Effects of sugar and mineral salts on the growth of *Aspergillus carbonarius* in carob pod solid state fermentation

M. LAMBRAKI[1], S. MARAKIS[1], L. HANNIBAL[2], AND S. ROUSSOS[2]

[1] Institute of General Botany, Biology Department, Athens University, 157 84 Ilisia, Greece.

[2] Biotechnology Laboratory, ORSTOM Centre, 911 Av. Agropolis, BP 5045, 34090 Montpellier cedex 1, France.

SUMMARY

The effects of sugar concentration and mineral addition on the growth of *Aspergillus carbonarius* in carob solid state fermentation were studied. Data on CO2 production, sugar levels and tannins levels, and optical observations, revealed when carob husk with additional mineral salts was used as a substrate, this led to high biomass production. On the other hand, removal of 52% of the water-soluble sugars, prior to fermentation, and elimination of the minerals resulted in a tannin degradation up to 55%, but poor biomass production.

Keywords: Solid state fermentation, carob pods, *Aspergillus carbonarius,* mineral salts enrichments, initial sugar concentration, biomass production, tannin degradation.

RESUME

Effets des sucres et de sels minéraux sur la croissance *d'Aspergillus carbonarius* cultivé sur farine de caroubes en fermentation en milieu solide.

LAMBRAKI, M., MARAKIS, S., HANNIBAL, L. ET ROUSSOS, S.

Les effets de la concentration initiale en sucres et en sels minéraux sur la croissance d'*Aspergillus carbonarius*, cultivé sur farine de caroube épuisée en fermentation en milieu solide ont été étudiés. Les mesures de production de CO2, les analyses des sucres et des tannins ainsi que les observations visuelles de l'évolution de la biomasse ont montré que l'utilisation de sucres contenus dans la farine de caroube additionnée de sels minéraux, est associée à une forte production de biomasse. Par contre, l'élimination préalable de 52% des sucres et de la totalité des sels minéraux contenus dans la gousse de caroube conduit à la dégradation de 55% des tannins et à une faible production de biomasse.

Mots clés: Fermentation en milieu solide, farine de caroube épuisée, *Aspergillus carbonarius*, addition de sels minéraux, concentration initiale en sucres, production de biomasse, degradation de tannins.

INTRODUCTION

Carob bean is the fruit of *Ceratonia siliqua*, L. The ripe deseeded carob pod (husk), although rich in water-soluble sugars (40-60%), has a very low protein level (3-5%) and contains appreciable amounts of total tannins (4-13%) (Marakis and Karagouni, 1985; Marakis *et al*, 1993). The tannins present are of mainly condensed types (Tamir and Alumot, 1970), which minimize the pod's nutritional value (Vohra *et al*, 1966; Tamir and Alumot, 1970).

This worldwide produced product could be variously upgraded (feed, enzymes, probiotics, etc.), after tannin degradation and husk protein enrichment. In spite of the wide use of solid state fermentation (SSF) for the upgradation of several agroindustrial products 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 exploited for tannin degradation. Hence, *A. carbonarius* strain, with high tanninolytic abilities (Lambraki and Marakis, 1993), has been used in SSF of carob pods.

This paper describes the effect of sugar and mineral salts on the growth of *A. carbonarius* in carob SSF.

MATERIALS AND METHODS

MICROORGANISM

A strain of *A. carbonarius* (Bainier) Thom, previously isolated from mouldy carob beans (Marakis, 1980), was used.

MEDIA

Ripe milled carob pods (hereafter referred as 'carob') and ripe milled carob pod from which 52% of their water-soluble sugars had been removed by stirring and filtration (hereafter referred as 'spent carob') were used. These were mixed with sugarcane bagasse (Roussos *et al*, 1991b) in a 5:1 (w/w) ratio. To achieve a humidity level of 65-67%, the above mixture was weted by distilled water or by a mineral solution containing (g/l) $(NH_4)_2SO_4$, 9.7; Urea, 2.4; KH_2PO_4, 5.0 and pH 4.4. Using the above, a total of 4 media were obtained, i.e.: A) carob + distilled water, B) spent carob + distilled water, C) and D) the same as A) and B), respectively, but contained mineral solution, instead of distilled water.

Each medium was inoculated with 10^7 spores/g initial dry weight (IDW) of substrate, incubated at 30°C and aerated at the rate of 1.8 l/column/h (Lambraki *et al*, 1994).

