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SOLID STATE FERMENTATION: ACID PROTEASE PRODUCTION IN CONTROLLED CO_2 AND O_2 ENVIRONMENTS

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ABSTRACT

The effect of the partial pressure of O_2 and CO_2 on the acid protease production in solid state fermentation by *Aspergillus niger* on wheat bran was studied. A fermentation system was used, which allowed on-line reactor measurements and continuous data acquisition of pH, temperature, gas flow, pressure drop and CO_2 production. Six paired combinations of CO_2 and O_2 concentrations were studied. The results showed a direct relationship between pressure drop, production of CO_2 and temperature increase. The pH evolution patterns were similar in all cases but different if the measurements were made on-line or on a liquid homogenate of the fermented substrate. Acid protease production was increased when the gas had 4% CO_2, (vol/vol), and it reached its highest level, a 43% increase over air, with a mixture of 4% CO_2 and 21% O_2. The protease production was strongly related to the mold metabolic activity as represented by the total CO_2 evolved.

KEY WORDS: Solid State Fermentation, *Aspergillus niger*, acid protease, wheat bran, controlled environments.

INTRODUCTION

A Solid State Fermentation, (SSF), is a process which involves the growth of microorganisms on porous solid substrates in the absence of free water. The required moisture is sorbed on the substrate matrix to assure growth and the biochemical activity of the cells without surpassing its maximum capacity to retain water. Growth and product formation occurs on the surface and/or the inside of the solid. This four phase system (insoluble support, water, biomass and air) makes non-destructive on-line monitoring more difficult than in the liquid fermentation. This constraint reduces the ability to effect control of the fermentation. Nevertheless, in SSF some parameters that affect growth or product formation, such as temperature, agitation, aeration rates and gas composition can be controlled throughout the fermentation.

The gas composition has been shown to be a relevant parameter for the production of enzymes by molds in SSF. The partial pressures of oxygen (pO_2) and carbon dioxide (pCO_2) influence the gaseous micro environment surrounding the microorganism and affect its regulatory system.[12] In SSF it has been suggested that the control of the gas composition may be used for regulating primary and secondary metabolism and could lead to process optimization with respect to product formation and energy consumption. Changes in the gas composition affects lignin and cellulose degradation by white-rot fungi growing on straw.[11] It has been reported,[4] for *Aspergillus oryzae* , that a-amylase increases gradually as the O_2 concentration changes from 12% to 40% when the total pressure was 1 atm. CO_2 levels of 2% were found to favor enzyme production under these

conditions. In the *koji* cultivation, O_2 and CO_2 concentrations of 40% and 1%, respectively, were optimal for glucoamylase and a-amylase production.[9]. Narahara et al.[13], reported that for the solid state culture of *Aspergillus oryzae* , controlling the CO_2 partial pressure between 2% and 5% during the early stage of growth (up to 30 h), increases the production of certain enzymes. The effect was more important for the acid protease than for the a-amylase. Desgranges and Durand [5], studied the influence of CO_2 on growth and cellulase, pectinase and amylase production by three fungi (two strains of *Aspergillus niger* and one of *Trichoderma viride*). It was found that growth was increased for the three strains under 10% CO_2 but the response in enzyme production did not show a clear-cut pattern, except for the polygalacturonase that was notably enhanced with 10% CO_2.

It is clear that there is great variability in the response of molds to changing gas composition. The problem is further complicated by the transfer between the gas and the mold which may be mediated by a liquid stagnant film on the support.[12] This effect may cause strong environmental differences between the mold that grows on the surface and that which grows in the interior of the support. To acquire more knowledge regarding the response to gas composition, the production of the acid protease of *Aspergillus niger* was studied. The fermentation was performed on an instrumented system with strict control on the environmental conditions.

MATERIALS AND METHODS

Microorganism.

Aspergillus niger ANH-15, an acid protease producer was used. The cultures were maintained on potato-dextrose agar. (PDA), (Bioxon, Mexico), at 6°C. Fungal spores were obtained from a 7 day culture grown on PDA at 33°C. The spores were collected with a 0.02% sterile solution of Tween-80 in water.

Substrate preparation.

Wheat bran was milled to reduce particle size. It was then sieved through a 20-40 mesh screen to obtain a particle diameter between 0.425 and 0.85 mm. The substrate was autoclaved for 15 min. at 121°C and then the pH was adjusted to 5.5. The bran was supplemented with $2.5x10^{-5}$ g $ZnSO_4$/ per gram dry matter, (gdm)and $4.0x10^{-4}$ $FeSO_4$ g/gdm.

