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Solid-State Culture of *Aspergillus niger* on Support

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The growth kinetics of *Aspergillus niger* on a solid support *i.e.* sugarcane bagasse, impregnated with a liquid glucose medium, were investigated under different culture conditions. The water activity of the medium, the amount of spore inoculum and the support particle size were shown to be critical factors for mold growth. The elevated rates of growth observed with high substrate concentration media demonstrated the feasibility of the method for culturing filamentous fungi.

Solid-state cultures (SSC) of molds have traditionally been limited to polymerized substrates (starch, cellulose) able to retain water in their porous matrix, and few attempts have been made to grow filamentous fungi on solid inert materials impregnated with nutritive solutions. When substrate and support are separate and an absorbed liquid medium is used, it becomes possible:
1) to use monomeric carbohydrates immediately available for the microorganism
2) to avoid the degradation of the solid matrix during growth and thus ensure constant geometric conditions
3) to make easier comparisons with submerged cultures.

In 1935, Cahn[1] achieved citric acid production by *Aspergillus niger* cultured on a sucrose solution absorbed on sugarcane bagasse or sugarbeet pulp. This kind of culture has also been studied by Terui *et al.*[2] and more recently by Lakshminarayana *et al.*[3] Mineral or synthetic materials such as vermiculite[4] and polyurethan foam[5,6] have also been used as supports for growth and enzyme production by *Aspergillus* or *Penicillium* sp.

SSC on wood pulp already constituted a model for estimating growth of yeasts[7] but no work has dealt with the environmental factors affecting mold growth kinetics on solid supports. In the present work, an attempt was made to describe the growth of *A. niger* with a liquid medium containing glucose and salts absorbed onto a lignocellulosic support *i.e.* sugarcane bagasse. The effects of the moisture content and water activity of the medium, amount of spore inoculum and support particle size on the growth kinetics were investigated.

Materials and Methods

Microorganism *Aspergillus niger* var. *hennebergi* (N° 10) described by Raimbault & Alazard[8] was used.

Pretreatment of raw material Sugarcane bagasse, free of sugars, was obtained from a sugar factory in Zacatepec (Mexico). Unless otherwise specified the 30–50 mesh fraction was used and the bagasse, moistened to 50%, was sterilized in an autoclave at 1 bar for 30 mn prior to the culture.

Preparation of spore inoculum Spore inoculum was obtained as previously described,[9] unless otherwise specified fixing the inoculum size at 2×10^7 spores per gram of solid support.

Solid state culture The culture was achieved under non-aseptic conditions as previously described.[8] Column fermentor units containing 10 g of moistened medium were incubated in a 35°C water bath. The inlet air was bubbled twice in water and the air flow was set to 2 $l.h^{-1}$; two columns were removed at each

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sampling time. Composition of the salts mixture was: KH_2PO_4, 5 g; $(NH_4)_2SO_4$, 9.8 g; Urea, 2.4 g per 100 g of glucose independantly of the glucose concentration in the liquid medium. Salts and glucose were solubilized and sterilized separately at 120°C for 20 mn. The pH of the suspension containing the spores in the nutritive solution was adjusted to 2.7, which resulted in a value of 4.5 for the solid material after inoculation.

Analysis The exit air of a 36 mm diameter column reactor containing 30 g of moistened material was dried by passing through a condensation and a Silicagel column. Then the gas composition was monitored by pumping it through a paramagnetic Servomex Oxygen Analyzer (Taylor Instruments). The oxygen uptake rate (OUR) is expressed in $ml \cdot h^{-1}$ per column containing 30 g of moistened material.

Sample analysis 4 g of sample were homogeneized in 96 ml of water with an Ultra-Turrax®. After centrifugation, glucose in the supernatant was measured by the Miller[10] method. For protein determination, samples were dried at 70°C for 24 h and then hydrolysed with HCl 6N for 20 min at 120°C. The hydrolysate was neutralized with NaOH and the amino-acids of the supernatant were determined by colorimetric reaction with ninhydrin[11] to avoid interference from lignin. Protein was related to biomass assuming that 40% of the biomass was protein.

Determination of water activity Water activity (a_w) defined as the relative humidity of the gaseous atmosphere in equilibrium with a sample was determined with a water activity detector Humidat IC II purchased from Novasina. 3 to 4 g of material were placed in a relative humidity captor thermostated at 30°C and the state of equilibrium was reached after 3 h.

Estimation of biomass from the oxygen uptake rate Cell mass was calculated from the oxygen uptake rate using the integration method described by Sato *et al.*[7] The values used for the cell (X) yield against O_2 (YO_2) and maintenance coefficient for oxygen consumption (mO_2) were 1.55 $gX \cdot g\ O_2^{-1}$ and 0.07 g $O_2 \cdot gX^{-1} \cdot h^{-1}$ respectively as calculated by Raimbault[12] for the growth of *A. niger* on solid cassava flour. The initial biomass content Xo was calculated to be 0.0006 g for 2×10^7 spores.

