MICOL. NEOTROP. APL. 7 : 23-34, 1994.

EFFECT OF TEMPERATURE AND AERATION FLOW ON CAROB TANNIN DEGRADATION BY *ASPERGILLUS CARBONARIUS* IN SOLID STATE FERMENTATION SYSTEM

M. LAMBRAKI[1], S. MARAKIS[1] and S. ROUSSOS[2]

[1] Institute of General Botany, Biology Department, Athens University, Panepistimiopolis, Ilisia, 15784 Athens, Greece.

[2] Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Centre de Montpellier, U. F. Biotechnologie PMC, 911 Avenue Agropolis, BP 5045, 34032 Montpellier Cedex 1, France.

EFECTO DE LA TEMPERATURA Y DEL FLUJO DE AIREACION SOBRE LA DEGRADACION DE TANINOS DE LA ALGARROBA POR *ASPERGILLUS CARBONARIUS* EN FERMENTACION EN MEDIO SOLIDO

Accepted for publication November 14, 1994

SUMMARY

The growth of *Aspergillus carbonarius* on carob pods rich in tannins, under different temperature and aeration conditions in a solid state fermentation system, revealed fungal preference for high humidity and aeration levels. Respiration parameters of the fungus (CO_2 production, and oxygen consumption) were not affected by the change of incubation temperature from 25° to 30°C. However, changes in the aeration flow significantly affected fungal growth. Higher sugar and tannin consumption were observed at 30°C, with an aeration flow of 30 ml/h. Under these conditions, sugar consumption began 10 h after incubation, and continued up to 24 h. At this point, an increase in sugar concentration of the medium was observed, probably due to tannin hydrolysis. A significant decrease in the pH of the culture also supported this hypothesis.

Key words: *Aspergillus carbonarius*, tannins, tannin degradation, carob, solid state fermentation, temperature, aeration flow.

RESUMEN

La presencia de taninos en altas concentraciones limita la utilización de la harina de algarroba para la alimentación de ganado. *Aspergillus carbonarius* tiene la capacidad de crecer sobre dicha harina y degradar específicamente los taninos por fermentación sólida. En este trabajo se presenta el efecto de la temperatura y de la aireación de los medios de cultivo sobre la respiración del hongo, así como la consecuente degradación de los taninos. Se demostró que aunque *A. carbonarius* crece de la misma manera a diferentes temperaturas (25° - 30°C), presenta un desarrollo y una respirometría muy diferentes cuando se cambia el flujo de aire. Por consecuencia, la degradación de los taninos está directamente relacionada con el flujo de aireación de los cultivos en medio sólido.

Palabras clave: *Aspergillus carbonarius*, taninos, degradación de taninos, algarroba, fermentación sólida, temperatura, flujo de aireación.

INTRODUCTION

The presence of polyphenols in nature, especially tannins (polymerized flavan-3-ols or flavan-3,4-diols, of 500-3500 molecular weight), has been attributed to their capacity of binding strongly to proteins and polysaccharides. Enzymes are totally or partially inactivated by the formation of complexes with tannins, while potential microbial substrates as polysac-charides and non-enzyme proteins become highly resistant to microbial attack, after binding to tannin molecules (Grant, 1976). This particular capacity of tannins is related to their protective role in plants preventing any attack, either animal or microbial. The relevant property is "astringency" (Bate-Smith, 1972), rendering the tissues unpalatable by precipitating salivary proteins, or by immobilizing enzymes impending the invasion of the tissues. According to this, tannins represent an important way of plant protection.

The presence of tannins has also been associated with control of bloat improved protein utilization, and reduced digestibility and palatability of the forage (Broadhurst and Jones, 1978). However, several organic materials have been used as animal feed, in spite of their poor nutritional value due to

the tannin content. This is the case of carob beans (fruit of *Ceratonia siliqua* L.), whose high concentration of tannins are limiting any further utilization.