Fermentation System.

A schematic diagram of the culture equipment used in this work is shown in Figure 1. Three different gases were used to obtain the mixtures needed in the experiments (1). The tanks contained 50% CO_2 in N_2 , 50% O_2 in N_2 and pure N_2. The gas composition and flow was provided by a mass flow controller (Dyna-blender Mod. 8250 Matheson Inst., USA). The gas flow was divided in two parts (A) one of them for a column fermenters system (8) and the second for the instrumented reactor (11). The instrumented reactor consisted of a glass cylinder with a diameter of 4 cm and a height of 13 cm with an acrylic jacket, this reactor was monitored throughout the fermentation. The column fermentors were those described by Raimbault et al. [15]. In the next step the flow for the column system was directed to a manifold (7) and distributed through needle valves, then the gas mixture was again humidified (9) at a constant temperature 30 °C before being fed to the fermentation column. From the manifold twelve separate column fermentations were performed. These fermenters were removed at different times for destructive (protease concentration from the enzyme activity, pH, sugars and humidity), analyses. The flow for the main reactor was humidified at 30 °C again before passing through the fermentation bed. The exit gas was desiccated (25) with silica and analyzed for CO_2 with an infrared analyzer (18). The temperature was monitored at the center and at the inlet of the reactor and at the bath. The pressure difference (23) was measured with a differential pressure transducer from two side arms at the bottom and the top of the reactor. A pH electrode (12) was placed in the middle of the reactor. A pump (14), recirculated the water from the bath through the jacket of the reactor. All the sensors (temperature, pH, CO_2, gas flow and pressure drop) were connected to the acquisition data system Mac 14 Cole Palmer, U.S., which was connected to a PC, (AT 286 Gama Computers, Mexico).

Fermentation Conditions.

The initial conditions were: pH: 5.5, moisture content: 50%, initial spore concentration: $2x10^{-7}$ spores/gdm. Temperature was controlled at 30°C. Aeration rate was 3.1 VVM (liters air/ liter reactor volume) for the instrumented bioreactor and the column fermenters system. The packing density was 0.3 g/cm^3 reactor.

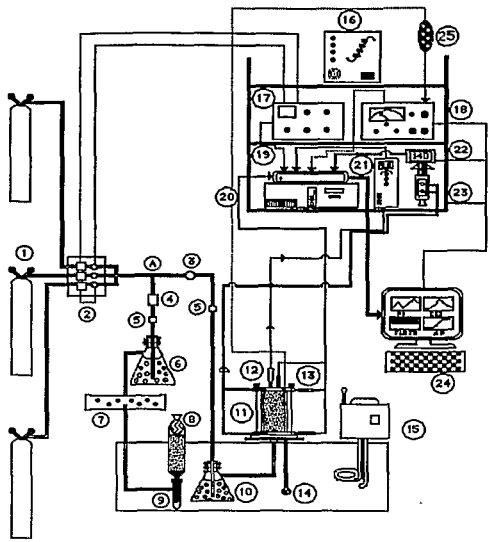

FIGURE 1. (1) Tanks. (2) Transducer and control valve., (A) Gas flow division, (3) Pressure regulator. (4) Flow valve. (5) Check valves. (6) Humidifier. (7) Manifold. (8) Fermentation columns. (9) Column humidifier. (10) Water bath. (11) Reactor. (12) Potentiometer electrode (13) Thermocouples. (14) Recirculation pump. (15) Thermoregulator. (16) Power source. (17) Mass Flow controller. (18) CO_2 gas analyzer. (19) Acquisition data card. (20) Hard disc. (21) Potentiometer, (22) Digital pressure meter. (23). Pressure transducer. (24) Computer monitor. (25) Silica gel desiccate.

Analytical procedures.

Enzyme extraction. One gram wet matter (gwm) was diluted with 10 ml tap water and homogenized with an Ultraturrax for 2 min. at maximum speed and filtrated through Whatman filter paper #2 and then diluted again 2:5 with a lactate buffer (0.05 M, pH 2.5).

Protease concentration determination. The protease concentration was determined from the enzyme activity of the supernatant and measured by the Anson method using casein as substrate.[2] It is reported in units/gram dry matter (U/gdm). One unit is 1 μg of tyrosine liberated per minute at pH of 2.7 and at 40°C.

