Brazilian Journal of Microbiology (2001) 32:15-19
ISSN 1517-8382

THE USE OF α-METHYL-D-GLUCOSIDE, A SYNTHETIC ANALOGUE OF MALTOSE, AS INDUCER OF AMYLASE BY *ASPERGILLUS* SP IN SOLID-STATE AND SUBMERGED FERMENTATIONS

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Submitted: June 26, 2000; Returned to authors for corrections: October 16, 2000; Approved: January 18, 2001

ABSTRACT

The use of α methyl-D-glucoside (αMG), a synthetic analogue of maltose, as carbon source and inducer of amylase synthesis to several species of *Aspergillus* was studied in submerged and solid-state fermentations. Among a group of ten species, *A. tamarii*, *A. fumigatus* and *A. flavus* were able to produce biomass and high specific amylolytic activity in submerged cultures containing αMG as the only carbon source. In solid state fermentation, the enrichment of basal wheat bran or corn cob medium with αMG increased up to 3 times the production of amylases. In both submerged and solid state fermentations, aMG was more effective inducer of amylases than maltose and starch.

Key words: amylase, *Aspergillus*, solid-state fermentation, submerged fermentation

INTRODUCTION

Many microorganisms, including bacteria, yeast and filamentous fungi are able to produce amylases (a term that refers here as α-amylase, β-amylase and glucoamylase) and are among the most important enzymes in present day biotechnology (8,16,17). Amongst the fungal amylases, those of species of *Aspergillus* such as *A. niger*, *A. awamori* and *A. oryzae* have received most attention because of their high productivity (20).

Fungal amylases can be produced using two main methods, solid state cultivation systems and submerged liquid cultivation systems. Although most research has used submerged culture, which allows the control of degree of aeration, pH, temperature of the medium and other environmental factors required for the optimum growth of the microorganisms, solid state fermentation has gained renewed interest for the production of these enzymes in view of several economic and engineering advantages. This procedure has been often employed to produce amylases (16).

Besides starch and starchy materials, maltose and other malto-oligosaccharides have been described as inducers of amylases in microorganisms (5,11,14,18,19). However, in some cases, the supplementation of cultures with maltose does not ensure the production of high levels of amylases, because maltose is readily hydrolysed to glucose and the production of enzyme is negatively affected (4,18,19). One of several experimental strategies used to overcome this type of problem is the enrichment of culture media with inert synthetic analogues of mono-, di- or oligosaccharides as gratuitous inducers. For example, a series of non-metabolizable alkyl-β-D-xylosides, especially methyl-β-D-xyloside (βMX) have been used to induce the production of xylanase more efficiently than xylan in many microorganisms (3,10,23). Then, the purpose of this study was to investigate the use of α methyl-D-glucoside (αMG), a synthetic analogue of maltose, as substrate for growth and inducer of production of amylase by *Aspergillus* sp., in both submerged and solid state fermentation.

MATERIALS AND METHODS

Microorganisms

Ten species of *Aspergilli* were used in this work: *A. glaucus*, *A. clavatus*, *A. ochraceus*, *A. oryzae*, *A. janus*, *A. niger*, *A. parasiticus*, *A. tamarii*, *A. fumigatus* and *A. flavus*. They were cultivated on potato dextrose agar at 30ºC for 5 days for spore production. The conidial suspensions were prepared by adding 10 ml of sterilized water to slant cultures and the surface gently rubbed with a sterilized wire loop.