The carob tree (*C. siliqua* L.) grows naturally on barren soils mainly near the coast (often unprofitable for any other type of crop), in the warmest regions of the Mediterranean. This species also occurs in Rhodesia, U.S.A., Australia, South America, and other regions with similar Mediterranean climate (Mitrakos, 1968; Imrie and Vlitos, 1975). Greece is the fourth largest producer of carob beans with an estimated production of about 35,000 tons/year (Marakis *et al.*, 1993). The production of this fruit in Spain, another important producer, is of about 200,000 tons/year (Calixto and Cañellas, 1982).

Carob fruit consists of about 90% pod and 10% seeds. The ripe deseeded carob pods of some greek varieties contain high levels (6-13%) of total tannins (Marakis *et al.*, 1993), while carob pods from Portugal, Italy, and Cyprus contain higher tannin concentrations of 20-27% (Würsch, 1987). Protein content of carob beans is low (3-5%), in comparison with the high concentration (±60%) of sugars (Marakis, 1992). If the tannin content is reduced or eliminated by microbial degradation, it could then be possible to use carob beans as animal feed, improving their protein content at the same time.

Previous research work (Lambraki and Marakis, 1993) has identified a strain of *Aspergillus carbonarius* with high tanninolytic abilities, which is capable of growing on carob pods in a solid state fermentation (SSF) system. However, this ability of *A. carbonarius* has not yet been exploited for tannin degradation and the resulting tannase production, in spite of the wide use of SSF for the valorization of agro-industrial products by the production of secondary metabolites (*e.g.* enzymes) and other microbial products (Lonsane *et al.*, 1982; Lonsane *et al.*, 1985; Roussos *et al.*, 1991a; Roussos *et al.*, 1994). This research work deals with the effect of two culture conditions (temperature and aeration) on tannin degradation of carob pods by *A. carbonarius* in a SSF system.

MATERIALS AND METHODS

Microorganism

A strain of *Aspergillus carbonarius* (Bainier) Thom was studied, which was previously isolated from mouldy carob beans (Marakis, 1980). This strain was maintained and subcultured on potato dextrose agar (PDA) at 4°C.

Substrates
Ripe deseeded carob pods (size <0.5 mm) were mixed with sugar cane bagasse in a 5:1 ratio, respectively (Roussos *et al.*, 1991b). 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. This substrate, without any other pretreatment, was the basal medium. No mineral solution was used, and distilled water was added when needed to reach an initial moisture of 60% on dry weight basis of the substrate.

Inoculation
Spore suspensions from fresh PDA cultures were prepared in 0.01% distilled-water solution of Tween 80 for inoculum preparation. The inoculum ratio was 1×10^7 spores per g of initial dry weight (IDW) of substrate. After inoculation, the substrate was placed in columns of 2.5 cm diameter and 20 cm height (Raimbault and Alazard, 1980). The total volume of the column was 100 cm^3, from which 60 cm^3 are filled up with substrate, representing the actual volume of the column.

Solid state fermentation system (SSF)
The design and control of SSF system has been previously described by Saucedo-Castañeda *et al.* (1993). The column reactors were incubated at two different levels of temperature (25° and 30°C), and aerated at the rates of 0.12, 0.9 and 1.8 L/h (Table 1) where the air passed through the actual volume of the column was 2, 15, and 30 times/h, respectively. These data

Table 1. Temperature and aeration conditions used for fermentation of the substrates. T= Temperature (°C), A= Aeration flow (L/h).

Aeration flow (L/h)	Temperature (°C)		Air renewal (times)
	25	30	
	Substrate names		
0.12	T25A2	T30A2	2
0.9	T25A15	T30A15	15
1.8	T25A30	T30A30	30

indicate that to achieve these aeration conditions in any size of column or even a large-scale fermentor, the air should be renewed 2, 15, and 30 times per hour. All air-providing instrumentation must be as accurate as in this work, even for small air quantities, otherwise the comparison of low aeration flow levels would not be possible. During SSF, the gas produced was analysed automatically by CPG 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
The fermented products were removed from the fermentation columns, homogenized, and weighed. A fresh sample (5 g) from this product was placed at 105°C for 24 h, to determine amount of dry matter. The pH of the extract was measured by adding 10 ml of distilled water to a 1 g sample, and mixing thoroughly. The fermented products were kept at -20°C until they were lyophilized. Portions from lyophilized products were homogenized and used for analytical studies.

