European J. Appl. Microbiol. Biotechnol. 9, 199–209 (1980)

European Journal of Applied Microbiology and Biotechnology

Culture Method to Study Fungal Growth in Solid Fermentation

Maurice Raimbault* and Didier Alazard

O.R.S.T.O.M. Laboratory of Soil Microbiology, B.P. 1386, Dakar (Sénégal)

Summary. A new culture method is described to study the growth of *Aspergillus niger* on cassava meal in the solid state. This method uses preparations of the cooked starchy substrate as a homogeneous granulated product containing spores, salts and water. An incubation device aerates the mass with humidified air at a controlled temperature. Homogeneous development of mycelia, without sporulation, occurred in the substrate mass. From physiological studies, optimal conditions for *A. niger* growth on cassava in the solid state were 50 - 55% moisture, 35°C, a nitrogen source comprising 60% ammonium and 40% urea (on a nitrogen basis) and 2×10^7 spores/g of substrate. Growth kinetics were established and changes in pH, protein, carbohydrate and water content were determined during the incubation. Growth rate and yield were quite similar to those described in the literature for *A. niger* cultivated in liquid media under optimal conditions.

Introduction

The development of liquid culture techniques has given rise to a number of important industrial applications (Smith and Berry 1975). However, liquid media are far from the natural environment of fungi. In particular, they decrease the importance of specific phenomena such as osmotic resistance or cytoplasmic transfer. Moreaux (1974) and Trinci (1971a) found apparent morphological and physiological differences in fungi cultivated in liquid and solid conditions.

Fungi play an important role in food spoilage as well as in traditional solid fermentations such as cheese, miso, tempeh or koji (Gray 1970; Hesseltine 1965; Stanton and Wallbridge 1969). However, because of the lack of specific methods of investigation, very little is currently known of the physiology and kinetics of such solid state fermentations. Previous studies concerned essentially microscopic examinations of natural

* Present address: Centre de Recherche I.R.CH.A. - B.P. n° 1 F-91710 Vert-le-Petit (France)

0171–1741/80/0009/0199/$ 02.20

products (Lloyd 1965), influence of environmental factors on fungal microflora (Moreaux 1974) and biochemical transformations observed only during traditional fermentations (Hesseltine and Wang 1967; Yong and Wood 1973). More recently studies of surface growth on artificial solid media were carried out by Trinci (1971b) and Pirt (1967) in which estimates of growth rates were made by colony diameters and optical elongation or branching rate.

In this report we describe a new culture method which facilitates the selective and homogeneous development of mycelia in starchy solid substrates and allows the study of the physiology and growth of fungi in such conditions. We chose a starchy substrate because a large variety of amylolytic fungi are capable of rapidly degrading it and because of the importance of starchy products as foodstuffs.

Material and Methods

Organism

We used a strain of *Aspergillus niger* (n° 10) which was isolated from rotting cassava tuber and was further selected for its high amylolytic activity and protein content. It was maintained by serial transfer at six monthly intervals on a medium containing cassava meal, 20 gl^{-1}; $(NH_4)_2SO_4$, 3 gl^{-1}; KH_2PO_4, 1gl^{-1} and Bacto-agar, 25 gl^{-1}.

Starchy Solid Substrate

Studies were conducted using cassava tubers (*Cassava utilissima*) as the initial raw material. Assays showed that potato could be used in the same way. Tubers were washed and cooked by steam in an autoclave (100°C for 20 min). After cooling they were frozen overnight and then dried in an oven at 50 - 60°C. The dry and friable product was milled into a coarse powder. This cooked cassava meal was used for our solid media preparations.

Spore Inoculum Preparation

Spores were obtained from 250 ml flasks containing 17 g of a doughy medium composed of 5 g of cooked cassava meal, 0.2 g of KH_2PO_4, 0.4 g of $(NH_4)_2SO_4$ and 83 ml of tap water. After autoclaving, flasks were inoculated with spores from a slant and incubated at 30°C for one week. Two hundred millilitres of water and a drop of Tween 80 were then added and the spores suspended by stirring for 15 min. The suspension was passed through a 0.5 mm sieve to eliminate mycelia and the spore concentration was estimated by direct microscopic counting using a Neubauer cell. Usually a single flask provided about 4×10^{10} spores which was enough to inoculate 2 kg of solid substrate.