SOLID STATE FERMENTATION SYSTEM

The design and control of the SSF system was, as described by Raimbault and Alazard (1980) and Saucedo-Castañeda *et al*, (1994). During SSF, the gas produced was analysed automatically by GC analyser and results were monitored by an integration programme in a computer. All fermentations lasted 50 h. Downstream processing was as described by Lambraki *et al* (1994).

SUGAR AND TANNIN DETERMINATIONS

Sugar and tannins extraction is reported elsewhere (Lambraki *et al,* 1994). Total sugars were determined by the method of Dubois *et al* (1956), using glucose as a standard. Total tannin measurement was carried out, according to the Swain and Hillis (1959) method, using gallic acid as the standard.

RESULTS AND DISCUSSION

The most significant growth parameters of *A. carbonarius* are shown in Table 1.

Table 1 Growth parameters of *A. carbonarius* cultured in media containing different sugar and mineral contents.

Media	Sugar conc (%)[a]	Mineral solution addition	Initial humidity (%)	Final humidity (%)	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	+	65.0	71.7	6.05	3.70	20	7.7
D	48	+	65.4		6.12	6.58	10	9.7

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

EFFECT OF SUGAR CONCENTRATION OF THE SUBSTRATE

Respiration of A. Carbonarius

The respirometry data of *A. carbonarius,* cultured in carob media of different sugar and mineral contents, are shown in Fig.1. Partial sugar extraction (48% of initial sugar concentration remained) resulted in a significant decrease in spore germination time. This probably indicates the inhibitory effect of high sugar concentration 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's growth (unpublished data).

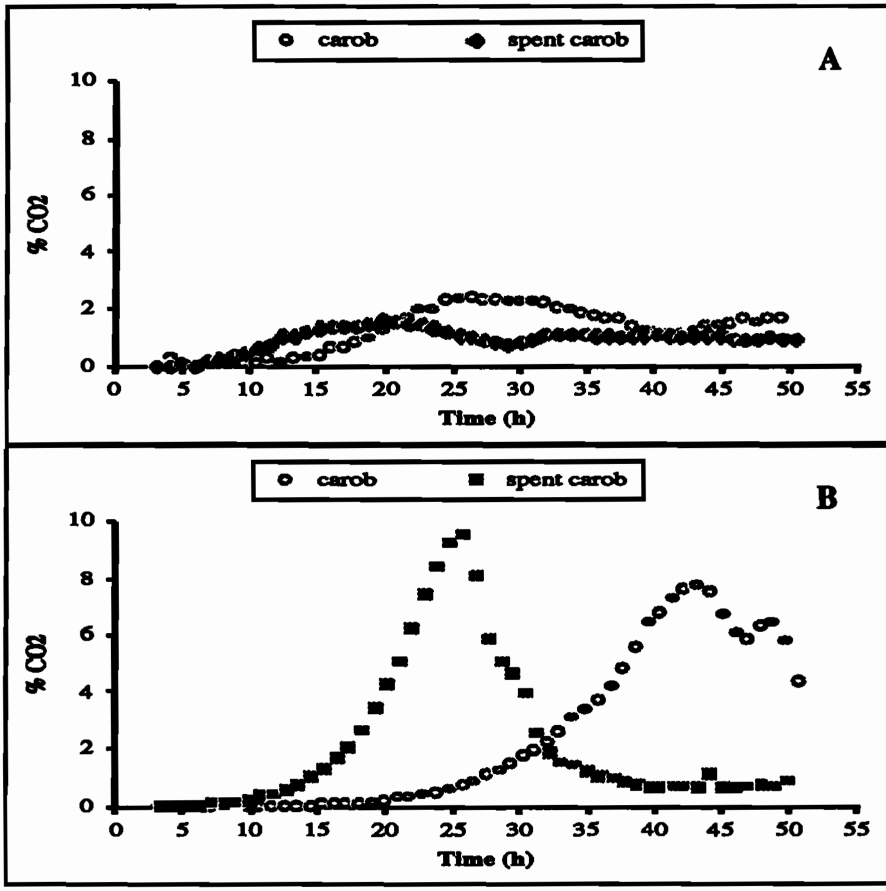

Fig. 1. Effect of sugar concentration of the substrate and mineral addition on the respiration of *A. carbonarius.* A: Without mineral addition, B: With mineral addition.