Analytical studies
Sugar and tannin extraction. A sample of dried and homogenized material (5 g) was mixed with 20 ml of distilled water, and centrifuged at 500 rpm for 30 minutes. The extract was filtrated, and the filtrate used for sugar analysis. Another sample (5 g) of dry product, after the addition of 20 ml of distilled water, was autoclaved (110°C, 1 atm, 45 min) to prepare tannin extraction. The extract was filtrated, and the filtrate was used for estimation of tannins.

Sugar and tannin determination. The determination of total sugars was made according to the method of Dubois *et al.* (1956), using glucose as standard sugar, while total tannins were determined according to the method of Swain and Hillis (1959), using gallic acid as standard phenol.

RESULTS AND DISCUSSION

Effect of temperature and aeration flow on fungal respiration
Respirometry data (% CO_2 production, % O_2 consumption) of *Aspergillus carbonarius* growing on substrate T30A30 (see Table 1), in which the fungus was best adapted, are shown in figure 1. No growth was observed before the first 25 h of fermentation. *A. carbonarius* started its respiration, *i.e.* to pro-

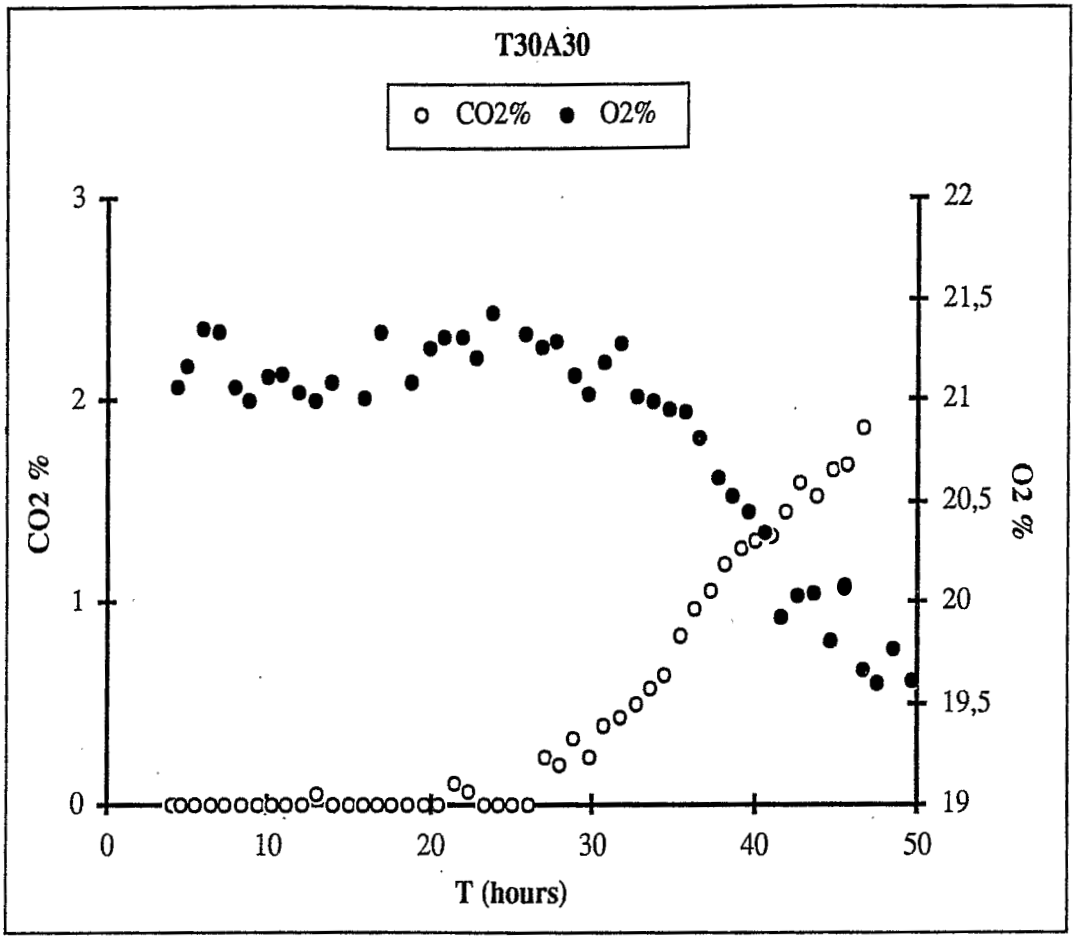