Solid Medium Composition

From preliminary experiments, the standard solid medium was as follows. Cooked cassava meal, 100 g; spores, 2×10^9; KH_2PO_4, 5 g; $(NH_4)_2SO_4$, 11.25 g; urea, 1.7 g and tap water, 100 ml. The pH of liquid containing salts and spores was adjusted to 2.7 before mixing with cassava meal, which resulted in a final pH of 4.5.

Culture Methods

When liquid was added to the cassava meal, small discrete granules formed spontaneously. In order to improve homogeneization of these granules, the product passed through a 2 mm sieve. This inoculated solid substrate was then transferred into incubators (Figs. 1 and 2) with gentle packing. The upper part of these incubators could hold 20 g of solid material which was enough for all laboratory analyses. Incubators were put into a regulated water bath and aerated with 4 - 6 l/h of air saturated with water. For our studies, we used equipment capable of holding 24 u (Fig. 2).

Sample Treatment

Prior to analysis, samples were treated as follows. All contents of the incubator were weighed and cut into small pieces. Moisture content was determined gravimetrically

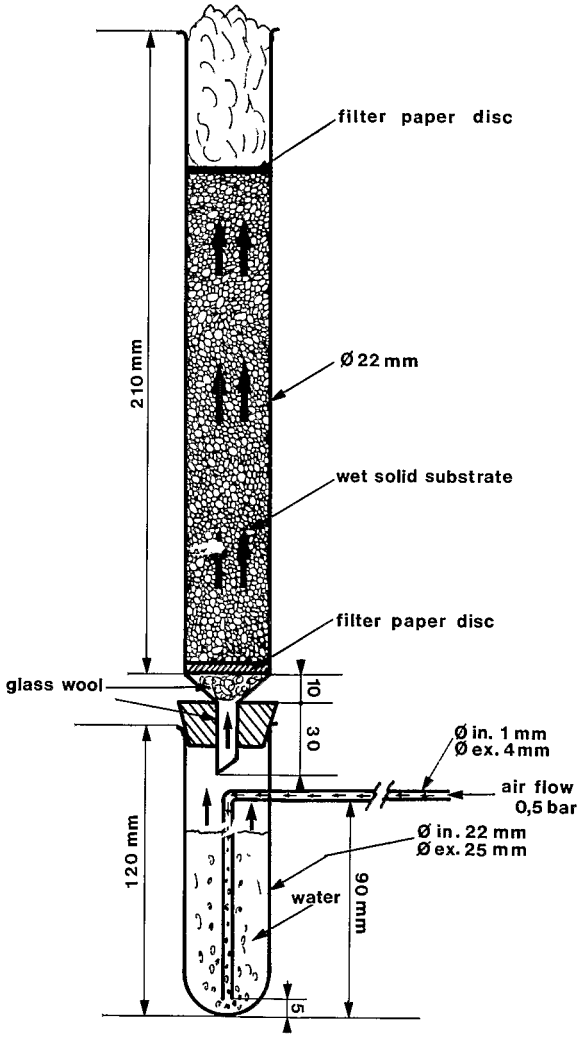

Fig. 1. Incubator device

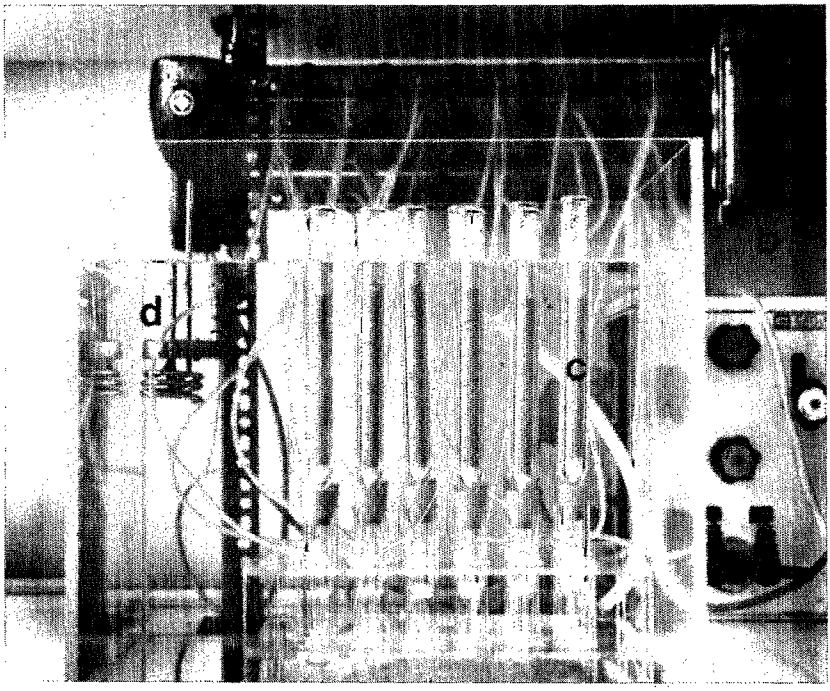

Fig. 2. A 24 unit apparatus for solid culture; *a*, needle valve to adjust air flow; *b*, flow-meter (0 - 12 l/h) to control exit air flow; *c*, incubator unit; *d*, regulated water bath

from 5 g of this homogeneous material. The pH, protein and residual carbohydrates were determined in the same way from an homogeneous suspension of 5 g of product in 50 ml of water after mixing in a Ultra-Turrax blender.

Protein

From the 5 g/50 ml suspension, suitable dilutions were made to 50 - 150 mg protein/ml. The suspension was then digested with alkali to break the cellular wall material and to solubilise protein (1 ml of 1 N NaOH to 1 ml sample at 100°C for 5 min). After cooling, the protein was determined by the method of Lowry (1951) using bovine serum albumin as standard.

Residual Carbohydrates

These represent reducing sugars after total hydrolysis of residual starch by glucoamylase. Thirty mg of Amygase 200 AGN (Seclin France) was added to 5 g/50 ml suspension and incubated for 30 min at 50°C. Reducing sugars of the diluted hydrolysate were determined by the Somogyi-Nelson method (Nelson 1944).

Results

Solid Culture Technique

