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# EFFECTS OF INITIAL SUGAR AND MINERAL CONCENTRATIONS OF CAROB SUBSTRATES ON THE GROWTH OF *ASPERGILLUS CARBONARIUS* IN SOLID STATE FERMENTATION SYSTEM

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## EFECTOS DE LA CONCENTRACION INICIAL DE AZUCARES Y SALES MINERALES SOBRE EL CRECIMIENTO DE *ASPERGILLUS CARBONARIUS* EN MEDIO SOLIDO SOBRE ALGARROBA

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## SUMMARY

The effects of initial sugar concentration and addition of salts to carob substrates on *Aspergillus carbonarius* growth were studied in a solid state fermentation system. Data on $CO_2$ production, sugar and tannin levels, and biomass optical observations revealed that the initial (= total) sugar con-

centration of the carob substrate, combined with mineral addition, led to high biomass production. However, removal (52%) of carob sugars prior to fermentation, and elimination of the mineral source, resulted in a tannin degradation up to 55%, but poor biomass production.

Key words: *Aspergillus carbonarius*, carob bean, total sugars, tannin degradation, solid state fermentation, mineral salts.

## RESUMEN

Se estudiaron los efectos de la concentración inicial de azúcares y sales minerales sobre el crecimiento de *Aspergillus carbonarius*, cultivado en medio sólido sobre harina de algarroba. Los análisis de producción de $CO_2$, consumo de azúcares y taninos, así como el desarrollo micelial del hongo indicaron que la concentración inicial de azúcares totales, combinada con la adición de una solución mineral variable, influyen de una manera importante en la producción final de biomasa. Sin embargo, cuando se mantuvo el contenido inicial de azúcares en 52% en la harina de algarroba antes de llevar a cabo la fermentación, y se eliminaron las sales minerales del medio de cultivo, se observó una degradación importante de los taninos (55%) y una baja producción de biomasa.

Palabras clave: *Aspergillus carbonarius*, algarroba, azúcares totales, degradación de taninos, fermentación sólida, sales minerales.

## INTRODUCTION

Carob beans are the fruits of *Ceratonia siliqua* L. The ripe deseeded carob pod (husk), although rich in water-soluble sugars (40-60%), has a very low protein content (3-5%) and contains high concentrations of total tannins (4-13%) [Marakis and

Karagouni, 1985; Marakis *et al.*, 1993]. The occurrence of tannins, mainly of condensed type (Tamir *et al.*, 1971), diminishes its nutritional value (Vohra *et al.*, 1966; Tamir and Alumot, 1970).

This product, which is produced worldwide, may be used in different ways (*e.g.*, feed and enzyme production), after tannin degradation and husk protein enrichment. Despite the wide use of solid state fermentation (SSF) for processing several agro-industrial byproducts, and the production of secondary metabolites (*e.g.*, enzymes), as well as other microbial products (Lonsane *et al.*, 1982, 1985; Roussos *et al.*, 1991a, 1994), SSF has not yet been applied for tannin degradation and tannase production. Accordingly, the authors have started studies on a strain of *Aspergillus carbonarius* with high tanninolytic abilities in SSF of carob pods.

This paper describes the effects of initial sugar concentration and addition of minerals to the carob substrate on the growth of *A. carbonarius*, in a SSF system.

## MATERIALS AND METHODS

### *Microorganism*

A strain of *Aspergillus carbonarius* (Bainier) Thom, isolated from mouldy carob beans according to Marakis (1980), was studied.

### *Substrates*

Ripe milled carob pod (hereafter referred to as carob), and ripe milled carob from which 52% of its water-soluble sugars had been removed by stirring and filtration (hereafter referred to as spent carob), were mixed with sugar cane bagasse (Roussos *et*

*al.*, 1991b) in a 5:1 ratio. The bagasse was autoclaved at 110°C (1 atm) for 20 min, whereas carob pods were not sterilized in order to avoid sugar caramelization. Under these conditions, no contamination was observed during the entire course of fermentation. Distilled water or a mineral solution [g/L: $(NH_4)_2SO_4$= 9.7; Urea= 2.4; $KH_2PO_4$= 5.0; pH= 4.4] were added to these mixtures to get a final moisture content of 65-67%. Four media were obtained with this simple pretreatment: A= Carob + distilled water, B= Spent carob + distilled water, C= Carob + mineral solution, and D= Spent carob + mineral solution.

