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EFFECT OF CO_2 CONCENTRATION ON THE MICELIUM GROWTH OF Rhizopus SPECIES

C.R. Soccol[1], M. Raimbult[2] & L.I. Pinheiro[3]

[1]Laboratório de Processos Biotecnológicos - Depto. de Engenharia Química da Universidade Federal do Paraná, 81531-970 - Curitiba-Pr.-Brasil. [2]Centre ORSTOM - Laboratoire de Biotechnologie, 911, Avenue Agropolis, 34032 Montpellier - France. Instituto de Tecnologia do Parana - TECPAR, Curitiba-Pr.

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Maurice Raimbault

ABSTRACT

The growth of 19 strains of *Rhizopus* from different species were evalued in environments with variable concentrations of CO_2. The results showed that most strains studied can tolerate concentrations of 5-10% CO_2. Even the radial growth of some strains is stimulated in such CO_2 concentrations. However, in concentrations of 16-20% CO_2, most strains have their growth significantly reduced. Some strains demonstrated to be able develop in anaerobiosis, though slowly.

Key Words : anaerobic growth, CO_2 effect, radial growth, *Rhizopus* .

INTRODUCTION

Rhizopus are microscopic filamentous fungi belonging to the Mucorales order. They are part of the phycomycetes class (primitive fungi) and of the Zygomycetes subclass (1,2). Many *Rhizopus* species present a great industrial interest. Particulary, they take part in the preparation of different fermented food, the production of organic acids and enzymes, the biotransformation of steroids, the protein enrichment of agricultural products, the biodegradation of pesticides and the bioabsorption of pesticides and the bioabsorption of heavy metals, radioactive materials, etc.

Recently, SOCCOL et al., (3,4) isolated *Rhizopus* strains capable of degrading starch to its natural state (without gelatinized) through culture in solid medium. The studies showed that the production of glucoamylase is 32 times more important in solid medium than in liquid medium.

Studies of influence of CO_2 on the growth and metabolism of filamentous fungi are still rare. TABACK et al., (6) reported an interesting review on the influence of the gaseous composition of the environment on the reproduction cycle of the fungi. ZADRASIL (7) demonstrated that CO_2 concentrations of 16-20% stimulate the growth of the superior fungi mycelium of *Pleurotus* genus. MUD-

GETT et al., (8) demonstrated that the production of enzymes by bacteria and superior fungi is stimulated by the enrichement of the environment with carbonic gas. SOCCOL (5), studying the action of CO_2 on the *Rhizopus* NRRL 395 metabolism, showed that the fungus produces high concentrations of L(+) lactic acid in aerobiosis and in the presence of a neutralizing agent in the culture medium ; however, in an environment rich with CO_2 its metabolism is deviated almost exclusively to the production of ethanol.

This work evaluates the effect of CO_2 concentration on the growth of varied *Rhizopus* species.

MATERIALS AND METHODS

Nineteen strains of different species were used (Table I). The strains came from different international collections provided by Dr. Kurtzman from Northern Regional Research Laboratory (NRRL - USA), Prof. G.L. Hennebert from the Mycotheque of Catholic University of Louvain (MUCL - Belgium) and American type Culture Collection (ATCC - USA).

Sporangiospores of the different strains tested were produced on potato dextrose agar (PDA) medium in Petri dishes after 8 days at 28°C. They were collected from the Petri dishes with a platinum rod and suspended in tubes containing 20 mL of sterile physiological saline solution with 0.01% Tween 80 and glass balls to extract as many sporangiospores as possible during shaking in a Vortex.

The study of *Rhizopus* spp. growth in environments with reduced amounts of oxygen and enriched with CO_2 were accomplished using Petri dishes of 9 and 13 cm in diameter and 1.5 cm heigth. The plates containing 15 and 25 ml of sterile PDA were surface inoculated in the central region with 150-200 sporangiospores with a Pasteur pipette. The plates were incubated at temperatures varying between 25 and 30°C during 24 to 168 hours.