Our solid culture method for mycelia is based on the homogeneous distribution of spores and salts in the mass of a suitable starchy substrate. The preparation of a porous granulated material with adequate pH and moisture content is essential to insure good aeration and fast mycelial growth. The method permits selective growth of the mold due to the acidic pH, a low water content and a heavy spore inoculum and does not require aseptic conditions.

After a germinating phase, hyphae surrounded starch granules and bound them together in a solid mass. In the last period of fermentation starch was digested and replaced by a porous mycelial mass mixed with residual initial material. In this way aeration remained efficient and aerobic conditions could be maintained at all times.

Microscopic examination of the products indicated that all spores had germinated after 6 - 8 h of fermentation and during the growth phase, all the mycelium was in development with no conidiation. At the end of fermentation (20 - 24 h) no spores could be found. A very low sporulation was observed only after more than 40 h of growth with a high aeration rate.

Physiological Studies

We used this technique to study the influence of environmental factors on the growth of *Aspergillus niger* in cooked cassava meal and to determine optimal conditions. After different times of incubation the pH, moisture, protein and sugar content were analysed in the samples.

Moisture

The influence of the initial water content of the solid medium on the growth, as measured by protein synthesis, is shown in Fig. 3. Enough water was necessary to insure good fungal development, but too much water decreased porosity and oxygen diffusion in the mass and could favour bacterial contamination.

Temperature

The influence of temperature on germination, growth and sporulation of fungi is complex (Moreaux 1974). The data in Fig. 4 indicate the optimum temperature is between 35 and 40°C. A high temperature inhibited germination of the spores, but did not significantly affect mycelium growth.

pH

The correct pH is critical for the success of solid state fermentations. It must be low enough at the beginning to avoid bacterial growth. However mycelial growth promoted rapid acidification which then stopped further growth when ammonium salts were used alone as the nitrogen source. Therefore we carried out experiments to determine the influence of different mixtures of ammonium sulfate and urea on the pH, protein synthesis and carbohydrate uptake (Fig. 5). Urea stimulated fungal growth when it represented up to 40 - 50% of the total nitrogen.