Fig. 1. Respirometry data of *Aspergillus carbonarius* grown on substrate T30A30 in a SSF system (kinetics of O_2 and CO_2). The substrate code is that of Table 1.

duce CO_2 and consume O_2, just after this period, showing as expected that CO_2 production and O_2 consumption were reverse phenomena.

Different temperature and aeration conditions had influence on the growth of *A. carbonarius*, as shown in figure 2. The total CO_2 production per g of initial dry weight of the substrate (IDW) is presented for the 6 substrates analysed. The highest CO_2 production was observed at the medium with the

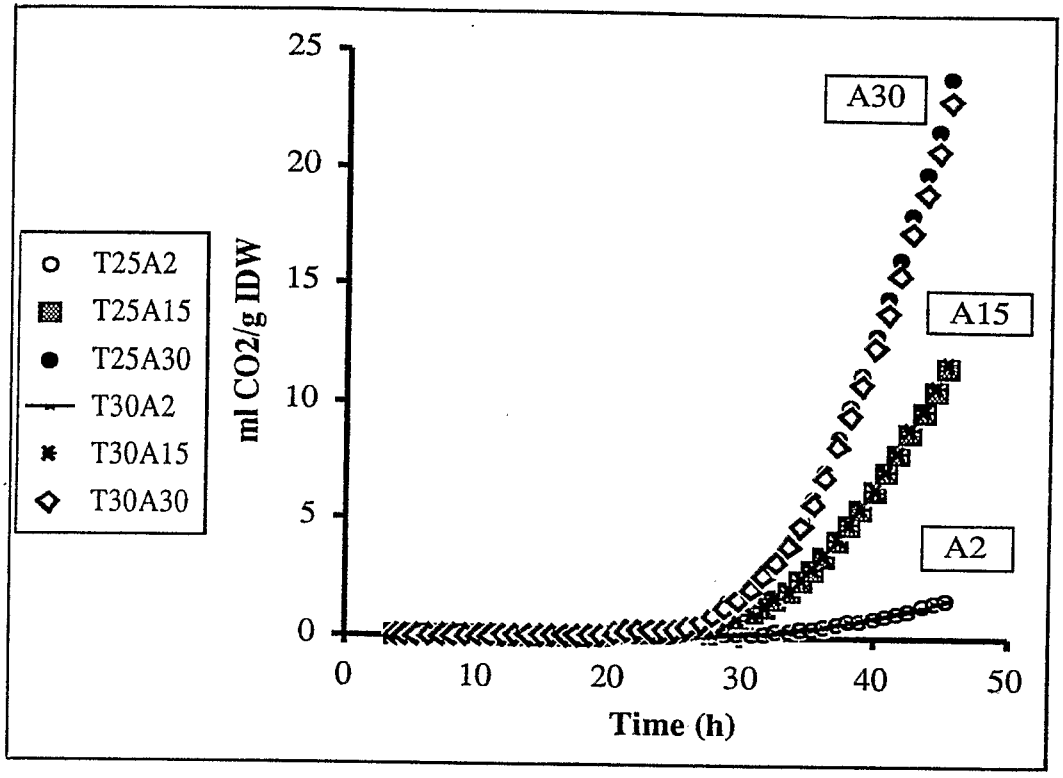

Fig. 2. Total CO_2 production by *Aspergillus carbonarius* grown under different temperature and aeration conditions (substrate codes are those of Table 1). IDW= Initial dry weight of the substrate.

maximum aeration flow (30 ml/h), while an increase of temperature from 25° to 30°C had no influence at all on fungal respiration. On the contrary, changes on the aeration flow, ranging from 2 to 30 ml/h, gradually improved the respiration of *A. carbonarius* from 2 to 25 ml CO_2/g IDW. This clearly indicated that the best growth of *A. carbonarius* is obtained with high aeration levels.