*Inoculation*

Spore suspensions from fresh PDA cultures were prepared in 0.01% distilled water solution of Tween 80 for inoculum preparation. Each medium was inoculated with 1 x $10^7$ spores/g initial dry weight (IDW) of substrate, incubated at 30°C, and aerated at a rate of 1:8 L/column/h (Lambraki *et al.*, 1994).

*Solid state fermentation system (SSF)*

Design and control of a SSF system have been previously described, by Raimbault and Alazard (1980), and Saucedo-Castañeda *et al.* (1993). During SSF, the gas produced was analyzed automatically by a GPC analyser and results were monitored by a programme integrated to a PC computer. $CO_2$ (%), $O_2$ (%), and the total volume of $CO_2$ (ml/IDW) were measured by this programme. All fermentations lasted 50 h.

*Downstream processing and analytical studies*

This process, as well as the experimental work for sugar and tannin extraction, were previously described by Lambraki *et al.* (1994). Total sugars were determined by the method of Dubois *et al.* (1956), using glucose as standard sugar, while total

tannins measurement was carried out according to Swain and Hillis (1959), using gallic acid as standard phenol.

## RESULTS AND DISCUSSION

Significant growth parameters of *Aspergillus carbonarius* are shown in Table 1.

### *Effect of initial sugar concentration of the substrate*

*Respiration of* A. carbonarius

Respirometry data of *A. carbonarius* cultured in carob media with different sugar and mineral concentrations are shown in Fig. 1. Partial extraction of sugars (48% of initial sugar concentration remained) resulted in a significant reduction of spore germination time. This probably indicates the inhibitory effect

**Table 1.** Growth parameters of *Aspergillus carbonarius* cultivated in media containing different sugar and mineral concentrations.

| Media | Sugar concentration (%)[a] | Mineral solution addition | Initial moisture (%) | Final moisture (%) | Initial pH | Final pH | Germination time (h) | Maximum $CO_2$ production (%) |
|---|---|---|---|---|---|---|---|---|
| A | 100 | - | 65.3 | 71.3 | 5.42 | 2.67 | 10 | 2.4 |
| B | 48 | - | 66.2 | 75.1 | 5.59 | 2.98 | 5 | 1.6 |
| C | 100 | + | 65.1 | 71.7 | 6.05 | 3.7 | 20 | 7.7 |
| D | 48 | + | 65.4 | 71.9 | 6.12 | 6.58 | 10 | 9.7 |

[a] Remaining sugar concentration, percentage of initial sugar on dry weight.