Results and Discussion

Moisture content, water activity (a_w) The three main ingredients of the medium were the support, the substrate and water. The initial moisture content could be set by varying one ingredient and water independently of the third. Two series of experiments were run; in the first series we changed the support/water ratio and in the second the substrate/water ratio.

Cultures with a constant substrate/water ratio (corresponding to a glucose

Table 1. Effect of moisture content and water activity on the growth parameters of *A. niger* cultured on support with an absorbed liquid glucose medium.

Bagasse/water ratio (w/w)	Glucose concentration ($g \cdot l^{-1}$)	Initial moisture content (%)	Initial A_w	Time (h)	Specific growth rate (h^{-1})	Final cell mass[a] (g/column)	Final cell mass[b] (g/column)	Carbohydrate consumption (g/column)	Rx[c] (gX/gS)
1.30	168	40	0.975	18	0.453	—	—	—	—
0.83	168	50	0.981	18	0.388	—	—	2.31	—
0.50	168	60	0.977	16	0.429	—	—	2.20	—
0.36	168	65	0.977	15	0.473	—	—	2.72	—
0.25	168	70	0.974	17	0.464	—	—	2.42	—
0.19	168	75	0.973	18	0.429	—	—	—	—
0.36	57	72	0.986	12	0.541	0.58	0.85	1.10	0.527
0.36	168	65	0.962	17	0.433	1.40	1.60	2.81	0.498
0.36	260	59	0.939	26	0.354	2.39	2.30	5.08	0.470
0.36	330	55	0.920	31	0.317	2.95	3.00	5.99	0.492
0.36	417	50	0.893	41	0.193	3.82	2.71	7.53	0.507

[a] calculated from the amino-acid content
[b] calculated from the OUR
[c] final cell mass/carbohydrate consumption

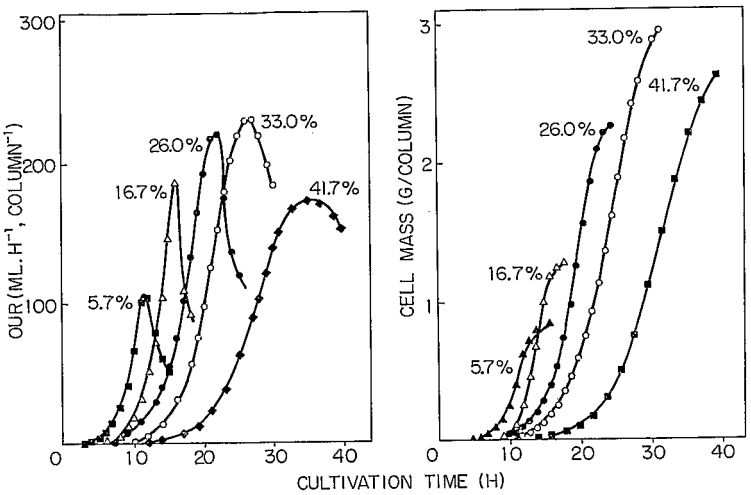

Fig. 1. Time course of OUR and cell mass calculated from OUR during growth of *A. niger* on bagasse with different glucose concentrations (% weight/volume) in the liquid phase. Culture conditions: bagasse/water (w/w) ratio, 0.36; inoculum, 2×10^7 spores/g of support; support particle size, 0.5 mm.

concentration in the liquid phase of 17% (w/v)) and increasing support/water ratios (corresponding to initial moisture contents ranging from 40 to 75%) showed little variation of growth kinetics, resulting in similar specific growth rates and fermentation times (Table 1).

On the other hand, trials carried out maintaining a constant support/water ratio and increasing the substrate concentration in the liquid phase showed differences in the fermentation profiles (Fig. 1). As may be seen in Fig. 2 the germination time increased drastically and the average specific growth rate decreased when the a_w of the medium was lowered. It may be hypothesized that the profiles observed in Fig. 2 are representative of the "xerotolerancy" of the considered species; as defined by Corry[13] as the ability to grow at low a_w. These results demonstrated that, as predicted by Sato *et al.*[7] with yeasts cultured on wood pulp, the water activity and thus the osmotic pressure of the medium are more important than the water content for the germination and the growth pattern of *A. niger* on solid phase with an absorbed nutritive liquid medium.