Evolution of moisture and pH

Kinetics of the moisture content of the substrate revealed a gradual increase during fermentation, being 4-6% higher than the initial moisture of the substrates (Fig. 3). A similar result was reported for *Trichoderma harzianum* growing on substrate containing sugar cane bagasse and wheat bran at a

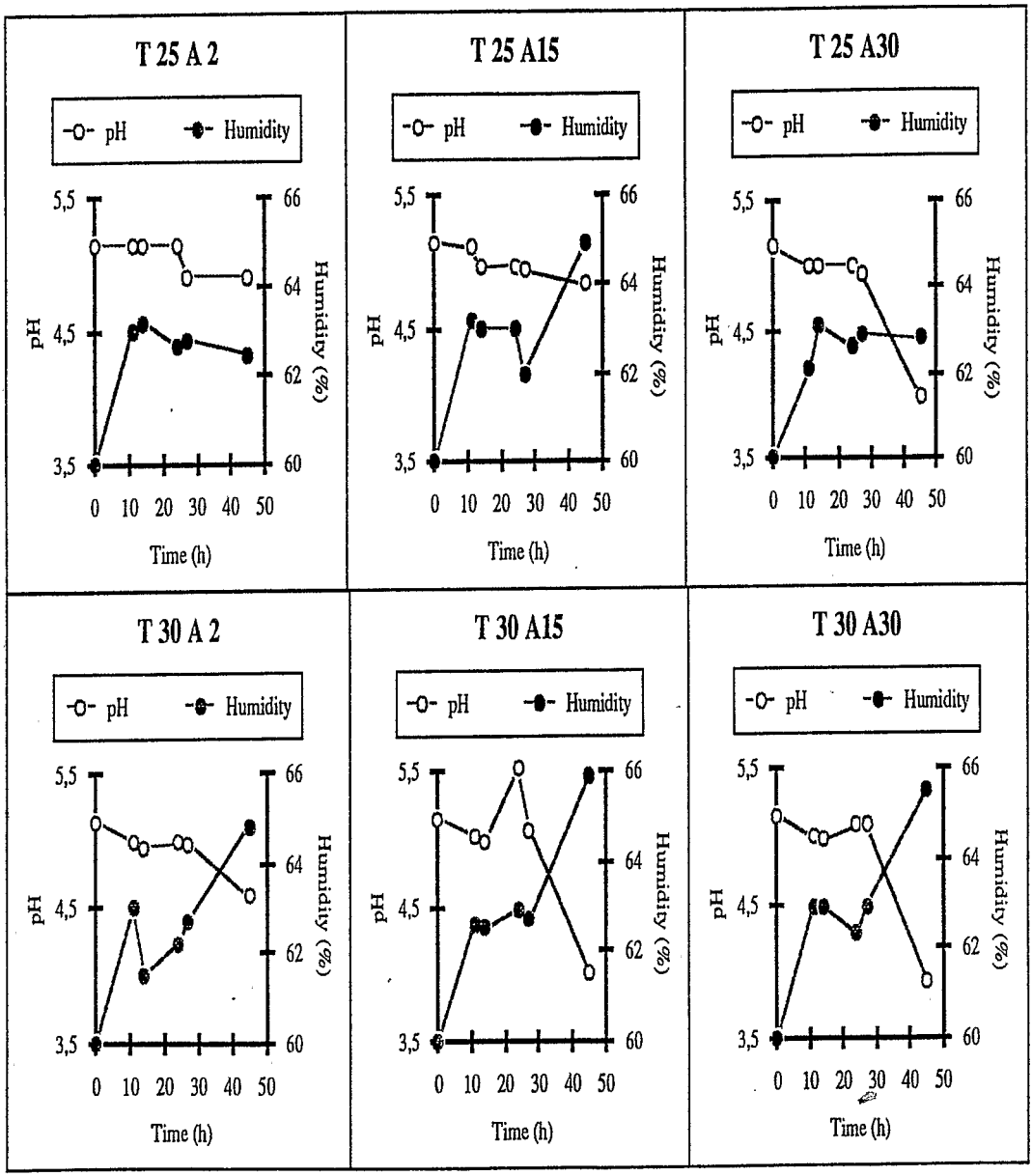

Fig. 3. Evolution of humidity and pH during the course of fermentation, under different temperature and aeration conditions (the substrate codes are those of Table 1).