The anaerocult P Merck Kit N° 13.807 was used for the studies of anaerobiosis. The anaerobiosis is formed by reaction of a mixture of iron powder, sodium carbonate and citric acid, wich rapidly fixes O_2 in the presence of water and releases CO_2, resulting an environement free of O_2 in the (anaerobiosis) in a atmosphere rich with CO_2. The anaerobiosis is controlled by change of the indicator from blue to white after 4 hours.

For obtaining different atmospheres with a reduced amount of CO_2 and enriched with O_2, the Anaerocult C Merck Kit N° 16.275 was used. The plates were put in an anaerobiosis jar. Concentrations of CO_2 in % (4 to 5, 8 to 10 and 16 to 20) were obtained in the jar, causing a variation of weight in the powder mixture of the kit, as well as in the volume of water of humidification. A perforation was done in the cap of the jar for the installation of plasma rubber cap so as to permit the determination of CO_2 concentration in the jar. The determination of the atmospheric composition ($\%CO_2$) in the jar was possible due to the use of a gas chromatograph from Delsi instruments provided with a thermal conductivity detector. Helium gas with a flux of 35 mL/min. was used as vector. The temperature of the oven was 60°C and tension in the detector adjusted to 160 mA. With the help of 2 mL syringe and needle, the jar cap was perforated and 1 mL gas sample was taken and manually injected in the chromatograph. The desired concentration of CO_2 in the jar was reached, on the average, after 4 hours of culture.

The growth of the fungi was estimated through two different methods : a) by measuring the diameter of the colony as a function of time using a vernier caliper; b) by weighing the dry biomass formed in the plates containing the growing mycelium ; aftewards the plates were taken to the microvane oven and heated to the complete fusion of the medium. The mycelium was separated from the culture medium by filtration in a Buchner funnel with 8μm filter paper previously weighed. After filtration the material was washed several times with boiling water for the total elimination of medium that could remain in the filter paper. The filter was dried at 105°C for 24 hours and the mycelial biomass calculated by the difference in weight. The results of each determination express the mean of three different plates. The maximum especific growth rate (μm) during the phase of exponential growth was calculated with the equation:

$$X_f = X_i\, e^{\mu m\, t} = \frac{\ln X_f - \ln X_i}{t_f - t_i} = \mu m$$

Where : X_i = value of biomass at the beginning of exponential growth (mg/dish)

X_f = value of biomass at the end of exponential growth (mg/dish)

t_i = initial time of exponential growth (h)

t_f = final time of exponential growth (h)

μm = maximum specific growth rate (h^{-1})

RESULTS AND DISCUSSION

Table I shows the effect of crescent CO_2 concentrations on the radial growth of different fungi species of *Rhizopus* genus. It can be obeserved that most *Rhizopus* strains can tolerate CO_2 concentrations of 5-10. Within these experimental conditions it is evident that strains have their radial growth slightly stimulated witch such CO_2 concentrations, as in the cases of *R. oryzae* 22580, *R. oryzae* 25976, *R. oryzae* 395, *R. oligosporus* 2710, *R. microsporus* 46436 and *R. microsporus* 9667. Figure I emphasizes such characteristic for *R. oligosporus* NRRL 2710.

Strain	Aerobiosis	4-5% CO_2	8-10% CO_2	16-20% CO_2	Anaerobiosis
R. formosa MUCL 28422	65.50	64.00	64.00	64.50	7.00
R. delemar NRRL 1472	49.50	48.50	49.25	34.50	2.70
R. oryzae MUCL 28168	78.50	73.50	70.00	33.00	4.50
R. stolonifer MUCL 28169	71.00	71.00	67.50	46.50	6.00
R. oryzae MUCL 28627	68.00	66.00	66.00	47.00	7.00
R. oryzae ATCC 22580	68.80	71.00	70.50	29.50	11.00
R. oligosporus ATCC 6203	71.00	66.00	71.30	45.00	5.00
R. sp NRRL 25975	84.00	72.00	71.00	34.00	7.50
R. circicans NRRL 1475	31.00	24.50	22.00	6.50	
R. arrhizus NRRL 1526	63.00	63.50	63.50	47.00	5.00
R. oligosporus NRRL 2710	30.50	31.50	30.50	18.00	4.00
R. oryzae NRRL 395	70.00	72.00	72.00	41.00	6.00
R. oryzae NRRL 25976	68.50	71.00	70.50	29.50	10.50
R. microsporus ATCC 46436	72.50	76.00	68.00	31.00	5.50
R. microsporus MUCL 9667	29.00	30.00	28.00	20.00	
R. stolonifer MUCL 28181	24.00	18.30			
R. delemar ATCC 34612	76.00	71.00	70.50	29.50	11.50
R. arrhizus MUCL 16179	72.00	72.00	67.00	24.50	5.00
R. arrhizus MUCL 28425	74.00	72.50	67.00	24.50	