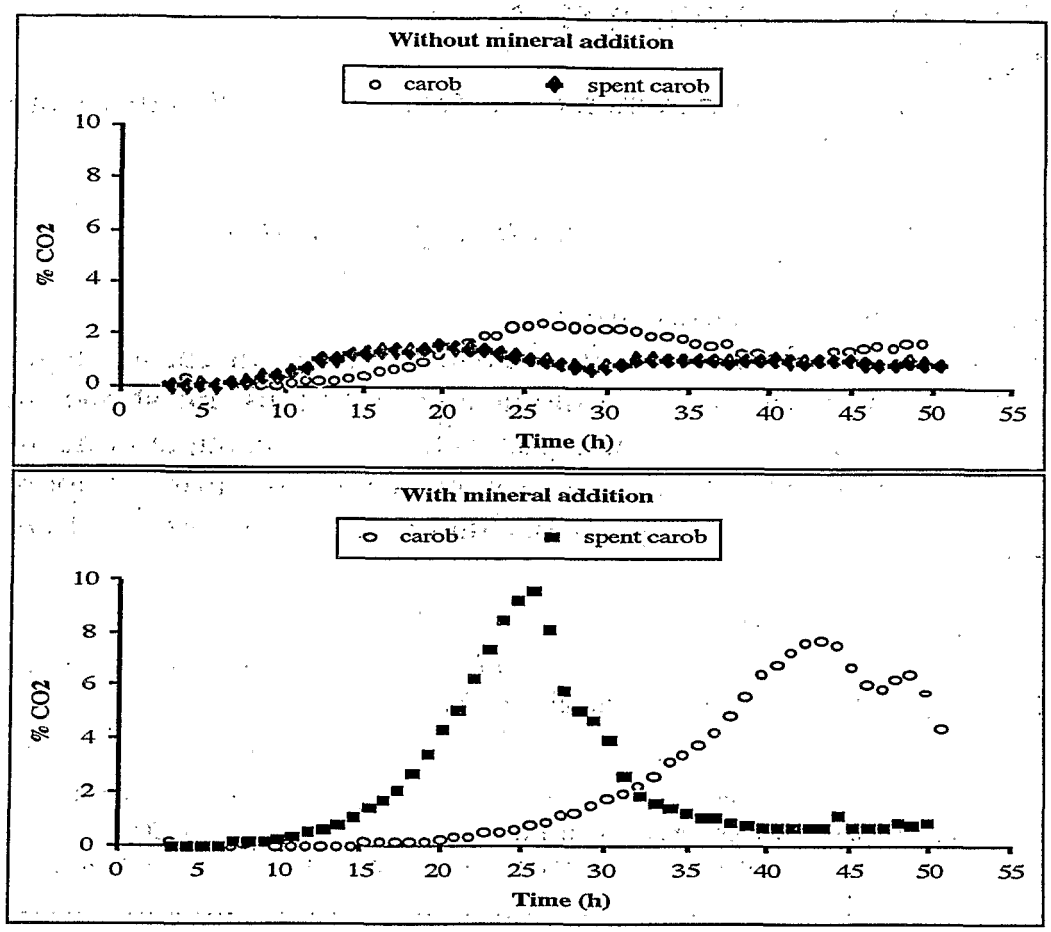


**Fig. 1.** Effects of initial sugar concentration of the substrate and mineral addition on the respiration (% $CO_2$ production) of *Aspergillus carbonarius*, under SSF conditions.

of high sugar concentrations on *A. carbonarius* growth. The same effect has been observed in liquid cultures, in which sugar concentration higher than 3% inhibits or delays this strain growth (unpublished data).

*Sugar and tannin consumption*

Sugar and tannin consumption are shown in Fig. 2. It can be easily seen that extraction of 52% of the initial sugar weight led to a better utilization of carbon source from the substrates, in terms of sugars or tannins. The only exception was medium D, in which the lowest sugar consumption was recorded. In this case, sporulation occurred after 25-30 h of incubation, revealing rather hard conditions for strain growth. Under the same conditions, a significant reduction of tannin content in the substrate was observed before the first 10 h of fermentation. However, this may be considered as an artifact, caused by absorption and binding of tannins on the spore surface. This hypothesis was confirmed by a comparison between spores originated from two different media: a) PDA, and b) Medium containing tannins. Microscopic observation of these spores showed a thick cover around the surface of the spores obtained from tannin-containing medium.

The highest tannin degradation occurred in medium B, which contained lower sugar concentration and no mineral addition. It seems likely that *A. carbonarius* degrades tannins in order to find necessary nutrients, as no external nitrogen had been added to the medium. The fact that only proteins (not tannins) contain nitrogen atoms in their structure, does not agree with this hypothesis. However, as tannins form complexes with proteins, fungal tannin degradation as a strategy for liberating and attacking proteins could be an alternative possibility.