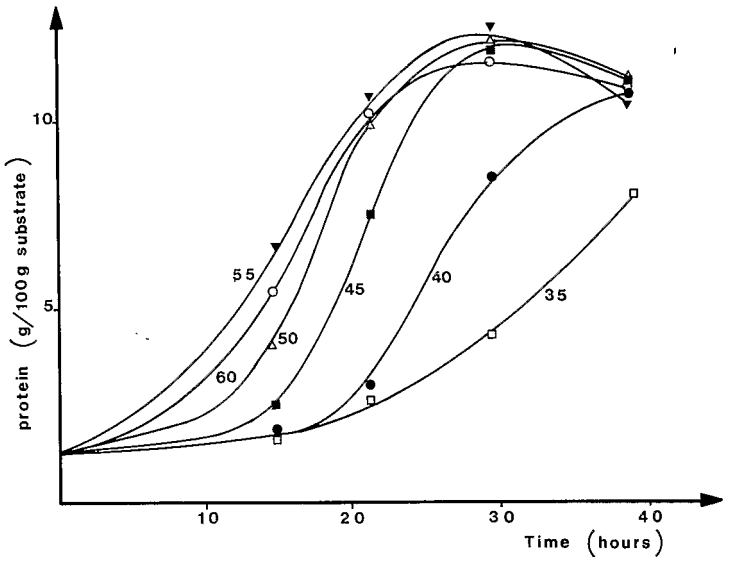

Fig. 3. Influence of moisture content of cassava solid medium on mycelial growth of *A. niger*. Numbers indicate the initial moisture content of the medium

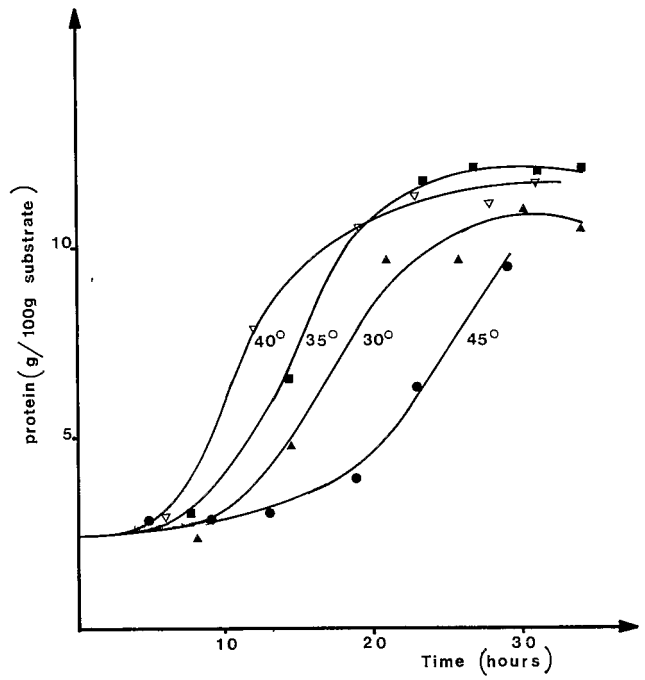

Fig. 4. Influence of temperature on mycelial growth of *A. niger* on solid substrate.