Sugar determination. An acid hydrolysis was performed. A 2.5-3 g of a dried sample, was washed while stirring with 50 ml of cold H_2O for an hour, filtered and again washed with 250 ml of cold H_2O. The insoluble residue was heated for 2.5 h with 200 ml of H_2O and 20 ml of HCl, (sp. gr. 1.19) with reflux. The hydrolyzate was refrigerated, neutralized with NaOH and discolored with the Carrez solution.[14] Sugar measurements were made with the phenol sulfuric method.[6]

pH. pH was measured by two methods. In the instrumented reactor by direct contact of the pH probe with the fermenting mass. Also it was measured from the columns by mixing 2.5 g of the fermented sample with 25 ml of destilled. The mixture was homogenized for 5 min and the pH measured.

Water content. The water content of the medium was obtained by drying at 110°C for 24 hours to constant weight.

CO_2 evolution. The gas effluent from the reactor was dried in a silica gel desiccator and then passed continuously to an infrared CO_2 gas analyzer, (model 864, Beckman Inst., USA). CO_2 production rate was obtained by multiplying the CO_2 by the volumetric flow.

Pressure drop. Pressure drop ($\Delta p/\Delta z$) across the fermentation bed from the instrumented reactor was measured with a pressure transducer (mod. 7350-30, Cole Palmer, USA). Silica gel traps were used

to protect the instrument. $\Delta p/\Delta z$ is reported as mmH_2O/ mm reactor.
Temperature. Temperature was measured continuously at different points in the instrumented fermenter with type K thermocouples. Temperature increase is reported as the difference between the temperatures at the geometric center (Tc) and the gas inlet (Te).

RESULTS AND DISCUSSION

The results are presented in three parts. The first one describes the evolution of the fermentation with atmospheric air (21% O_2 and 0% CO_2) as control. In the second part protease production, CO_2 production, temperature gradient, pressure drop and the total sugars concentration are analyzed as a function of six different gas environments. Finally, protease production is related to the mold response under the conditions studied.

Evolution of the fermentation under atmospheric air conditions.

Figures 2 through 5 show the evolution of the fermentation of *A. niger* under (21% O_2, 0% CO_2). The protease concentration increased gradually until a maximum value of 52 U/gdm was attained after 36 h of fermentation time, a steep decrease was then observed. Sugar consumption was slow during the germination phase but increased as growth proceeded, (Figure 2). After 40 h of fermentation 37% of the total sugars remained in the medium. Apparently the process was not limited by the carbon source.

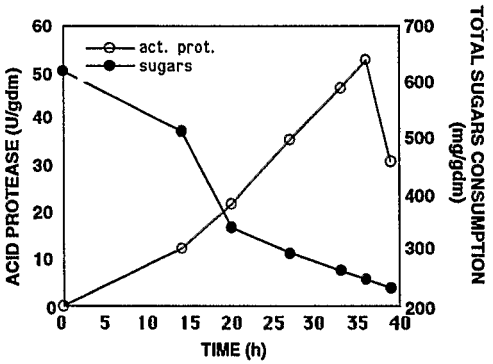

FIGURE 2. Protease concentration evolution and sugar consumption

In Figure 3 the CO_2 production rate (CDPR) and the temperature difference as a function of time are represented. Both measurements are directly linked to the metabolic activities of the mold and follow the same pattern in the fermentation. Both have maximum values between 14 and 15 hours from inoculation. Maximum values were 7 ml CO_2/h/gdm and 4.5 °C.

Figure 4 shows the variations in pressure drop ($\Delta p/\Delta z$) and the integrated value of CO_2 obtained from the data in Figure 3. The $\Delta p/\Delta z$ has been related to the free space occupation by the mold.[3] For the case of the wheat bran, the increase in the $\Delta p/\Delta z$ cannot be attributed exclusively to the biomass increase because there is also substrate utilization which compensated partially for the reduction in the flow area. In the case of the growth of *A. niger* on Amberlite IRA-900, a synthetic resin, the mold grows only on the surface and the inerparticular space and the $\Delta p/\Delta z$ attained a fifteen fold value as compared with wheat bran. The increase in the $\Delta p/\Delta z$ was linked to hyphae elongation (by microscopic observations) up to about 15 hours. After 27 hours conidiophore formation is reflected in a new steep increase in the $\Delta p/\Delta z$. The integral CO_2 does not reflect this morphological change of the mold.