ABREVIATIONS USED : ATCC = American Culture Collection (Rockville, Maryland, USA) ; MUCL = Catholic University of Leuven, Belgium) ; NRRL = Northern Regional Research Laboratory (U.S. Department of Agriculture, PEoria, Illinois, USA)

- No growth

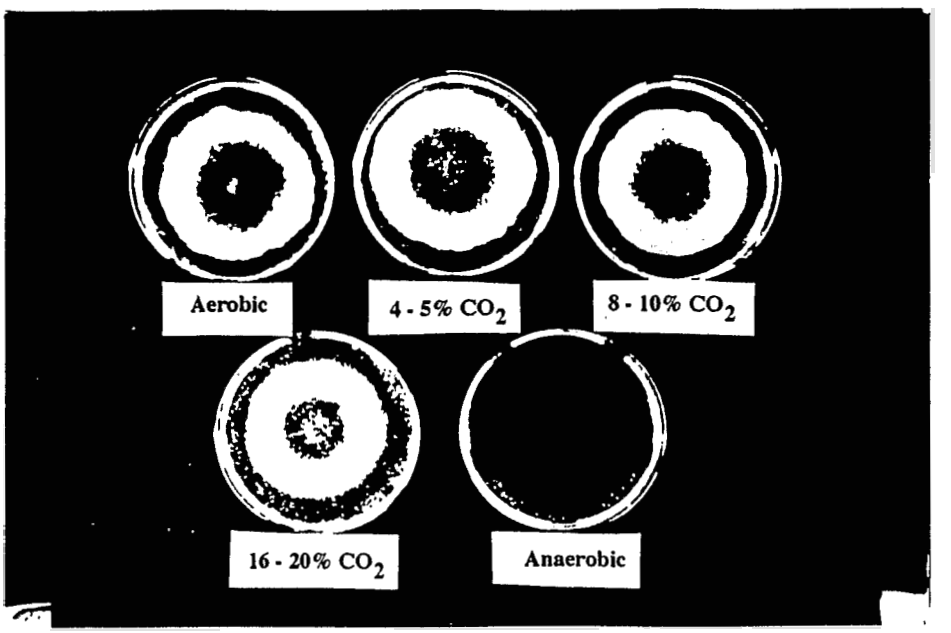

Figura I - Effect of different gaseous environements on radial growth of *R. oligosporus* NRRL 2710 after 72 hours at 30°C.

It was observed that concentration of 16-20% CO_2 lead to a reduction of growth in most strains studied ; only *R. formosa* 28422 strain mantains an elevated growth in such concentrations. It was also verefied that *R. stolonifer* strains is very sensible to CO_2 ; besides, its growth is inhibited when the concentration exceeds 5% .

Concerning the growth in environments without O_2 (anaerobiosis) and enriched with CO_2, it was noticed that most *Rhizopus* strains are capable of growing in anaerobiosis, although their growth is significantly reduced when compared to growth in aerobiosis or in environments with 5-10% CO2 concentrations. *R. circicans* 1475, *R. microsporus* 9667 and *R. arrhizus* 28425 strains do not grow in anaerobiosis.