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Enzyme production in submerged fermentation (SF)

For production of enzymes in SF, two different forms of culture were used. In the first, spores of each fungus species were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of mineral medium (13) supplemented with glucose at 1% (w/v). Incubation was carried out on an orbital shaker at 120 rpm and 30ºC for 3 days. The mycelia were then recovered by filtration, washed twice with distilled water and fractions of 1 g (wet weight) were transferred to the same medium with starch, maltose or αMG at 1% (w/v) as the carbon source. Flasks were harvested at periodic intervals, the contents filtered through filter paper and the mycelia dried to constant weight at 60ºC. The culture filtrates were analysed for amylase activity and substrate residual concentration. In the second form of culture spores of each fungus species were inoculated in 250 ml Erlenmeyer flasks containing 50 ml of mineral medium supplemented with starch, maltose or αMG at 1% (w/v) as the carbon source. The cultures were developed at 30ºC on a rotary shaker at 120 rpm. At periodic intervals, the mycelia were removed from the media by filtration and the enzymatic activities and substrate residual concentration were analysed in the culture filtrates. In both cases, the results were expressed as the mean of at least three different cultures.

Enzyme production in solid-state fermentation (SSF)

For production of enzymes in SSF, the fungi were grown at 30ºC in 250 ml Erlenmeyer flasks containing 5 g of wheat bran or corn cob powder enriched or not with glucose, maltose, starch or αMG at 1% (w/w). Vogel solution (13) was used to adjust the moisture content to 75%. Dry weight of the substrate and moisture content were determined gravimetrically after drying samples at 60ºC. In order to extract the enzyme, 50 ml of cold water was added and the mixtures were shaken for 30 min at 4ºC and centrifuged. The supernatants were assayed for amylase. Results were expressed as the mean of at least three different cultures.

Enzyme activity and other analytical methods

Amylase activity (a term that refers here to α-amylase plus β-amylase plus glucoamylase activities) was estimated by analysis of reducing sugars released during hydrolysis of 0.5% (w/v) starch in 0.05 M phosphate buffer, pH 6.0, at 40ºC by the dinitrosalicylic acid method (12). One unit of amylase activity was defined as the amount of enzyme that releases 1 µmol of reducing sugar as D-glucose per min under the assay conditions. Reducing sugar concentration was estimated using dinitrosalicylic acid reagent. Starch, maltose and extracellular αMG concentrations were estimated using the anthrone reagent (9). Glucose concentration was determined by the glucose oxidase peroxidase method (2). The intracellular αMG were extracted with ethanol (mycelia were boiled for 20 min in 70% ethanol) and the ethanol-soluble extracts were subjected to paper chromatography on Whatman nº 1 paper in 1:5:3:3 benzene:butanol:pyridine:water, and developed with a silver nitrate reagent (22).

Chemicals

Starch, maltose, αMG and glucose were obtained from Sigma Chemical Corp. (St. Louis, Mo). All other reagents were of analytical grade.

RESULTS AND DISCUSSION

The pH values of all cultures in this work were monitored and no significant difference among pH values were observed in the cultures supplemented with different sugars (data not shown). High levels of amylase were found in submerged culture filtrates with starch, maltose or starchy materials, while very low levels of amylase were detected in submerged culture filtrates with glucose, cellobiose, lactose or sucrose (data not shown). Thus, the amylases from *Aspergillus* sp are inductive enzymes. To examine whether αMG was applicable to the amylase production as an available inducer, washed glucose-grown mycelia were transferred to media containing starch, maltose or αMG at 1.0% as substrate (Fig. 1A). An effective induction by αMG, more efficient than starch and maltose induction, was found in *A. flavus*, *A. tamarii* and *A. fumigatus*, while no induction by αMG was observed in the other seven species. The determination of residual concentrations of starch, maltose and αMG in the culture filtrates, showed that αMG was not consumed by *A. glaucus*, *A. clavatus*, *A. ochraceus*, *A. oryzae*, *A. janus*, *A. niger* and *A. parasiticus*, while the determination of residual αMG in the culture filtrates of species induced by αMG (*A. flavus*, *A. tamarii* and *A. fumigatus*), indicated that more than 70% of αMG was taken up by the cells (Fig. 1B). As some synthetic analogues of sugars have been described as non-metabolizable inducers (3,15,23), the intracellular αMG was extracted with ethanol and the ethanol-soluble extracts were subjected to paper chromatography. The experiment showed that αMG was present, but did not accumulate inside the mycelia (data not shown). Thus, these three species of *Aspergillus* were apparently able to use αMG as carbon source. To confirm this idea, spores of *Aspergillus* sp were inoculated into mineral medium containing αMG as the only carbon source and the production of biomass by these cultures was compared with those obtained from starch and glucose cultures (Fig. 2). Three species, *A. flavus*, *A. tamarii* and *A. fumigatus* were able to produce biomass when αMG was offered as the only carbon source. However, αMG was consumed more slowly by the cells than starch and glucose. In fact, the residual starch and glucose concentrations in the culture filtrates were less than 5% after 4 days of cultivation, while 10 days were necessary for the cells to consume 90% of αMG (data not shown).