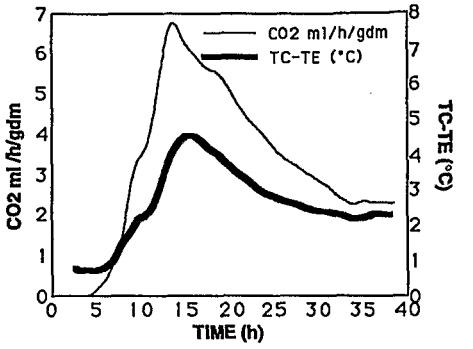

FIGURE 3. CO_2 production rate and temperature difference as a function of time.

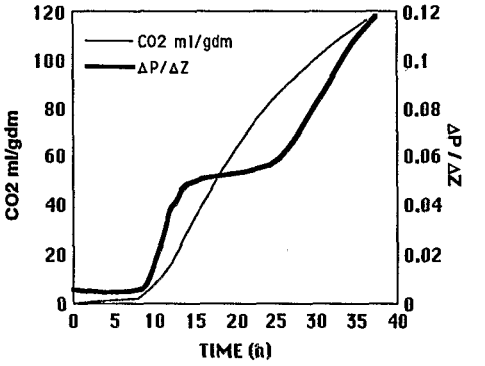

FIGURE 4. Evolution of pressure drop and the integrated value of CO_2 with time

Figure 5 shows the evolution in on-line and external pH measurements. In general the variation of pH by the growth of microorganisms is linked to the ionic balance established by substrate uptake and product formation. The pH, as measured from the homogenate, reflected the overall ionic balance and was strongly related to the buffer capacity of the substrate. The on-line response showed steeper variations. Microscopic observations of the pH electrode probe showed that the hyphae grew on the porous glass of the tip. The pH reflected, thus, solely the micro environment of the mold. Despite the fluctuations, the response proved to be highly reproducible.

Evolution of the fermentation under different CO_2 and O_2.

The evolution of the fermentation was studied under five different sets of CO_2 and O_2 gas mixture, besides atmospheric air conditions: (4% CO_2 and 21% O_2); (8%CO_2, 21% O_2); (0% CO_2, 10% O_2); (0% CO_2, 5% O_2); and (4% CO_2, 10% O_2).

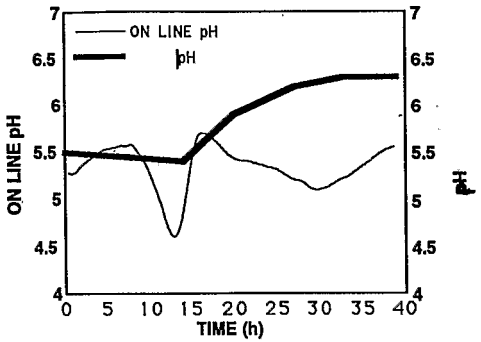

FIGURE 5. On - line and external pH measurements during fermentation process

In Figure 6 it can be observed that an increased protease production was obtained under 4% CO_2. For a (4% CO_2, 21% O_2) gas mixture, protease concentration increases up to 74.3 U/gdm at 36 hours which is 43% higher than in air. Initial production of the enzyme was favored by low O_2. Higher CO_2 provoked a steeper loss in enzyme concentration after 28 hours. These results are in accordance to those reported by Narahara. [13]

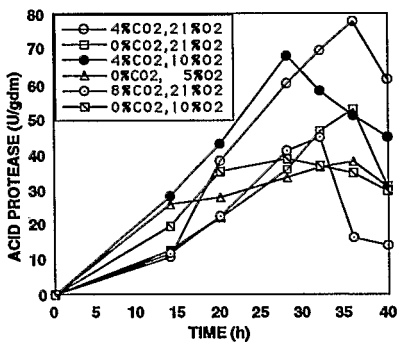

FIGURE 6. Effect of CO_2 and O_2 on protease production.

CO_2 evolution is related to the overall metabolic activities of the mold. It reflects growth, respiration and lysis. Figure 7 describes the integrated CO_2 production. The two experiments with a 4% CO_2 produced much higher amounts of CO_2 than the others. The maximum CDPR was 18.5 ml/h/gdm for (4% CO_2, 10% O_2). An increase of 46% in total evolved CO_2 was found on the (4% CO_2, 10% O_2) case as compared with air.

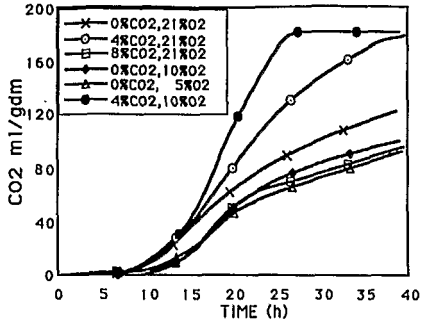

FIGURE 7. Accumulated CO2 production under different CO_2 and O_2.