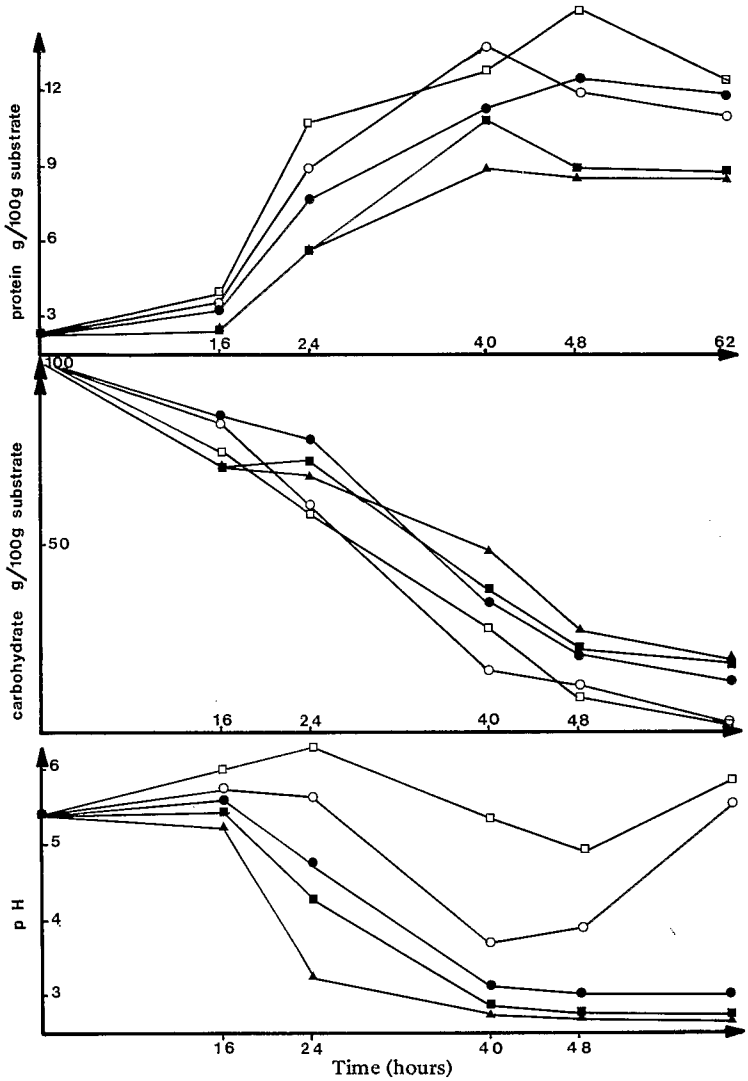

Fig. 5. Influence of urea-ammonium mixtures on *A. niger* growth. ▲—▲; urea 0%, NH_4 100%; ■—■; urea 10%, NH_4 90%; ●—●, urea 20%, NH_4 80%; ○—○, urea 40%, NH_4 60%; □—□; urea 60%, NH_4 40%

Aeration

Experiments were conducted with different aeration rates from 0 to 10 l/h per incubator (20 g of wet product). Only 1 l/h was sufficient to ensure good growth; this indicated a very efficient aeration in this design of fermentation. An air flow of 4 l/h was routinely used.