Figure II.A shows the growth kinetics of *R. oryzae* NRRL 395 strain in aerobiosis as well as in different CO_2 concentration. It can be observed that as the CO_2 concentration increases, the weight of the biomass formed decreases, although the diameter of the colony is slightly superior for the 5-10% CO_2 concentrations (Table I). It is probable that an increase in CO_2 concentration makes the mycelium more aerial and less dense. Calculating the nepierian logarithm of the biomass formed per plate, it is observed in figure II.B that the fungus presents three distinct grwth phases, independent from CO_2 concentration of the environment. In the first phase with an ap-

proximate duration of 12 hours, it is observed a period of latency in which spores germinate and the biomass starts forming. In the sequence there is an exponential and linear growth period that lasts 36 hours. It is in the phase exponential growth that specific growth rate is maximal. In the third phase it can be seen a reduction of growth due probable fall in then nutrient concentrations

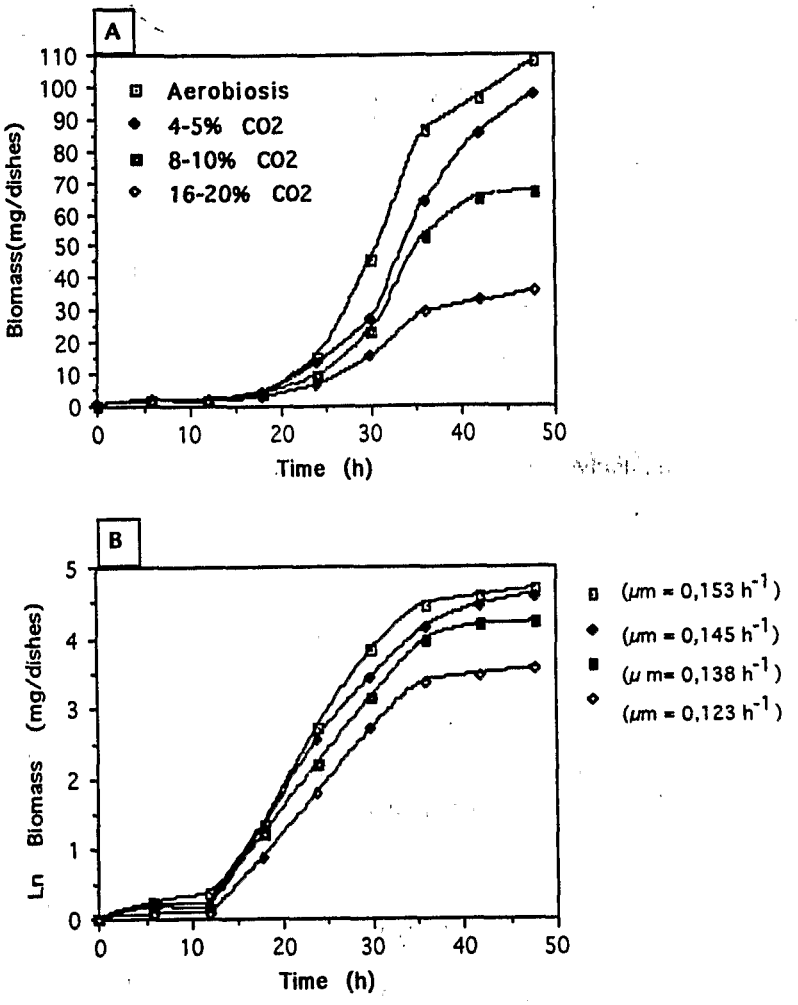

Figura II - Growth Kinetics of *Rhizopus oryzae* NRRL 395in different gaseous environments at 30°C. A : Evolution of the biomass formed; B : Evolution of ln of the biomass formed.

and also because the more extreme hyphas of the colony reach the edges of the plate. HSU et al., (9) studied the growth of 3 different *Rhizopus* species in plates and demonstrated that after germination of spores the colony formed goes through an important phase of exponential radial growth. The results of this work evaluating the biomass of the colony formed in the plate as a function of time confirm such hypothesis.