Sugar and tannin consumption

Sugar and tannin consumptions are shown in Fig. 2. It can be seen that the removal of 52% of the initial sugar weight led to a better utilization of the carbon source of the substrates, in terms of sugars or tannins. The only exception was the medium D), in which the lowest sugar consumption was recorded. In this case sporulation occured, after 25-30 hours incubation, thereby revealing rather harsh conditions for the microorganism's growth. Under the same conditions, a significant reduction of the medium tannin content was observed, before the first 10 h fermentation, but this is considered as an artifact, caused by absorption and binding of tannins on the spore surface. This hypothesis was confirmed by comparison of spores originating from two different media: a) PDA and b) tannin-containing medium. The microscopic observation of these spores revealed a thick cover around the surface of the spores, obtained from the tannin medium.

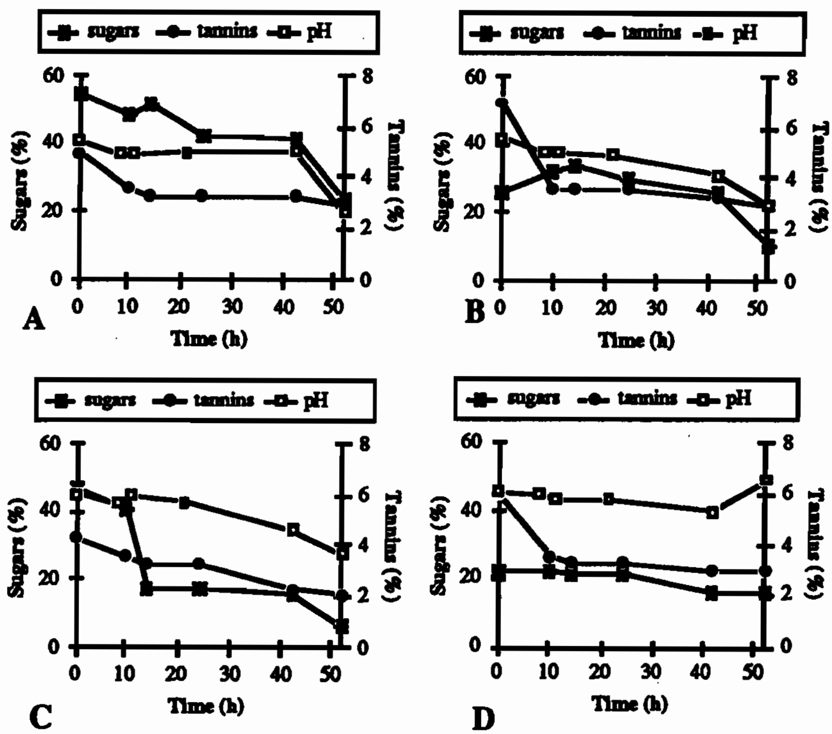

Fig. 2. Kinetics of sugar and tannin consumption (%, initial dry weight of the substrate) and pH evolution in solid state fermentation of 4 carob substrates: A = carob pod, B = spent carob, C and D = carob and spent carob, respectively, enriched with mineral solution.

Highest tannin degradation occurred in medium B, which contained a lower sugar concentration and no mineral addition. It seems that *A. carbonarius* degrades tannins in order to find the necessary nutrients, as no external nitrogen was added to the medium. The fact, that only proteins (and not the tannins) contain nitrogen atoms in their structure disproves the above hypothesis. Since tannins form complexes with proteins, fungal tannin degradation for liberating and attacking proteins is a likely possibility.

pH evolution

pH values during the course of fermentation in the 4 different media revealed that the removal of 52% of the initial sugars, from the carob pods, did not significantly affect the initial pH of the substrates. The pH values increase of 0.17 and 0.07 were recorded in the low sugar media, without and with minerals, respectively (Fig. 2). The pH remained almost stable or rarely decreased until 42 hours' fermentation and then sharply decreased, probably due to tannin degradation and liberation of acidic products. This result has also been observed in 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 on the basis of high sugar concentration in the media up to 40 hours' incubation. Under these experimental conditions, there was no need for pH regulation, as the substrate itself behaved more or less as buffer and maintained the pH at desirable levels. A similar result was reported by Roussos *et al* (1991a).