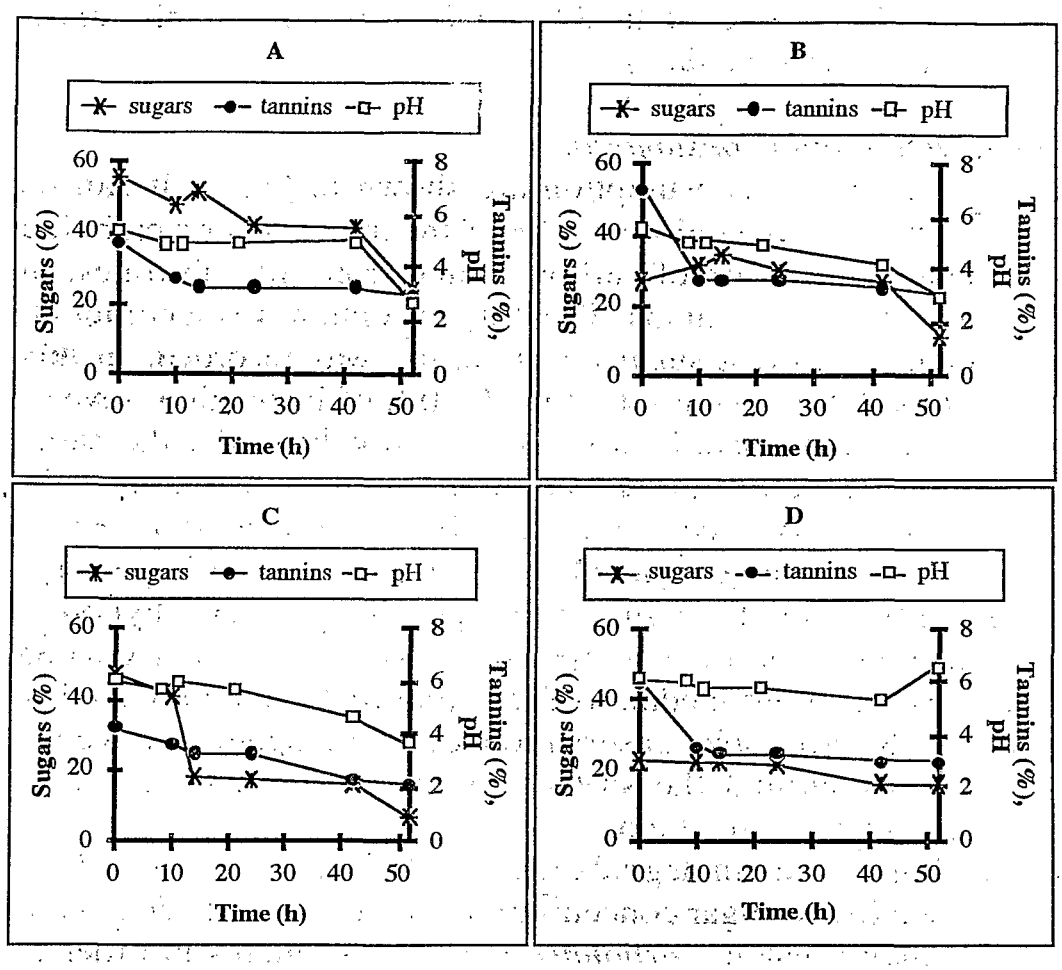


**Fig. 2.** Kinetics of sugar and tannin consumption (% on initial dry weight of the substrate), and evolution of pH during solid state fermentation of 4 carob substrates: A= Carob pod + distilled water, B= Spent carob + distilled water, C= Carob pod + mineral solution, and D= Spent carob + mineral solution.

*pH evolution*

pH values, during the fermentation course of media with 4 different carbon and nitrogen concentrations, revealed that the extraction of 52% of initial sugars from carob pods did not affect significantly the initial pH of the substrates. A pH increase of 0.07 and 0.17 was recorded for the low sugar media with and without minerals, respectively (Fig. 2). The pH remained almost stable, or decreased rarely until 42 h of fermentation, and then it decreased sharply, probably due to tannin degradation and liberation of acidic products. The same result was also observed in a previous work (Lambraki *et al.*, 1994). The fact that tannin degradation occurs from the beginning of fermentation, while pH decreases just before the end, can be explained by a high sugar concentration present in the media until 42 h of incubation. Under these experimental conditions, there was no need for pH regulation as the substrate, itself, behaved more or less as a buffer, and maintained the pH at desirable levels. A similar result was reported by Roussos *et al.* (1991a).

*Effect of mineral addition*

*Respiration of* A. carbonarius

The respirometry data (Fig. 1) of carob and spent carob fermentations, in media with added minerals, showed a significant increase according to the quantity of $CO_2$ production, which was about 5 times higher than that observed with substrates lacking minerals. This can be interpreted either as a high biomass production (medium C) or as a result of stress (medium D, in which the strain could hardly grow and, for this reason, sporulation occurred). Spore germination time was also affected by mineral addition, as it took twice as long in compari-

son with that from media lacking minerals, independently of the sugar concentration in the substrates.

*Sugar and tannin consumption*

External nitrogen significantly improved sugar consumption in medium C, in which the highest sugar consumption occurred, as compared to medium A without minerals (Fig. 2). Medium D did not follow this trend, because sporulation took place. The highest biomass production was observed in medium C, in which *A. carbonarius* can easily find both carbon and nitrogen sources needed for its growth. Under these conditions, there is no need for tannin degradation, which would mean an extra effort for the strain.

