic pulp treatment in which the biocatalyst has already been incorporated into commercial bleach sequences *(3)*.

The bioconversion of lignocellulosic materials to fermentable sugars for fuel alcohol production has been hindered by economical and technical aspects, as well as the existence of more competitive sources of carbohydrates such as starch and sucrose. Nevertheless, there has been much effort to develop efficient bioprocesses using such raw materials, particularly those deriving from agricultural residues, which have no production cost attached to them, although costs for collection and transportation of these residues to a centralized processing location may be incurred.

Considering the industrial importance of xylanases, and the importance of nitrogen nutrition on fungus metabolism, in the present study we evaluated the effect of nitrogen sources and C:N ratio on xylanase production by *A. awamori* using milled sugarcane bagasse as the carbon source. Experiments were carried out in solid-state fermentation.

Materials and Methods

Maintenance and Propagation of Organism and Preparation of Inoculum

A. awamori NRRL 3112 was used as the xylanase-producing microorganism. Czapeck agar slants, incubated at 30°C for 5 to 6 d were used for conidia production to serve as inoculum in all fermentations. The conidia suspension was prepared by adding 4 mL of sterile water to a slope with a dense sporulation. Conidia enumeration was determined using a Neubauer camera (Assistent, West Germany). A standard inoculum of 5×10^6 conidia/g of bagasse was used in all cases.

Growth Media and Culture Conditions

Fermentations for enzyme production were carried out in 500-mL conical flasks, plugged with cotton corks, containing 4 g of roughly 1-mm-sized dry sugarcane bagasse, ground in a disc mill (Perten Laboratory mill 3600, Perten, SW). The sugarcane bagasse was moistened with 50 mL of an aqueous solution composed of 0.2 g of NaCl, 0.2 g of KH_2PO_4, 0.04 g of $MgSO_4 \cdot 7H_2O$, and different nitrogen sources at a C:N ratio of 14:1. Other

Table 1
Nitrogen Composition
of Different Media (C:N = 14:1)

Nitrogen source	Quantity (g/4 g bagasse)
NH_2CNH_2	0.24
NH_2CNH_2[a]	0.14
$(NH_4)_2SO_4$	0.54
$(NH_4)_2SO_4$[a]	0.30
$NaNO_3$	0.69
$NaNO_3$[a]	0.39

[a]These media contained 0.5 g of yeast extract/4 g of bagasse.

C:N ratios were also investigated. The control experiment contained 0.5 g of peptone plus 0.5 g of yeast extract, providing 113.5 mg of total nitrogen, which is equivalent to a C:N ratio of 14:1. Fermentations were carried out in media containing individual nitrogen sources such as sodium nitrate, ammonium sulfate, urea, and combined nitrogen sources supplemented with yeast extract to the same total nitrogen content (Table 1). The flasks were incubated in a stove (FANEM, Brazil) at 30°C for 60 h, after which their whole contents were extracted by adding 100 mL of distilled water. After a period of 30 min under agitation at 150 rpm and at room temperature, the supernatant was separated by filtration to obtain the crude enzyme preparation.

Analysis

Extracellular protein was measured by the modified Lowry method *(4)* using bovine serum albumin as the standard. Elemental composition of sugarcane bagasse was determined using an Elementar Perkin-Elmer analyzer 2400 CHN.

Enzymatic Assays

Endoxylanase activity was measured by the formation of reducing sugars according to the following procedure. A 0.9-mL solution of 1% birchwood xylan (Sigma) in 50 m*M* citrate-phosphate buffer, pH 5.0, was preincubated for 2 min at 60°C followed by the addition of 0.1 mL of the diluted crude enzyme. The reaction was stopped after 2 min by the addition of 1 mL of the dinitrosalicylic acid reagent. One unit of activity was defined by the release of 1 µmol of reducing sugar (as xylose)/min. β-Xylosidase activity was determined by measuring the formation of *p*-nitrophenol according to the following procedure. A 0.5-mL solution of 1.25 m*M* *p*-nitrophenyl β-xylanopiranoside in 50 m*M* phosphate-citrate buffer, pH 5.0, was preincubated for 2 min at 55°C followed by the addition of 0.1 mL of the diluted crude enzyme. The reaction was stopped after