The production of amylases in cultures using maltose and αMG as the substrate was monitored daily (Fig. 3A-C). The residual concentration of the substrate in the maltose-culture filtrates was less than 10% after 3 days, while production of amylases by the three species was maximum in the 5-day-cultures. After a lag of 2 days, where apparently the cells did not take up any αMG, the residual concentration of this source decreased

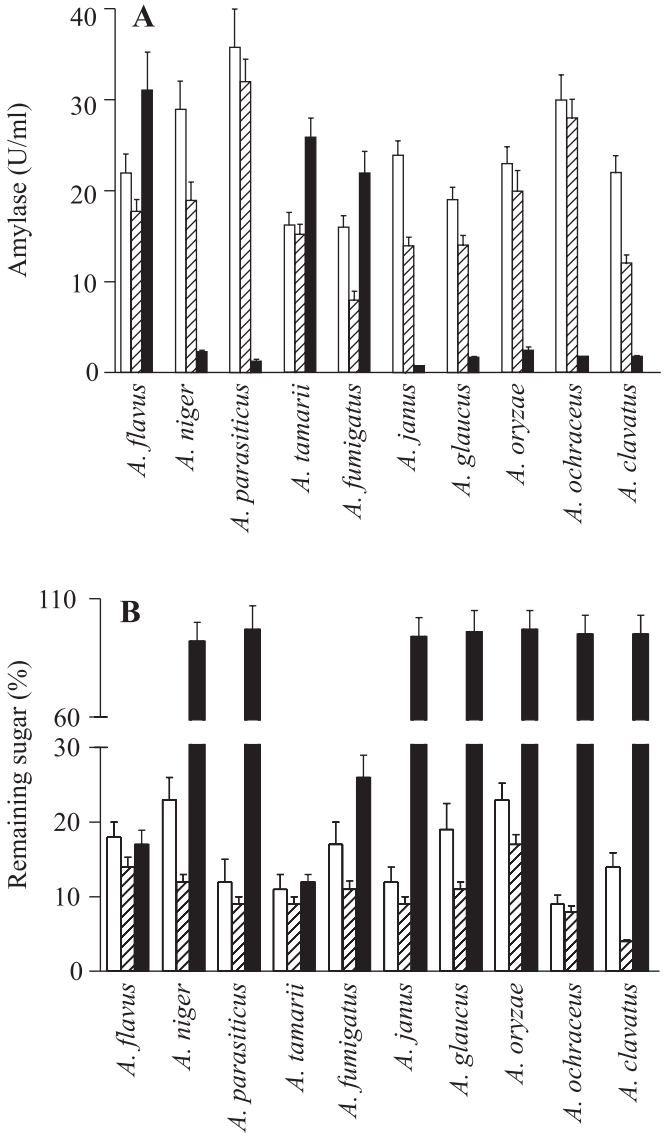

Figure 1. Production of amylases by *Aspergillus* sp. Washed glucose-grown cells were transferred to the inducing culture medium containing starch (□), maltose (▨) or αMG (■) at 1% (w/v). The cultures were carried out at 30ºC and 120 rpm. After 4 days, the levels of amylase (A) and the remaining inducer concentration (B) in the culture filtrates were determined. Error bars represent S.D. from the mean values.