EFFECT OF MINERAL ADDITION

Respiration of the fungus

The respirometry data (Fig. 1) of carob and spent carob fermentations, in media with added minerals, showed a significant increase in the quantity of CO2 production, which was about 5 fold higher than that observed with substrates containing no minerals. This can be interpreted as either due to high biomass production seen in 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; in these media its duration doubled compared to those with no minerals. This effect was independent of the sugar concentration of 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 result, because of sporulation. The highest biomass production was observed in medium C, in which the micro-organism could easily find both the carbon and nitrogen sources needed for its growth. Under such conditions, there is no need for tannin degradation, which would require an extra effort for the strain.

pH evolution

pH increases of 0.53 and 0.63 were observed in carob and spent carob media, respectively, after mineral addition. pH evolution followed the same pattern, as those mentioned above. The only exception was observed in medium D, in which the pH increased slightly after 42 hours' fermentation. This result is directly connected to tannin degradation, as no degradation occurred in medium D, and hence there is no reason for pH decrease.

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 parameters and behaviour of *A. carbonarius* . The fungus seems to be well adapted to varying C/N ratios, even for those of very high or very low values. Nevertheless, its behaviour depended strongly on changes in sugar and nitrogen concentrations, and led either to biomass production or tannin degradation. The former occurred, when all the necessary nutrients (carbon and nitrogen sources) were plentiful. The latter occurred, when no external nitrogen sources were added. As tannin degradation started before 10 hours' incubation, it was assumed previously (Lambraki *et al,* 1994), that nitrogen limitation might lead to tannin degradation. The results of the present work confirm this assumption, as tannin degradation occurred in the medium with no available nitrogen and with reduced sugar concentration. A possible explanation could be that the fungus needs a nitrogen source, which is not readily available in the substrate.

Taking into consideration that, the basic carbon sources are sugars and tannins, and, as the tannin content remained almost stable during the pre-treatment of the substrates, the reduction in sugar concentration is considered the factor responsible for the 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 quite low (0.68% on carob pod dry weight) (Marakis, 1980). Even though it is not easy to calculate the exact C/N value for the media, because their tannin content, is not yet very clear, an estimation of this value can be obtained, considering the carbon of sugars and the carbon of tannins as constant. On this basis, C/N ratio can be estimated by the following equation:

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

where, Cs : carbon of sugars=6.895, Ct : carbon of tannins, Ncp: nitrogen of carob pod=0.68 and Nm: nitrogen of mineral solution=2.056

After extraction of sugar, the equation becomes:

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

Where, Cr : carbon of reduced sugars=3.577

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

Medium A: (1) $= 6.895 / (0.68 + 0) + Ct / (0.68 + 0)$

$$= 10.14 + Ct / 0.68 \quad (3)$$

Medium B: (2) $= 3.577 / (0.68 + 0) + Ct / (0.68 + 0)$

$$= 5.26 + Ct / 0.68 \quad (4)$$

Medium C: (1) $= 6.895 / (0.68 + 2.056) + Ct / (0.68 + 2.056)$

$$= 2.52 + Ct / 2.736 \quad (5)$$

Medium D: (2) $= 3.577 / (0.68 + 2.056) + Ct / (0.68 + 2.056)$

$$= 1.31 + Ct / 2.736 \quad (6)$$

Equations (3), (4), (5) and (6) indicate that there is a gradual decrease in the C/N ratio from medium A to medium D, i.e. A > B > C > D

Case (4) proved to be unsatisfactory for the fungal growth and resulted in sporulation. Thus, the C/N ratio in this case is too low. The growth of the strain in medium A was not very satisfactory and resulted in low biomass production. Media B and C, containing intermediate C/N ratios, were most advantageous, each one for a different reason. In medium B, the micro-organism succeeded in the highest tannin degradation, while in medium C, the biomass production was highest (optical observation). It seems that the optimum C/N ratio for both of these procedures lies between the very high and very low values.

CONCLUSION

The solid state fermentation of carob pods containing different amounts of sugars and nitrogen sources resulted either in a high level of biomass production or in tannin degradation, depending on the experimental conditions. Thus, by proper selection of the carbon and nitrogen content of the medium, the desired goal can be achieved.

AKNOWLEDGMENTS

This work was financially supported by PLATON programme N° 92 217, under a French-Greek co-operation.

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FMS-95 : Solid State Fermentation : International Symposium, 2., Montpellier (FRA), 1997/02/27-28. ISBN 0-7923-4732-3