ratio of 80:20, respectively (Roussos *et al.*, 1991b). Further studies on the initial humidity (%) of the substrate should be done.

The moisture of the substrates increased gradually during the first 10 h of incubation, then remained stable up to 25 h, and thereafter began to increase sharply until the end of fermentation course. The only exceptions were observed in substrate T25A2, in which the microorganism hardly could grow, as well as in substrate T25A30, in which moisture remained stable after 10 h of incubation. We believe that these two cases are artifacts.

The evolution of the pH is similar to that of humidity (Fig. 3). With a starting value of 5.15, the pH remained almost stable during the first 25 hours of fermentation and then, in the media where the fungus could easily grow and tannin degradation occurred, decreased rapidly probably due to the release of acidic substances as products of tannin degradation (*e.g.* tannic and gallic acids).

Sugar and tannin consumption

The solid fermentation of carob pods under 2 different temperatures (25° and 30°C) and 3 different aeration conditions (0.12, 0.9 and 1.8 L/h), revealed that the consumption of sugars and tannins from the substrates was hardly influenced by the changes of temperature, whereas significant effect was observed by the changes in aeration flow. As the structure of carob pods did not allow the addition of the suitable amount of water for SSF, the initial humidity level was adjusted to 60% by the addition of sugar cane bagasse, which is an inert support for fungal growth. This bagasse also supported homogeneous aeration of the substrates, and did not affect significantly their composition.

The maximum sugar consumption and tannin degradation was observed in the substrates T25A30 and T30A30 (Fig. 4). The aeration flow is the most important parameter regarding fungal metabolic activity: under low aeration conditions, *A. carbonarius* consumes the sugars of the substrate, without being capable of tannin degradation; whereas under an aeration flow higher than 15 ml/h, tannin degradation occurs and as a result sugar concentration increases. In this case, sugar consumption began after 10 h of incubation, and continued up to 24 h when an increase in its concentration was recorded, probably due to tannin degradation. This hypothesis is supported by a parallel decrease in the pH value. Culture conditions of 30°C and 30 ml/h aeration