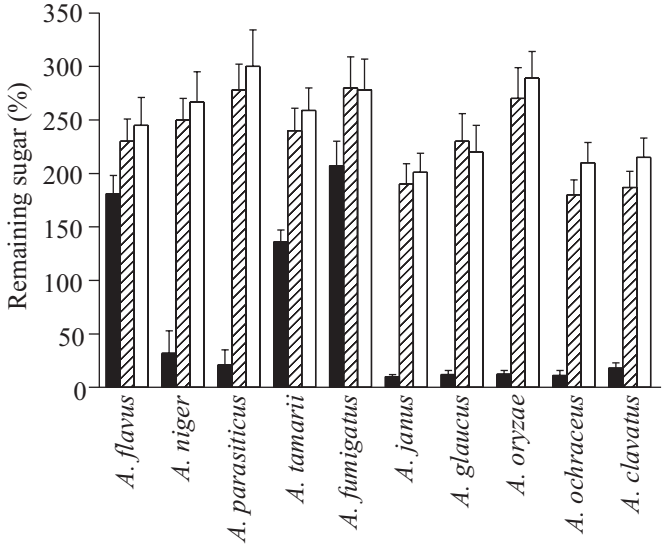

Figure 2. Production of biomass by *Aspergillus* sp. Spores of *Aspergillus* were inoculated in mineral medium supplemented with maltose (□), starch (▨) or αMG (■) at 1% as the only carbon source and the cultures were developed at 30ºC and 120 rpm. Starch and maltose cultures were carried out for five days and aMG cultures were carried out for 10 days. Error bars are S.D. from the mean values.

slowly, and less than 10% was present in the culture filtrates after 10 days. The delay in the consumption of αMG suggests the requirement for a specific inducible transport system.

The inducer efficiency of some oligosaccharide synthetic analogues has been suggested before in submerged cultures of species of *Aspergillus*. The most used, β-methyl-xyloside, was a non-metabolizable inducer of xylanases from *A. ochraceus* (3) and *A. sydowii* (6) and a metabolizable inducer of xylanases from *A. tamarii* (21). The use of synthetic analogues of malto-oligosaccharides is less frequent, but an efficient induction of amylases by αMG has already been described in submerged cultures from *A. fumigatus* (7) and *Aspergillus* sp. K-27 (1).

To test the ability of αMG to improve the production of amylases in solid-state fermentation, the fungi were cultivated under solid-state conditions using two different substrates: wheat bran (a starchy medium, where high levels of amylases are produced) and corn cob (very poor in starch and for this reason not adequate to produce amylases) enriched or not with αMG at 1% (w/v) (Fig. 4A-B). The addition of αMG increased about 1.5-2 times the production of amylase in wheat bran medium (Fig. 4A). The increase in the production of amylases can not be explained by the simple presence of an additional source of nutrients, considering that the addition of glucose, maltose, and starch did not result in a significant increase in the enzyme production. The inductive efficiency of αMG was more evident from the results with corn cob cultures. Very low levels of amylase were produced using basal corn cob media, 15, 13 and 9 U/g substrate respectively to *A. flavus*, *A. tamarii* and *A. fumigatus* (Fig. 4B). The enrichment of the medium with starch or maltose increased about twice the production of amylase (40, 28 and 16 U/g substrate, respectively), while the addition of αMG in the medium increased the production of amylases about four times or more (90, 70 and 60 U/g substrate, respectively).