SSC provided unique conditions for the microorganism/air/water interface and allowed growth at a high glucose concentration (42% w/v in the liquid phase) and thus high tonicity media (A_w: 0.88) with satisfactory specific growth rates (0.20 h^{-1}). When using greater glucose concentrations (superior to

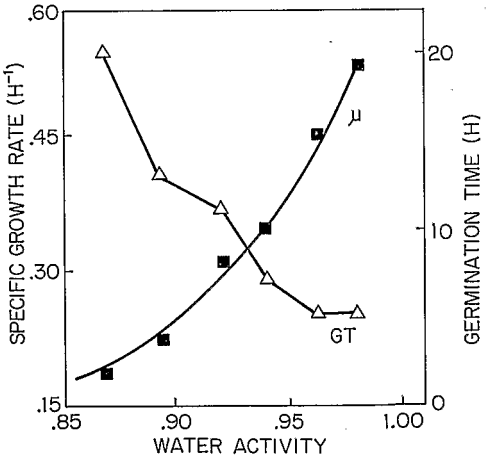

Fig. 2. Germination time (△) and specific growth rate (■) as a function of the initial A_w of the medium.

450 g·l^{-1}) in the liquid medium, condensation of water at the lower and upper parts of the column resulted in locally higher a_w, and gradients of growth were observed. Nevertheless, some average specific growth rates calculated here for low tonicity media (Table 1) are higher than those normally reported with fungi in submerged cultivations[14] (0.3 h^{-1}), showing that solid state culture conditions are more suitable than liquid ones for the growth of filamentous fungi.

Due to the difficulty of precisely determining cell mass concentration in SSC, some uncertainty still remains concerning the value of mO_2. Watson[15] reported 10-fold increases of the maintenance coefficient for the growth of yeasts when adding 1.0 M NaCl to the cultivation medium. In the experiments mentioned above, values of final cell mass as determined by amino-acid analysis are in good agreement with those calculated from the OUR (Table 1–3), assuming a constant mO_2 coefficient *i.e.* 0.07 g O_2·gX^{-1}·h^{-1}. Discrepancy between the values was only observed for the higher tonicity solution; this is a point needing further investigation.

Spore inoculum size The support/water ratio and the substrate concentration in the liquid phase were maintained constant and the spore inoculum size was changed from 8×10^5 to 1×10^9 spores per g of support *i.e.* 2.7×10^5 and 3.4×10^8 per ml of liquid medium. The evolution of the OUR and of cell mass as calculated from the OUR are represented in Fig. 3. Contrary to what has been reported in the literature with high density spore inocula in submerged cultures,[16] no inhibition of growth was observed on solid support. When the culture time was plotted against the decimal logarithm of the inoculum (Fig. 4), a linear relation was observed. These results confirmed those obtained previously by Oriol *et al.*[17] during microcalorimetric studies under similar cultivation conditions, accounting for an enhancement of the germination process when the amount of spore inoculum was increased.

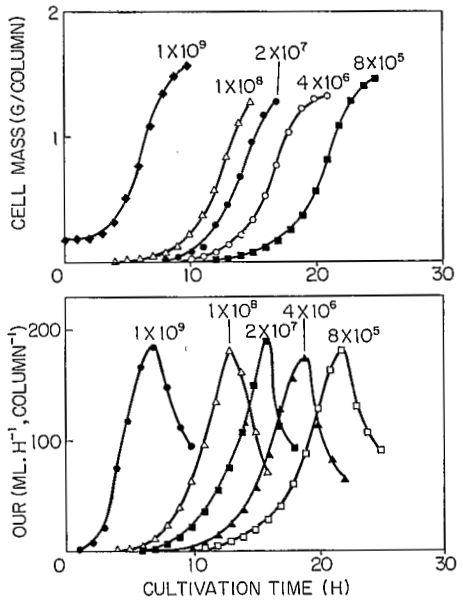

Fig. 3. Time course of OUR and cell mass calculated from OUR during growth of *A. niger* on bagasse with different amounts of spore inoculum (spores/g of support).
Culture conditions: bagasse/water (w/w) ratio, 0.36; glucose concentration in the liquid phase 16.8% (w/v); support particle size, 0.5 mm.

For the culture containing 1×10^9 spores/g of support, even if ungerminated spores remained, substrate (16% w/v in the liquid phase) was almost exhausted as soon as 11 h after inoculation. As may be seen in

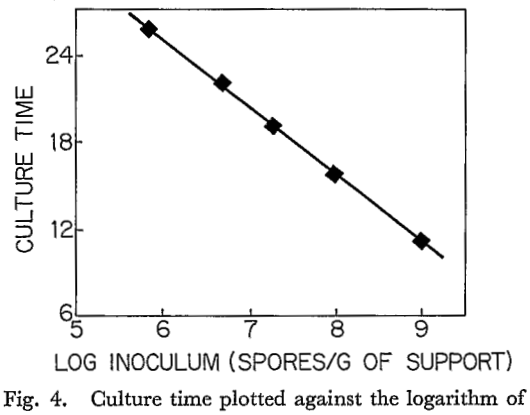

Fig. 4. Culture time plotted against the logarithm of the amount of spore inoculum.