10 min by the addition of 4 mL of 0.25 *M* Na_2CO_3. One unit of enzyme activity was defined by the release of 1 µmol of *p*-nitrophenol/min.

pH Stability Assays

Endoxylanase and β-xylosidase stability experiments were carried out in phosphate-citrate buffer at different pH values. In each case, the supernatant of filtrated samples from *A. awamori* cultivation on peptone + yeast extract medium was adjusted to the selected condition by dilution with the corresponding buffer and incubation at 30 and 35°C, respectively. Enzymatic activities were measured after 1 h for endoxylanase and 0.5 h for β-xylosidase.

Assays were performed in duplicate, except for bagasse elemental composition, which was performed in triplicate. Each experiment was repeated twice.

Results and Discussion

Sugarcane bagasse, an abundant lignocellulosic residue produced in Brazil, was shown to be an adequate raw material for the production of xylanases. Its partial elemental composition was 40% carbon, 6% hydrogen, and 1.9% nitrogen, allowing the estimation of different C:N ratios for different nitrogen sources employed in the media composition.

According to the data presented in Fig. 1, the highest endoxylanase activity, approx 100 U/mL, was obtained when nitrate supplemented with yeast extract was used as the nitrogen source. However, the use of $NaNO_3$ as the sole nitrogen source provided similar results. The superiority of this inorganic source is also evidenced by the specific activity values as well as by the volumetric productivity (Table 2), pointing out that both media induce highest production of endoxylanases. Also, as judged by the specific activity figures, a lower amount of contaminant proteins is excreted. The majority of filamentous fungi are autotrophic with respect to nitrogen sources and are able to grow in synthetic media consisting basically of glucose, NH_4^+ or NO_3^-, and minerals. Therefore, *A. awamori* seems to have taken advantage of this widespread ability. The pathway for the reduction of nitrate to ammonium involves two enzymes: nitrate reductase and nitrite reductase *(5,6)*. In *Aspergillus nidulans*, synthesis of nitrate reductase and nitrite reductase is controlled by both nitrate induction and ammonium repression. Furthermore, nitrate reductase has been postulated to play an autogenous regulatory role, controlling its own synthesis and that of nitrite reductase *(7)*.

On the other hand, the medium with ammonium sulfate without yeast extract supplementation displayed the lowest activity value (13 U/mL). In this case, the crude enzyme preparation had a pH value of 2.9 (Table 2). This could have been in response to the low enzyme activity owing to pH inactivation. It is well known that ammonium is transported by the fungus as ammonia, leaving the hydrogen ion behind, resulting in acidification of

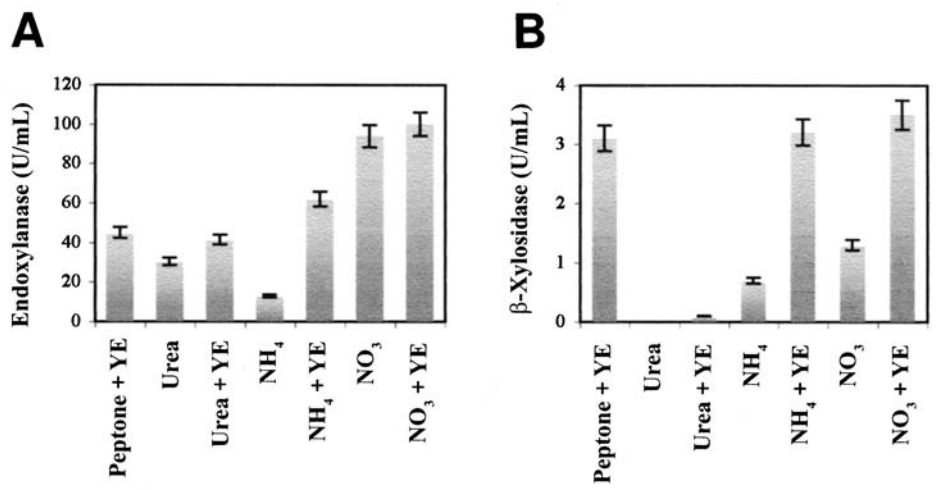

Fig. 1. Effect of organic and inorganic nitrogen sources on the production of **(A)** endoxylanase and **(B)** β-xylosidase (C:N = 14:1). Fermentations were carried out in media containing individual N sources such as sodium nitrate, ammonium sulfate, urea, and combined nitrogen sources supplemented with yeast extract (YE) to the same total nitrogen content.