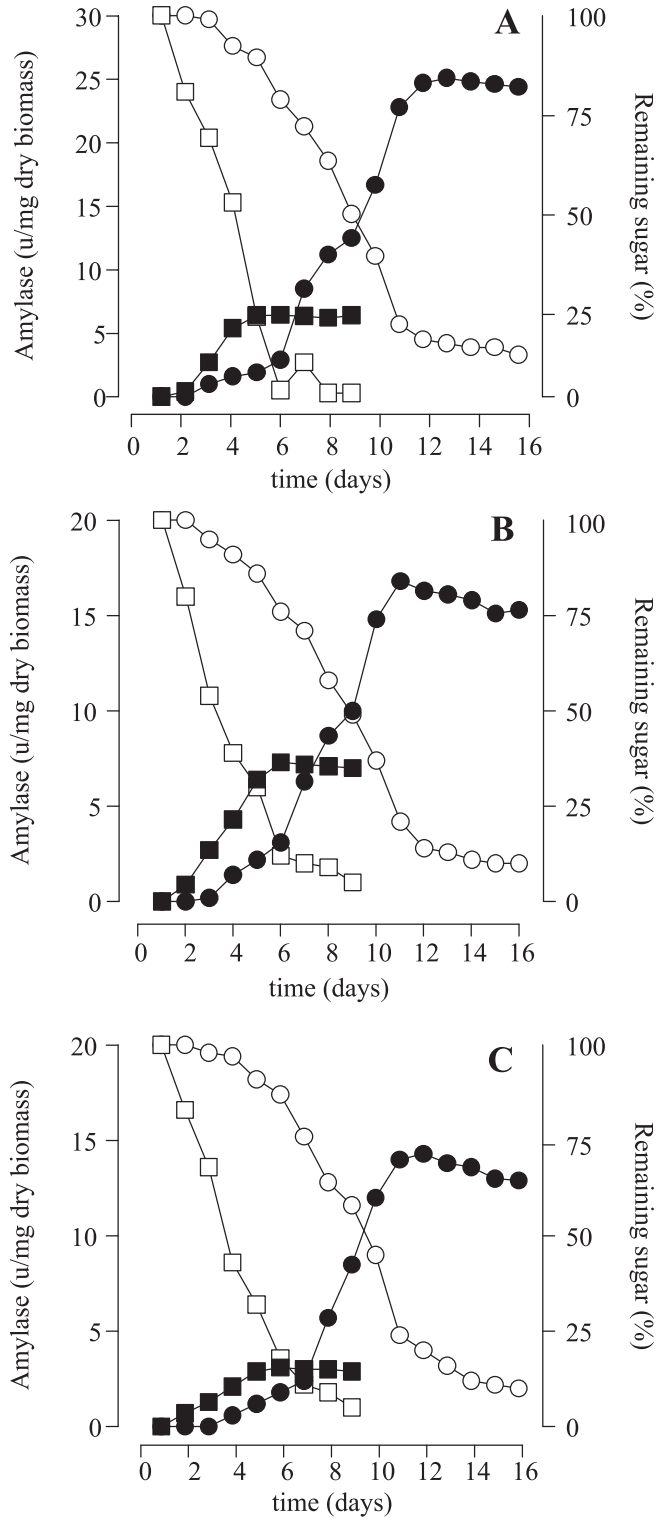

Figure 3. Profile of amylase production by *Aspergillus* sp. A: *A. flavus*; B: *A. tamarii*; C: *A. fumigatus.* Amylase production in maltose cultures (■); amylase production in αMG cultures (●); maltose (□); αMG (○).