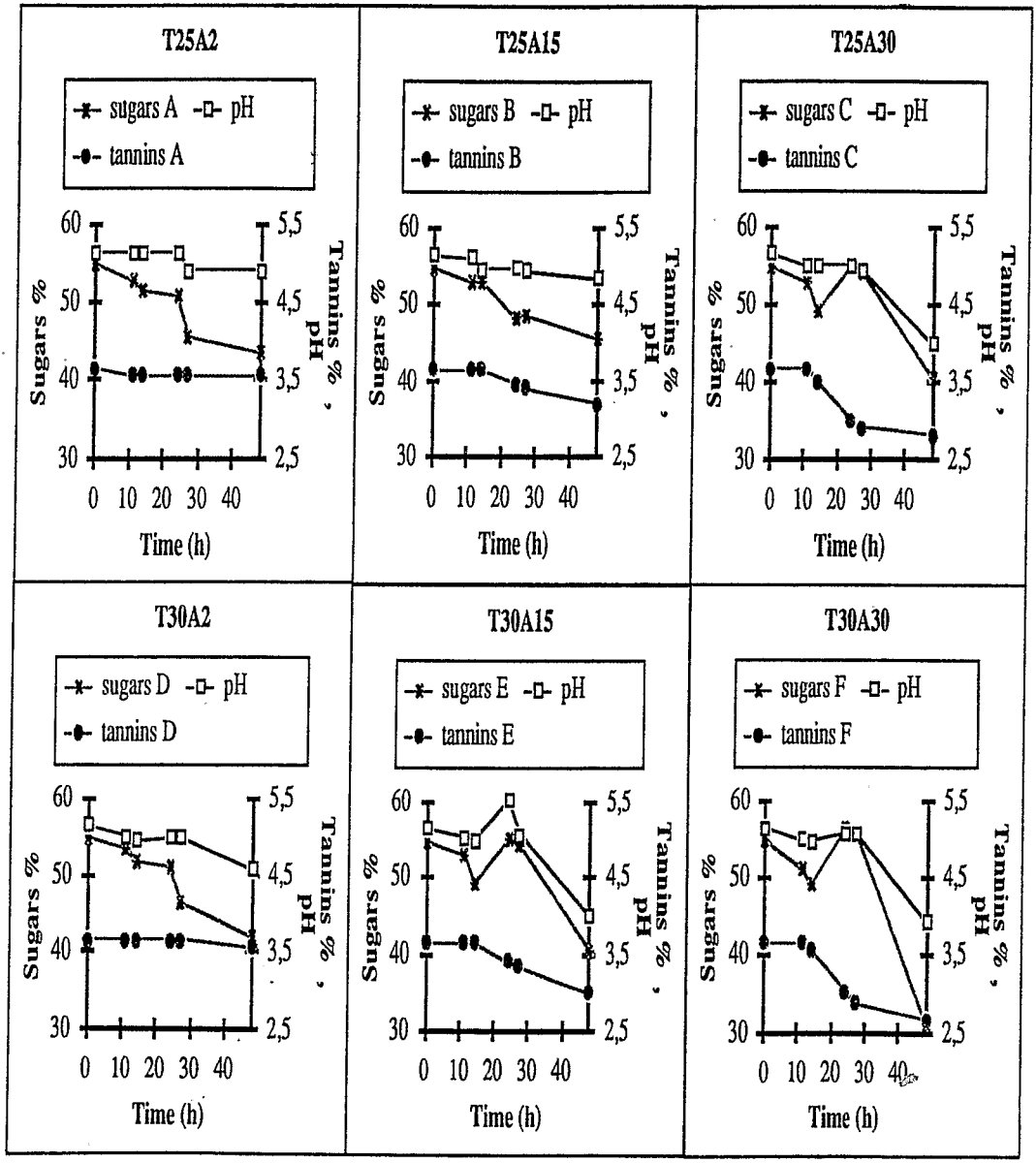

Fig. 4. Sugar consumption and tannin degradation in the substrate studied, under different temperature and aeration conditions (the substrate codes are those of Table 1).

flow (T30A30) showed the highest proportion of tannin degradation (almost 30% on initial tannin dry weight), which seems to be very low in comparison with liquid cultures (Lambraki and Marakis, 1993). These data suggest that humidity can be another significant factor in tannin degradation.

It is important to mention that tannin degradation started after the first 10 h of incubation, even earlier than the respiration of the fungus. We suppose that tanninolytic enzymes are involved either before or after germination of the spores, which are liberated in the surrounding medium in order to look for nitrogen sources, initially unavailable in the substrate. If this assumption is correct, nitrogen limitation might lead to tannin degradation.

Finally, it is possible to establish that aeration is an important parameter not only for the growth of *A. carbonarius*, but also for tannin degradation. On the contrary, changes in the incubation temperature from 25° to 30°C have no influence on fungal growth or on tannin degradation. Future research in our laboratory will be focused on the effects of initial humidity level in the substrate, as well as the addition of different nitrogen sources.

ACKNOWLEDGEMENTS

The authors are grateful to Mme. Laure Hannibal for technical assistance. This work was financially supported by the PLATON programme No. 92 217, under a French-Greek cooperation.

LITERATURE CITED

BATE-SMITH, E. C., 1972. Haemanalysis of tannins: the concept of relative astringency. *Phytochem. 12*: 907-912.

BROADHURST, R. B. and W. JONES, 1978. Analysis of condensed tannins using acidified vanillin. *J. Sci. Food Agric. 29*: 788-794.