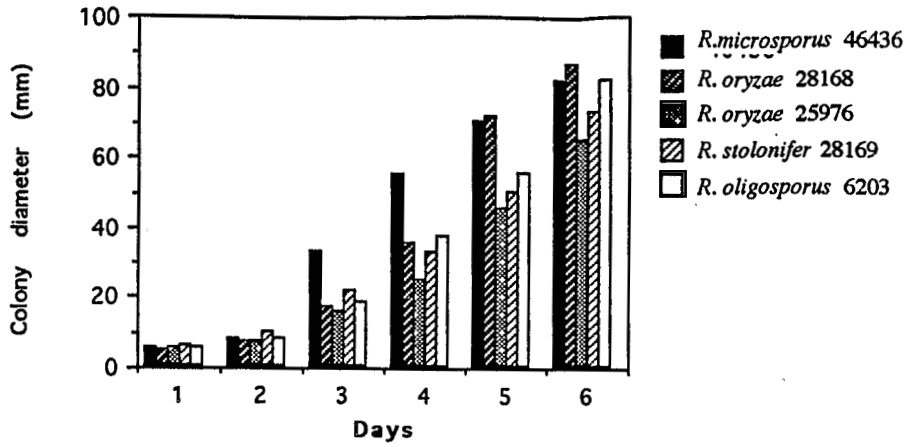

Figure III - Radial growth kinetics of different *Rhizopus* species cultivated in anaerobiosis at 30°C.

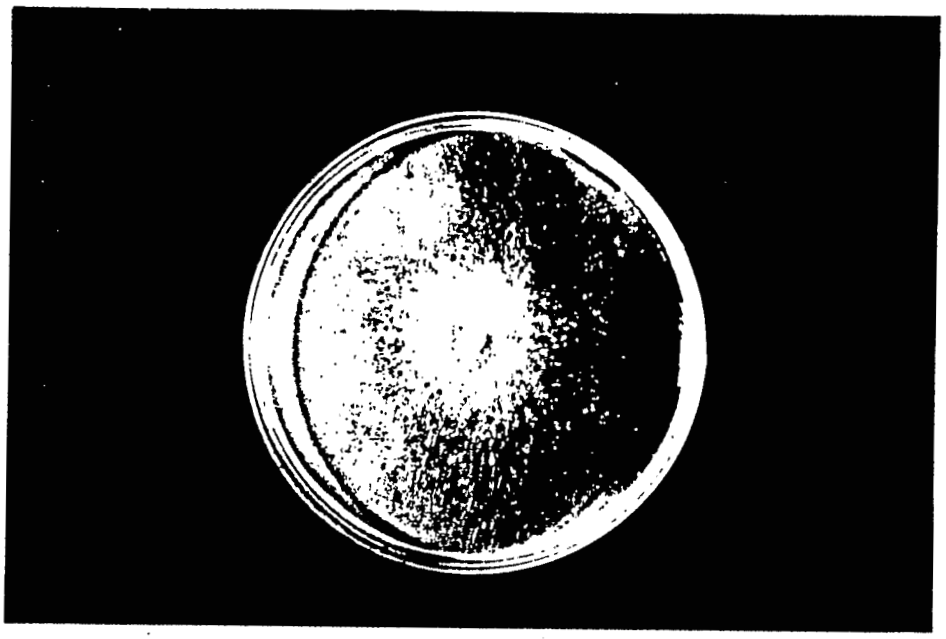

Figure IV - Characteristics of *R. oryzae* NRRL 25976 cultivated at 30°C during 7 days in anaerobiosis

The study of the radial growth kinetics of 4 different *Rhizopus* strains in anaerobiosis showed that some species of this fungus are capable of developing in environment without O_2 and rich with CO_2, although very slowly (figure III). It can be observed that during the first two dys the growth is practically constant ; after the third day a very important increase in growth rate occurs. figure IV shows the characteristics of *R. oryzae* NRRL 25976 after a week of culture in anaerobiosis. It is seen that in these conditions the fungus forms a very thin mycelium film, not much dense and aerial. HESSELTINE et al., (10) isolated some fungi species from starters of different fermented food from The East and observed that certain species of the *Rhizopus* , *Mucor* and *Amylomyces* were capable of growing in anaerobiosis.

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