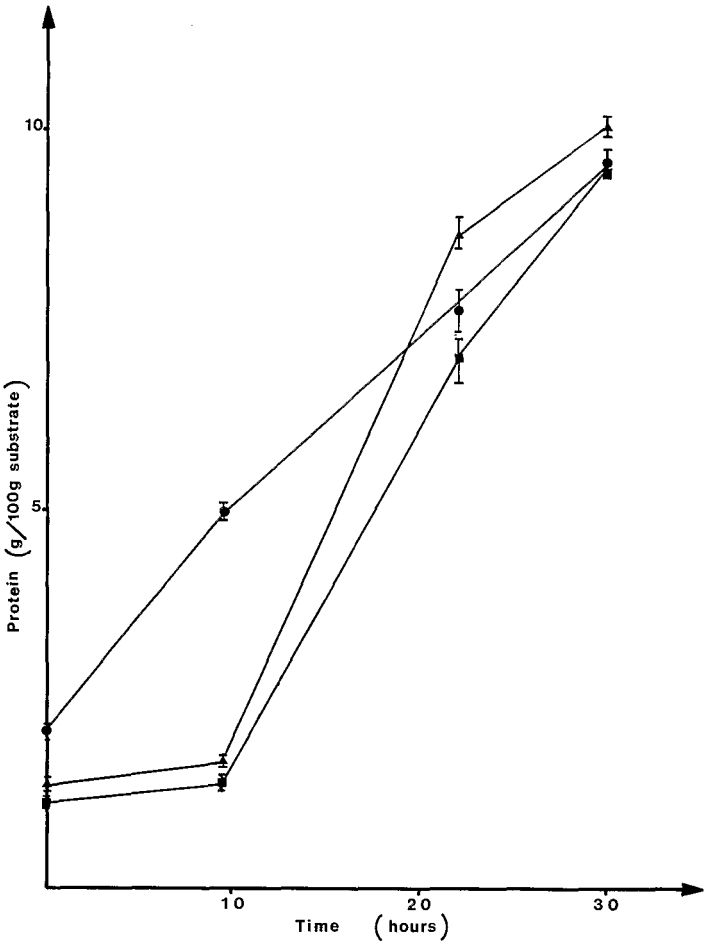

Fig. 6. Influence of spore inoculation on growth of *A. niger* in solid medium. Tests were made in duplicate from 3 samples. Curves represent the mean of these six analysis with bars indicating the dispersion of data. ●—●; 4 x 10^8 spores/g of cassava meal; ▲—▲; 4 x 10^7 spores/g; ■—■; 4 x 10^6 spores/g

Spore Inoculum

Figure 6 shows the protein synthesis in solid substrates inoculated with 4 x 10^8, 4 x 10^7 and 4 x 10^6 spores per g of cassava meal. A high spore concentration gave rapid initial growth but growth was limited; microscopic examination of the product after 30 h of fermentation indicated a large number of non-germinated spores. From 4 x 10^7 and 4 x 10^6 spores/g, the kinetics were similar and all spores germinated. Therefore 10^6 - 10^7 spores/g inoculum was adequate and was used in all further studies.

Growth Kinetics Studies

From the physiological data, the optimal conditions to study growth kinetics were as follows: Initial moisture content 50%, pH 4.5, the nitrogen source was a mixture of

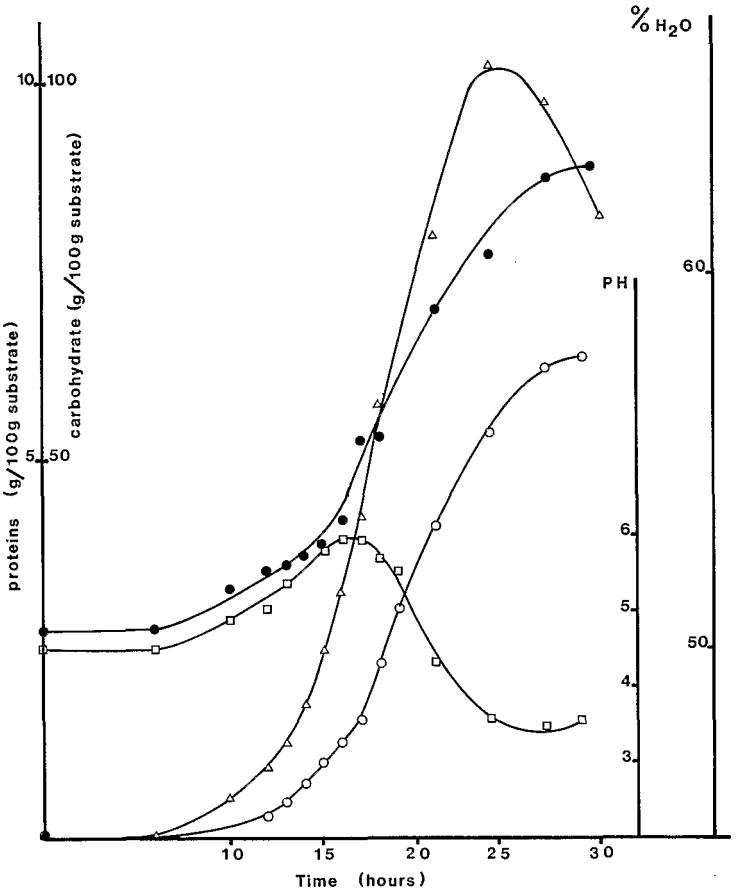

Fig. 7. Growth kinetics of *A. niger* in solid cassava substrate. Conditions are described in text. □—□; pH; ●—●; Water content in wet product; △—△; Protein synthesis; ○—○; Carbohydrate consumption

ammonium sulfate and urea (60% and 40% respectively of nitrogen content); an inoculum of 2 x 10^7 spores/g of cassava meal, the aeration rate was 4 l/h per incubator and the temperature 35°C.