A similar pattern, Figure 8, was observed for the consumption of total sugar: the two experiments with a CO_2 of 4% utilized more substrate than the other experiments. For this case no significant difference was observed between 10% and 21% O_2 with 4% CO_2. An increment of 30% uptake in sugar consumption was observed for both cases with respect to air. After 32 hr sugar conversion yield to CO_2 reached its maximum with an environment of (0% CO_2, 21 %O_2), where it reached a value of 0.26 mg CO_2/mg of sugars. The yield with a mixture of (4% CO_2, 21 %O_2) and (4% CO_2, 10% O_2) was 0.24 mg CO_2 produced/mg of sugars.

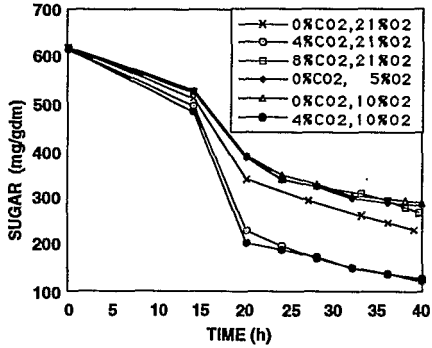

FIGURE 8. Total sugar consumption under different CO_2 and O_2.

The evolution of the $\Delta p/\Delta z$ for the six situations studied is depicted in Figure 9. Except for one case, the $\Delta p/\Delta z$ started to increase from 9 hours which corresponded to the beginning of mycelial growth. The evolution did not show the same pattern as the sugar consumption or the CO_2 evolution. This is particularly true for the (4% CO_2, 10% O_2) case The changes in morphology that may be brought by different environments plays a very important role on the response of the $\Delta p/\Delta z$.[3]

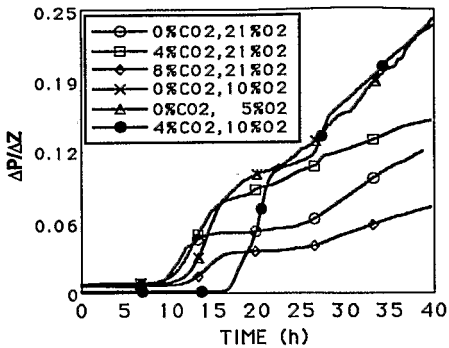

FIGURE 9. Evolution of the Pressure Drop for the six experiments

Figures 10 and 11 show the evolution for the on-line and the externally measured pH. Figure 10 shows a similar trend in the pH variation for the six experiments. The pH showed first a steep decrement (up to 0.25 pH units/h) at about the same time that the spore germination ceased (≈8 hours). Then the pH increased up to 18-20 hours, which corresponded to the time of the reduction in the growth rate. A second reduction and then an increase was observed thereafter. The first pH change was less marked when the pH was measured after homogenization of the sample, while the second change was not observed in the homogenate. While it is difficult to interpret the variations in the on-line pH measurement, it was very interesting to observe the repeatability of the patterns, even with the variations in the gas composition. Although the on-line pH could not be correlated with the enzyme production it may be used as another indicator of the development of the fermentation.

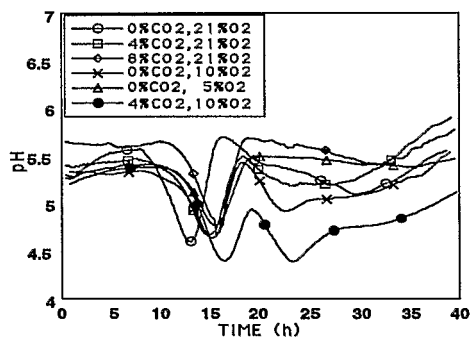

FIGURE 10. pH value registered with on-line sensor

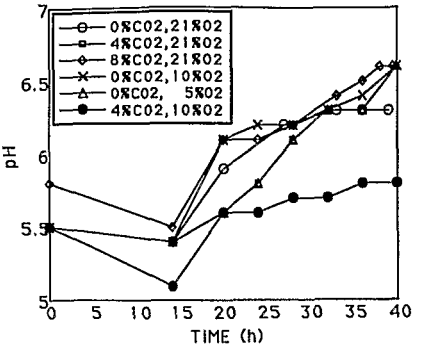

FIGURE 11. pH measurement in the homogenate

The increase in the temperature in the reactor is another indicator of the metabolic activities of the mold, (Figure 12). It is linked to the heat evolved by respiration but it is also affected by heat transfer characteristics of the system. Qualitatively, the temperature increase reflects higher heat liberation for the case when the gas had 0% CO_2 (which is inverse to the sugar consumption pattern), suggesting that 4% CO_2 may favor better substrate utilization.