Table 2
Effect of Nitrogen Sources on Production of Xylanases (C/N = 14/1)

	Endoxylanase		β-Xylosidase			
Nitrogen source	Volumetric productivity (U/[L·h])[a]	Specific activity (U/mg)	Volumetric productivity (U/[L·h])[a]	Specific activity (U/mg)	Initial pH	Final pH
Peptone + yeast extract	754	21.8	51.9	1.5	5.8	6.1
Urea	511	22.3	0.0	0.0	4.5	7.8
Urea + yeast extract	696	21.7	1.8	0.0	5.5	7.9
$(NH_4)_2SO_4$	216	20.9	11.5	1.1	4.4	2.9
$(NH_4)_2SO_4$ + yeast extract	1034	39.0	53.4	2.0	5.5	6.5
$NaNO_3$	1571	86.8	20.9	1.2	4.3	5.9
$NaNO_3$ + yeast extract	1666	60.5	57.7	2.1	5.5	6.1

[a]Calculated at 60 h of cultivation.

medium *(6)*. Nonetheless, the results obtained by Fernández-Espinar et al. *(8)* using different nitrogen sources on the production of α-arabinofuranosidase by *A. nidulans* showed that ammonium sulfate and ammonium chloride gave rise to notably higher enzyme activity than that reached in the presence of the other inorganic and organic compounds. Thus, it is clear that the ability of a cell to utilize a particular compound is a complex

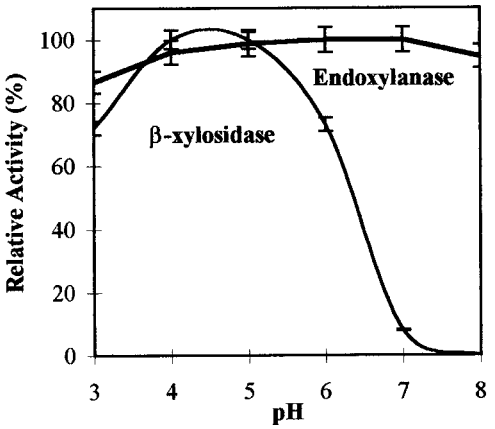

Fig. 2. pH stability of endoxylanase and β-xylosidase. Experiments were carried out with supernatants from *A. awamori* cultivation on peptone + yeast extract medium. The corresponding 100% of the relative activity was 42 U/mL for endoxylanase and 2.8 U/mL for β-xylosidase.

affair, depending on the genetic ability of the organism to synthesize the requisite enzyme in the first place, and then on its capacity to respond to induction.

In the present study, when ammonium sulfate was supplemented with yeast extract, the pH value of the enzyme preparation was about 6.5, indicating that the organic nitrogen source, of which approx 50% exists as free amino acid, was used preferentially. According to studies on *Saccharomyces cerevisiae* nitrogen consumption, ammonium is not absorbed from the wort until disappearance of the amino acids glutamate, glutamine, aspartate, asparagine, serine, threonine, arginine, and lysine *(9)*. The different effects of amino and ammonium nitrogen on product formation is 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. This fact could be beneficial for all growth and enzyme production. Regarding ammonium, its use is limited by the rate at which it is incorporated into its organic counterparts *(10)*.