CALIXTO, F. S. and J. CAÑELLAS, 1982. Components of nutritional interest in carob pods (*Ceratonia siliqua*). *J. Sci. Food Agric. 33*: 1319-1323.

DUBOIS, M., K. A. GILLES, J. K. HAMILTON, P. A. REBERS and F. SMITH, 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chemistry 28*: 350-356.

GRANT, W. D., 1976. Microbial degradation of condensed tannins. *Science 193*: 1137-1139.

IMRIE, F. K. E. and A. J. VLITOS, 1975. Single-cell protein II. *In*: Tannenbaum, S. R. and D. I. C. Wang (Eds.). MIT Press, Massachusetts.

LAMBRAKI, M. and S. MARAKIS, 1993. Screening of tanninolytic fungi. *15th Symposium of the Hellenic Society of Biological Sciences* (H.S.B.S.), Florina and Kastoria, Greece.

LONSANE, B. K., N. P. GHILDYAL, S. BUDIATMAN and S. V. RAMAKRISHNA, 1985. Engineering aspects of solid state fermentation. *Enzyme Microbiol. Technol. 7*: 258-265.

LONSANE, B. K., N. P. GHILDYAL and V. SRINIVASA MURTY, 1982. Solid state fermentation and their challenges. *Symposium on fermented foods, food contaminants, biofertilizers and bioenergy* (Technical Brochure), *Association of Microbiologists of India, Mysore.*

MARAKIS, S., 1980. New fungal strains for microbial protein production from carob beans. Ph. D. Thesis, Athens University, Greece.

MARAKIS, S., 1992. Sucrose syrup from carob pod. *Biotechnol. Letters 14*: 1075-1080.

MARAKIS, S., M. LAMBRAKI and S. DIAMANTOGLOU, 1993. Tannin chemistry of nine cretan carob varieties. *Chimica Chronica (New Series) 22*: 213-224.

MITRAKOS, K., 1968. The carob. A report to Tate and Lyle, Ltd, University of Reading, England.

RAIMBAULT, M. and D. ALAZARD, 1980. Culture method to study fungal growth in solid fermentation. *Eur. J. Appl. Microbiol. Biotechnol. 9*: 199-209.

ROUSSOS, S., L. HANNIBAL, A. DURAND, M. DIEZ, G. SAUCEDO-CASTAÑEDA, D. MONTET and J. GRAILLE, 1994. Enrichissement en proteins du tourteau de coprah: sélection de champignons filamenteux en FMS. *Oléagineux 49(5)*: 235-247.

ROUSSOS, S., M. RAIMBAULT, G. SAUCEDO-CASTAÑEDA, G. VINIEGRA-GONZALEZ and B. K. LONSANE, 1991a. Kinetics and ratios of carboxymethyl cellulase and filter paper activities of the cellulolytic enzymes produced by *Trichoderma harzianum* on different substrates in solid state fermentation. *Micol. Neotrop. Apl. 4*: 19-40.

ROUSSOS, S., M. RAIMBAULT, G. VINIEGRA-GONZALEZ, G. SAUCEDO-CASTAÑEDA and B. K. LONSANE, 1991b. Scale-up of cellulases production by *Trichoderma harzianum* on a mixture of sugar cane bagasse and wheat bran in solid state fermentation system. *Micol. Neotrop. Apl. 4*: 83-98.

SAUCEDO-CASTAÑEDA, G., M. TREJO, B. K. LONSANE, J. M. NAVARRO, S. ROUSSOS, D. DUFOUR and M. RAIMBAULT, 1993. On-line automated monitoring and control systems for CO_2 and O_2 in aerobic and anaerobic solid state fermentation. *Process Biochemistry 29*: 13-24.

SWAIN, T. and W. E. HILLIS, 1959. The phenolic constituents of *Prunus domestica*. I. The quantitative analysis of phenolic constituents. *J. Sci. Food Agric. 10*: 63-68.

WÜRSCH, P. (Ed.), 1987. *Proceedings of II International Carob Symposium*, Valencia, Spain.