Table 2. Effect of the amount of spore inoculum on the parameters of growth of *A. niger* on support with an absorbed liquid medium containing 168 g·l^{-1} (w/v) of glucose.

Spore inoculum (Sp./g support)	Final cell mass [a] (g/column)	Final cell mass [b] (g/column)	Substrate consumption (g/column)	Specific growth rate (h^{-1})	Time (h)
8×10^5	1.50	1.38	2.65	0.415	26
4×10^6	1.31	1.38	2.85	0.429	22
2×10^7	1.35	1.41	2.71	0.433	19
1×10^8	1.40	1.35	3.41	0.464	16
1×10^9	1.65	1.66	3.44	0.420	11

[a] calculated from the amino-acid content
[b] calculated from the OUR

Table 2, little variation of either the final mycelium content or of the average specific growth rate was observed when the inoculum size was varied.

Support particle size Given a standard bagasse/water ratio (0.36), substrate concentration in the liquid phase (28% w/v) and inoculum size (2×10^7 spores/g of support), trials were run varying the average support particle size from less than 10 to more than 150 mesh *i.e.* approximately 2.5 to 0.1 mm. In Fig. 5 the OUR and the cell mass production, as calculated from the OUR for three different average particle sizes, are reported. An increase of the average particle size produced a decrease of the growth rate in the deceleration phase of growth and, for the largest fibers, led to lower final mycelium contents (Table 3).

As mold growth observed with a microscope appeared to occur only on the support surface, the depressive effect observed with a large bagasse particle size might be due to a limitation of growth caused by intraparticular diffusion of nutrients.

Small average support particle size (0.1 mm) produced high density packed material in the column (0.5 kg·l^{-1} against about 0.3 kg·l^{-1} with larger particles), but no variation in the kinetics of growth nor in the final mycelium content was detected (Table 3). Biomass concentrations of 40 g (dry weight)·l^{-1} of reactor could be obtained, values about 30% superior to those assumed as limits for SSC of molds by Laukevics

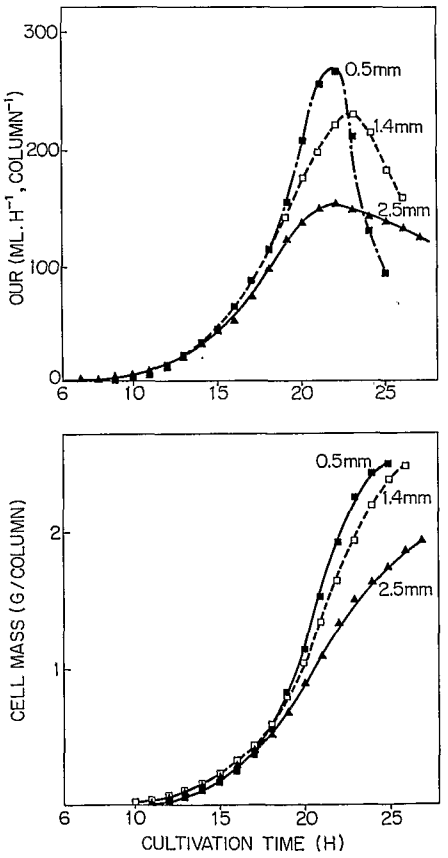

Fig. 5. Time course of OUR and cell mass calculated from OUR during growth of *A. niger* on bagasse with different bagasse particle sizes.
Culture conditions: bagasse/water (w/w) ratio, 0.36; glucose concentration in the liquid phase, 28% (w/v); inoculum, 2×10^7 spores/g of support.

Table 3. Effect of the support particle size on the parameters of growth of *A. niger* on support with an absorbed liquid medium containing 280 g·l^{-1} (w/v) of glucose.

Support particle size (mm)	Initial density (kg·l^{-1})	Final cell mass [a] (g/column)	Final cell mass [b] (g/column)	Substrate consumption (g/column)	Specific growth rate (h^{-1})	Time (h)
2.50	.230	2.16	2.05	4.75	0.323	27
1.43	.295	2.66	2.60	4.95	0.330	26
0.79	.295	2.52	2.40	5.42	0.348	26
0.51	.295	2.76	2.60	4.76	0.400	25
0.10	.485	2.53	2.33	4.58	0.375	25

[a] calculated from the amino-acid content
[b] calculated from the OUR

et al.[18]

Conclusion

The culture method involving a synthetic liquid medium absorbed on a solid support was demonstrated to be suitable for the growth of filamentous fungi, and it allowed the utilization of high-concentration substrate solutions. The water activity of the liquid phase, the support particle size and the amount of spore inoculum were found to be critical factors for the growth of molds, the latter accounting for different spore germination patterns between solid and submerged cultures. This kind of cultivation method might broaden the use of solid state cultures for producing fungal metabolites with low cost technology.

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