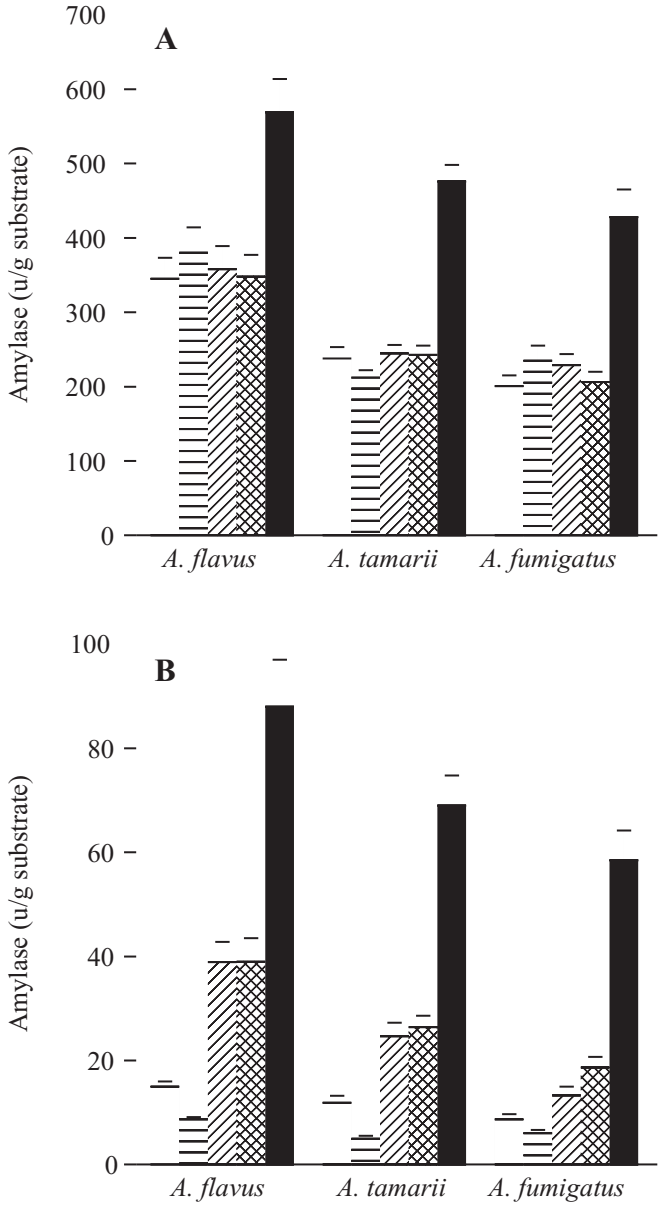

Figure 4. Production of amylase in solid state fermentation. A: wheat bran cultures; B: corn cob cultures. Control cultures (□); 1% glucose supplemented cultures (▤); 1% maltose supplemented cultures (▨); 1% starch supplemented cultures (▩); 1% αMG supplemented cultures (■).Error bars represent S.D. from the mean values.

In spite of *A. tamarii, A. flavus* and *A. fumigatus* were able to use the synthetic analogue of maltose, αMG, as the only carbon source, the very large lag phase presented by the αMG cultures when compared with maltose cultures, do not stimulate the use of αMG as carbon source. However, the results presented in this paper support the conclusion that αMG is an effective inductor of

production of amylase by *A. tamarii*, *A. flavus* and *A. fumigatus* in both submerged and solid-state fermentation and may be added in the starchy cultures to increase the production of amylases. As solid state fermentation holds tremendous potential for the production of industrial enzymes (16,17), the enrichment of conventional solid state media with synthetic inductor may be an attractive alternative to improve the efficiency of processes.

ACKNOWLEDGMENTS

This work was supported by the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Universidade Estadual de Maringá. The authors are grateful to M.A.F. Costa and A.Chaves for their technical assistance.

RESUMO

Uso de α-metil-D-glucosídeo, um análogo sintético de maltose, como indutor de produção de amilases por *Aspergillus* sp em fermentação submersa e fermentação em estado sólido

O uso de α-metil-D-glucosídeo (αMG), um análogo sintético de maltose, como fonte de carbono e indutor das amilases por *Aspergillus* sp., foi estudado em fermentação submersa e fermentação em estado sólido. De um grupo de dez espécies, *A. tamarii*, *A. fumigatus* e *A. flavus* foram capazes de produzir biomassa e alta atividade amilolítica em culturas submersas contendo αMG como única fonte de carbono. Em fermentação em estado sólido, o enriquecimento do meio basal de farelo de trigo ou sabugo de milho com αMG aumentou em 3 vezes a produção das amilases. Tanto em fermentação submersa quanto em fermentação em estado sólido, αMG induziu mais eficientemente a produção de amilases que maltose e amido.

Palavras-chave: amilase, *Aspergillus* sp., fermentação em estado sólido, fermentação submersa

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