*pH evolution*

A pH increase of 0.53 and 0.63 was observed in carob and spent carob media, respectively, after mineral addition. The pH evolution followed stable results, the only exception was observed in medium D, in which pH increased after 42 h of fermentation. This result is directly related to tannin degradation as no degradation occurred in medium D, hence there is no reason for pH decrease.

*Comments on C/N ratios of the substrates*

The solid state fermentation of carob pods, in media containing different concentrations of carbon and nitrogen, revealed a significant effect of C/N ratio on growth and behaviour of *A. carbonarius*. This fungus seems to be well adapted to varying C/N ratios, even for those of very high or very low values. Nevertheless, the behaviour of *A. carbonarius* strongly depended on changes of sugar and nitrogen concentrations, and

led to either biomass production or tannin degradation. Biomass production occurred when all necessary nutrients (carbon and nitrogen sources) were available. Tannin degradation occurred when no external mineral sources were added. As tannin degradation started before 10 h of incubation, it was assumed previously that nitrogen limitation might lead to tannin degradation (Lambraki *et al.*, 1994). The results of the present work confirm this assumption, as tannin degradation only occurred in the medium lacking available nitrogen and with reduced sugar concentration. A possible explanation could be that the fungus needs nitrogen sources, which are not easily available in the substrate.

Taking into consideration that the basic carbon sources of carob pods are sugars and tannins, and that tannin content remained almost stable during the pretreatment of the substrates, the sugar concentration reduction is considered to be a responsible factor for changes in the carbon levels. Furthermore, the nitrogen concentration of the media increased due to the addition of the mineral solution, as the nitrogen content of the carob pod is low (1% on carob pod dry weight) [Marakis, 1980]. It is not easy to calculate the exact C/N value for the media due to their tannin content, which has not yet been thoroughly estimated. However, an estimation of this value can be obtained considering the carbon content of sugars (by the contribution of their profile to carob pod), and the carbon content of tannins as constant *Ct*. On the basis of this, a C/N ratio can be estimated by the following equation:

$$C/N = (Cs + Ct)/(Ncp + Nm) = Cs/(Ncp + Nm) + Ct/(Ncp + Nm) \quad (1)$$

Cs= Carbon of sugars (6.895).
Ct= Carbon of tannins.
Ncp= Nitrogen of carob pod (1).
Nm= Nitrogen of mineral solution (2.056).

After partial sugar extraction, the equation becomes as:

$$C/N = (Cr + Ct)/(Ncp + Nm) = Cr/(Ncp + Nm) + Ct/(Ncp + Nm) \quad (2)$$

Cr= Carbon of reduced sugar concentration (3.577).

Using equations (1) and (2), the C/N ratio of the media can be expressed as:

I) Medium A: $(1) = 6.895/(1 + 0) + Ct/(1 + 0) = 6.895 + Ct/1$ (3)
II) Medium B: $(2) = 3.577/(1 + 0) + Ct/(1 + 0) = 3.577 + Ct/1$ (4)
III) Medium C: $(1) = 6.895/(1 + 2.056) + Ct/(1 + 2.056) = 2.256 + Ct/3.056$ (5)
IV) Medium D: $(2) = 3.577/(1 + 2.056) + Ct/(1 + 2.056) = 1.17 + Ct/3.056$ (6)

Equations (3), (4), (5), and (6), clearly indicate that there is a gradual decrease in the C/N ratio from medium A to medium D, *i.e.* A>B>C>D. Medium D (case IV) proved to be unsatisfactory for fungal growth, and resulted in sporulation. The C/N ratio in this case is too low. The growth of the strain in medium A was not very satisfactory either, as there was low biomass production. Media B and C, which had intermediate values of C/N ratios, were more suitable. In medium B, *A. carbonarius* showed the highest tannin degradation, while in medium C it had the highest biomass production (optical observation). It seems likely that optimum C/N ratio for the best results is between very high and very low values.

Solid state fermentation of carob pods containing different amounts of sugar and nitrogen sources resulted either in high levels of biomass production or in tannin degradation, depending on the experimental conditions. Therefore, a careful selection of carbon and nitrogen sources of the medium is necessary to manage the SSF process.

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