Figure 7 shows changes in pH, moisture, protein and residual carbohydrates during the growth of *A. niger* on cassava under these conditions. First the usual fungal growth and germination occurred (0 - 6 h), followed by exponential growth (6 - 18 h), deceleration (18 - 24 h) and finally autolysis.

During fermentation the moisture content increased because of the loss of dry matter and metabolic water during carbohydrate oxidation. However, in spite of this increase, free water in the medium might be a limiting factor since total water content did not exceed 65% and the constitutive water of the mycelium should represent about 80% of the wet biomass.

During the first stage, the pH increased as the urea was hydrolysed. During rapid growth, ammonium uptake exceeded the hydrolysis rate of urea and the pH decreased. After the growth stopped, the pH increased again when the protein levels fell indicating the begining of mycelium autolysis.

Since the protein content is related to the biomass, this parameter was used to estimate the growth rate, and plotted on a semi logarithmic scale. Thus, we calculated a doubling time of 3.5 h for the mycelial biomass during the exponential growth, a value quite compatible with the available literature for *A. niger* (Carter and Bull 1969; Fencl and Novak 1969).

After the exponential phase, growth decreased and then stopped leaving 30% of the carbohydrate. Further investigations to determine if growth is limited by oxygen diffusion, pH, free water or starch hydrolysis, would be necessary in order to improve the utilization of carbohydrate.

Discussion

Our culture method made possible the study of fungal growth of *A. niger* on a starchy substrate in the solid state. Kinetics were quite comparable with those usually obtained from classical liquid culture techniques.

These experimental results were reported only as an illustration of investigations which can be carried out using this solid phase culture method. As the growth conditions are close to the natural ones, it may be useful for investigating biodegradation in natural fermented food and to improve traditional techniques, food quality and to increase our knowledge of fungal growth on solid substrates. Starting from this simple culture technique, studies were undertaken in our laboratory to design a new process for the production of protein enriched fermented food (Peff) by direct solid fermentation of starchy substrates (Raimbault and Germon 1976). Data already obtained in this field are very promising for the large scale production of animal feed (Raimbault et al. 1977; Senez 1978).

Acknowledgements. The authors thank Professor J.C. Senez (C.N.R.S. Marseille) for scientific assistance. This work was supported by the French D.G.R.S.T. (P.O.U. N° 74.7.1234).

References

Carter BLA, Bull AT (1969) Biotechnol Bioeng 11:785–804
Fencl Z, Novak M (1969) Folia Microbiol (Prague) 14:314–321
Gray WD (1970) Critical reviews in food technology. Chemical Rubber Co. 1(2):225–329
Hesseltine CW (1965) Mycologia 57:1–148
Hesseltine CW, Wang HL (1967) Biotechnol Bioeng 9:275–288
Lloyd AO (1965) Int Biodeterior Bull 1:10–13
Lowry OH, Rosebrough NJ, Farr AL, Randall RJ (1951) J Biol Chem 193:265–275
Moreaux C (1974) Moisissures toxiques dans l'alimentation. Masson & Co, Paris
Nelson N (1944) J Biol Chem 153:375–380
Pirt SJ (1967) J Gen Microbiol 47:181–197

Raimbault M, Deschamps F, Meyer F, Senez JC (1977) Direct protein enrichment of starchy products by fungal solid fermentation. Vth Internation conf on global impacts of Appl Microbiol, Bangkok

Raimbault M, Germon JC (1976) Fr Demand n° 76.06677

Senez JC (1978) Solid state fermentation of starchy substrates. U.N.U., Intern Conf Guatemala City

Smith JE, Berry DR (1975) The filamentous fungi. I. Industrial mycology. Edward Arnold Ltd, London

Stanton WR, Wallbridge A (1969) Proc Biochem 4(4):45–51

Trinci APJ (1971a) J Gen Microbiol 67:325–344

Trinci APJ (1971b) J Gen Microbiol 67:345–348

Yong FM, Wood BJB (1973) Adv Appl Microbiol 17:157–194

Received October 15, 1979