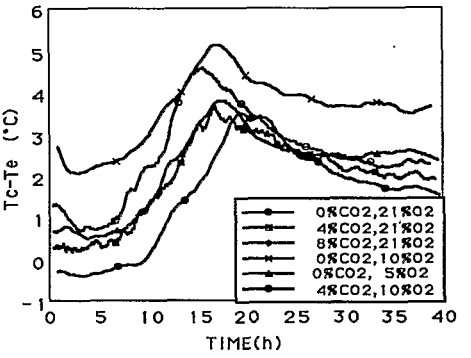

FIGURE 12. Temperature gradient as related to different gas environments.

Relation between the protease concentration and the CO_2 evolution.

Figures 13 and 14 show the relation between the total production of CO_2 and the maximum protease concentration for the conditions of CO_2 and O_2. studied. These figures show the close relation between the total production of CO_2, which is related to the total metabolic activities of the mold, and the production of the enzyme. The linear relation between the two variables is depicted in Figure 15. This was the best correlation found between the maximum protease concentration and the other studied variables. This relation can be expressed empirically as:

Max. protease conc. = 0.3686 (CO_2 total production) + 5.15. (r^2= 0.951)

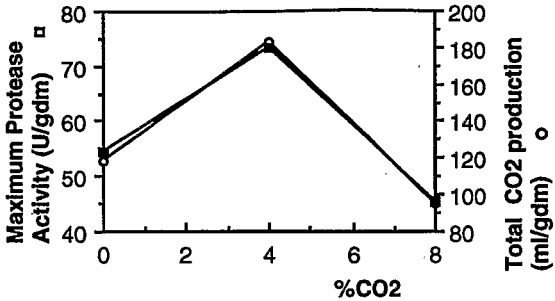

FIGURE 13. The total production of CO_2 and the maximum protease concentration as function of CO_2.

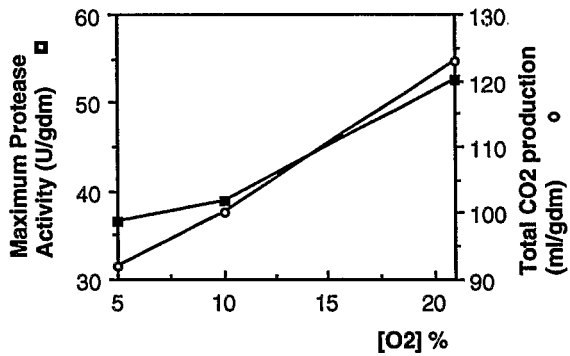

FIGURE 14. Total production of CO_2 and maximum protease concentration as function of O_2.

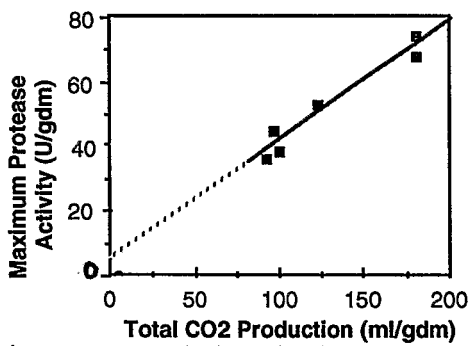

FIGURE 15. Maximum protease production as function of total CO_2

CONCLUSIONS.

The results showed that modification of the gaseous environment had a strong influence on mold growth, sugar consumption, CO_2 production, maximum protease concentration and pressure drop. Of the above mentioned , CO_2 production and maximum protease concentration showed a clear relation, suggesting that the enzyme production might be linked to the overall metabolic activity of the mold. While low O_2 favored higher initial enzyme production, maximum final concentrations were obtained at a gas composition of 4%CO_2 and 21% O_2.

The on-line measurement of pH showed no direct relation to the pH analysis on the homogenate. This was due to the growth of mycelia on the tip of the probe. Nevertheless the response was reproducible and can be used as an indicator of the evolution of the fermentation. The continuous measure of the $\Delta p/\Delta z$ correlated with the initiation and development of growth, the stationary phase and the initiation of sporulation. The quantitative changes were not correlated to the protease production.

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