A rise in pH was observed when *A. awamori* was cultivated on urea and urea plus yeast extract. Urea is cleaved by urease and ammonia appears as result of the enzymatic hydrolysis, raising the pH of the medium *(11)*. It can be observed that the differences in pH were more disadvantageous for β-xylosidase activity than for endoxylanase when urea or urea plus yeast extract was used. This fact can be related to the lack of stability seen for β-xylosidase at basic pH (Fig. 2). To evaluate the possible effect of low and high pH values on the enzyme production, an experiment was conducted, and the results showed that β-xylosidase activity was greatly increased in media containing citrate-phosphate buffer, pH 5.0 (Fig. 3). However, the production of endoxylanase remained nearly constant in the same media, and its stability was higher and more constant in a broader pH

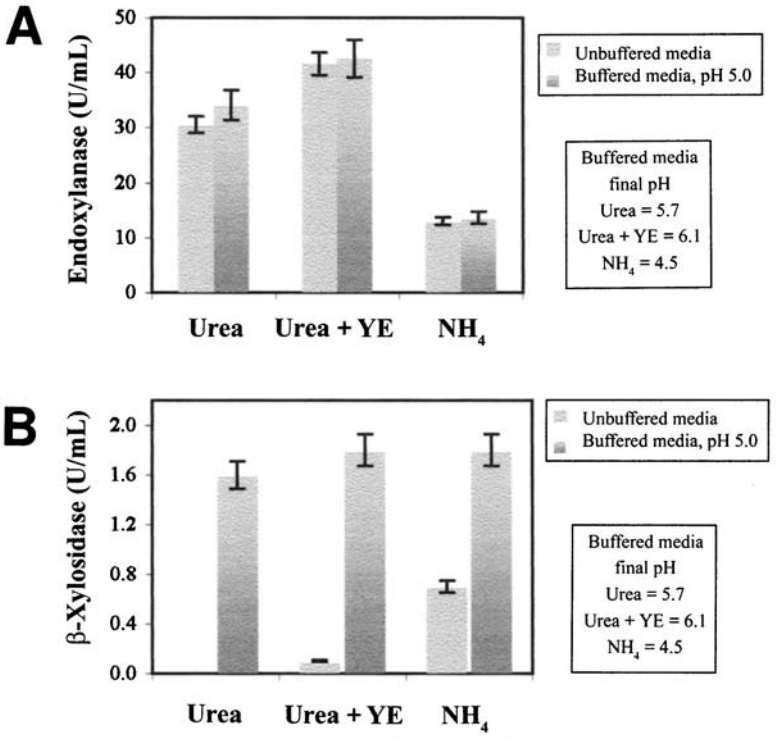

Fig. 3. **(A)** Endoxylanase and **(B)** β-xylosidase production in buffered (citrate-phosphate buffer, pH 5.0) and unbuffered media. YE, yeast extract.

range than β-xylosidase stability. Hence, the endoxylanase production did not increase as was expected in the buffered medium.

Peptone plus yeast extract has also been used to study the influence of organic nitrogen sources on endoxylanase and β-xylosidase activities. Our results show an endoxylanase activity comparable with that obtained in the presence of urea plus yeast extract. Nevertheless, both experimental data represent half of the activity obtained using $NaNO_3$ plus yeast extract medium. Generally, all fungi grow faster with complex organic nitrogen sources, but the effectiveness of these compounds varies considerably. In earlier studies, different organic nitrogen sources were found to improve xylanase production in several xylanase-producing fungi *(12–14)*. Their results are in disagreement with ours, which can be ascribed, particularly, to different species and even to variation in strain.

Concerning β-xylosidase production, $NaNO_3$, $(NH_4)_2SO_4$, and peptone, all supplemented with yeast extract, displayed the highest and similar results (3.5, 3.2, and 3.1 U/mL, respectively), with specific activity slightly better for the former.

Sodium nitrate plus yeast extract was used in further experiments, because this presented the most satisfactory results. These nitrogen sources were added on the basis of carbon percentage in bagasse (40%). Although this lignocellulosic residue contains nitrogen in its composition (2%),

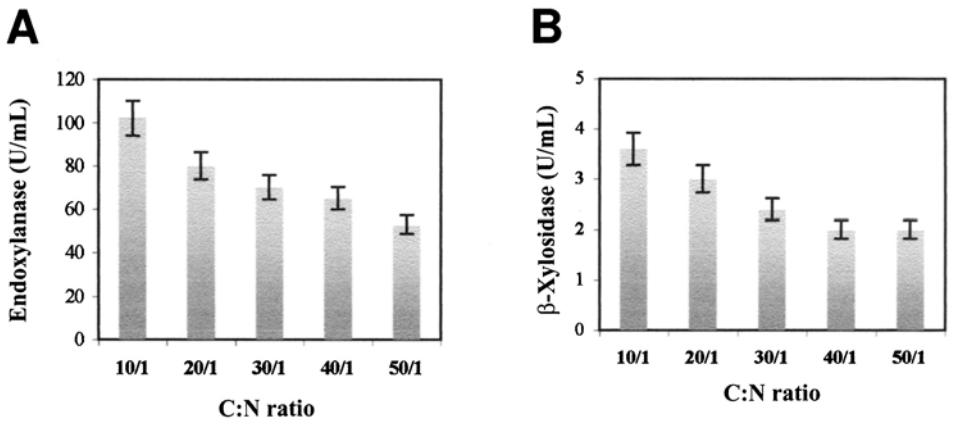

Fig. 4. Influence of C:N ratio on the synthesis of **(A)** endoxylanase and **(B)** β-xylosidase activities using $NaNO_3$ plus yeast extract. Of the total nitrogen added in each experiment, 57% proceeded from $NaNO_3$ and 43% from yeast extract.

A. awamori was unable to synthesize either endoxylanase or β-xylosidase from the basal medium, denoting the necessity of fortifying these media with nitrogen sources. The production of xylanolytic enzymes was maximized when the C:N ratio was adjusted to 10:1, as indicated in Fig. 4. The results show the importance of the C:N ratio on enzyme production. Considering the role of the nitrogen sources, one can observe that under nitrogen-limited conditions xylanase biosynthesis was less favored.

To determine the best proportions of sodium nitrate and yeast extract used as combined nitrogen sources, an experiment was performed using the best C:N ratio (10:1) obtained in the last experiment (Fig. 4). The highest values for xylanolytic enzyme activity (Fig. 5) were obtained for an initial sodium nitrate proportion of 40%, considering its nitrogen content. These results confirmed that yeast extract has an important role in enzyme synthesis by *A. awamori*, probably because a complex nitrogen source was essential for good growth and high enzyme activity, or because of some important elements contained in yeast extract that are necessary for the metabolism of fungus.

In conclusion, these results demonstrate that for the production of xylanases, sodium nitrate (to an initial proportion of 40%) supplemented with yeast extract appeared to be the best nitrogen source when added at a C:N ratio of 10:1, indicating that nitrogen source limitation is the cause for the decrease in xylanase yields. Considerations between organic and inorganic nitrogen sources are important, because their use is subject to metabolic versatility, as well as economical implications. The variation in pH media was shown to depend on nutrient composition, especially on nitrogen sources. Media containing proteins or related compounds showed a smaller variation in pH than those in which organic or inorganic nitrogen was used as the sole nitrogen source, except for $NaNO_3$. pH seems to have affected more β-xylosidase than endoxylanase activity.

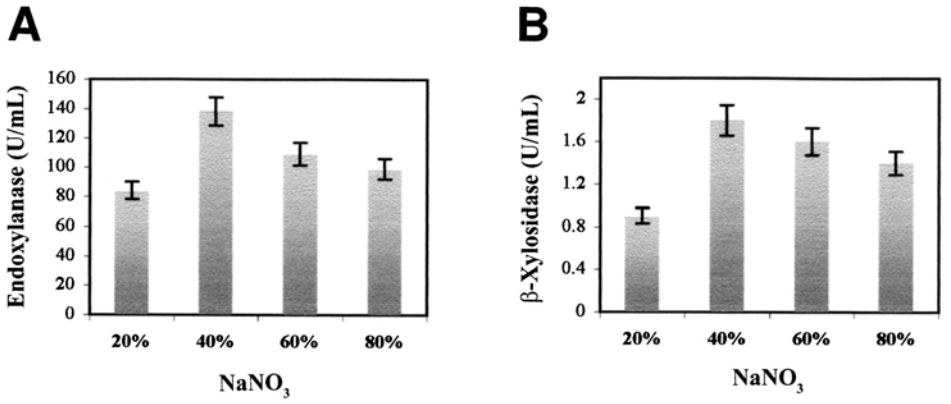

Fig. 5. Effect of proportions of sodium nitrate and yeast extract in **(A)** endoxylanase and **(B)** β-xylosidase production (C:N = 10:1).

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