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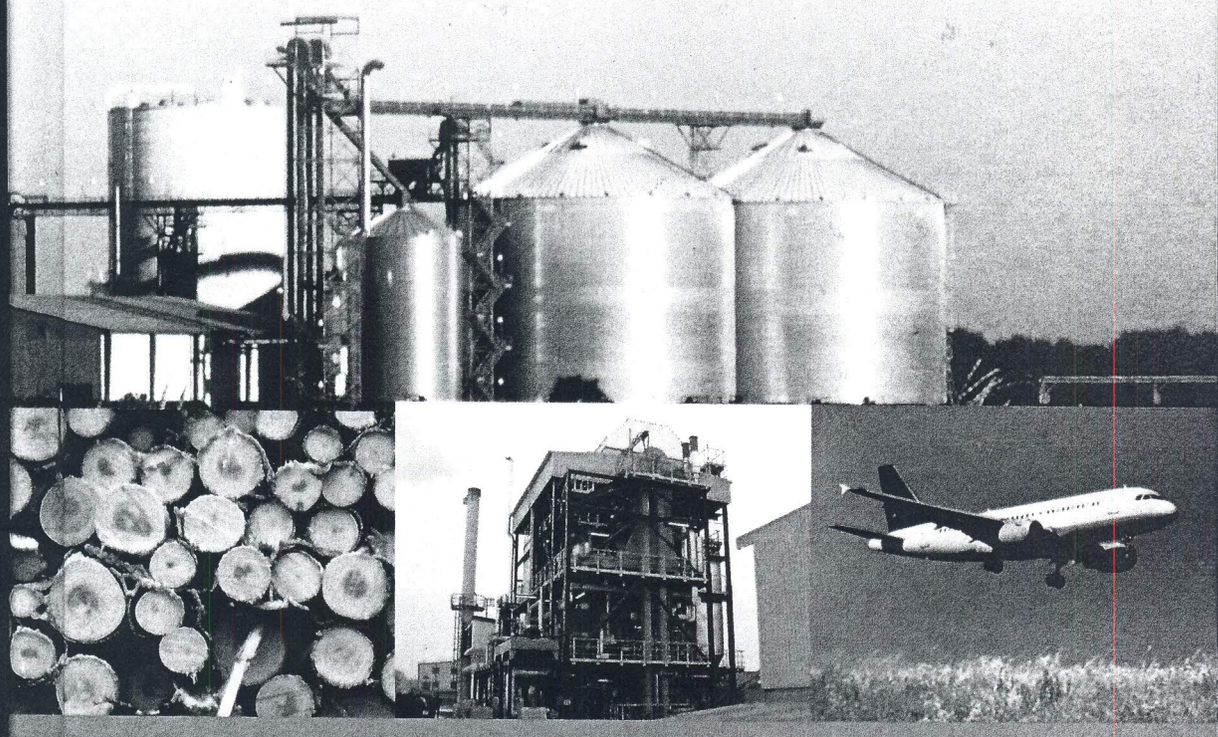

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SOLID STATE FERMENTATION PROCESS: AUTOMATION AND INSTRUMENTATION AIMING FOR THE CHARACTERIZATION OF CELLULASE PRODUCTION

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ABSTRACT: Production of enzymes by means of Solid State Fermentation (SSF) processes is one of the themes with regard to the renewed interest that has occurred in the last years mainly due to the search for more efficient Ethanol production, usually called Second Generation Ethanol (SGE) production process. Despite the increasing number of publications in the area it is difficult to draw general conclusion from the presented data. By the other hand, SSF processes performed in bench scale appear to be superior when compared to Submerged Fermentation processes in several aspects, but the difficulties frequently arise when is tried a scale up experiment. The increased interest on the SGE processes has led to the developing of more efficient SSF-based processes aiming to increase Cellulase production. Many different fungi species and solid substrates have been used and the productivity reported. The lack due to the absence of standardization gets difficult to properly compare these results. In this work it is shown that automation and proper instrumentation applied to SSF processes can be a reasonable tool to overcome these difficulties. By automatically measuring and controlling some parameters such as air humidity, air flow, and air temperature it is possible to analyze many different conditions of fungal cultivation aiming for optimal growing conditions. The proper analysis and understanding of these experimental results can be very useful during the scale-up design. The developed system is composed of control and data acquisition of air flow, humidity, and temperature that can be set automatically in different conditions. The resulting conditioned air flows through the SSF reactor and the CO_2 concentration is also measured at the output. All parameters and adjusts are performed by hardware and software commanded via personal computer. As example of Cellulase production it was chosen the Endoglucanase activity production compared in cultivation under different physical conditions.

INTRODUCTION

Solid State Fermentation (SSF) refers to the growth of microorganisms on solid materials without the presence of free liquid [1]. The concept of using SSF is probably the oldest method used by man to make microorganisms work for him [2], and in [3] is proposed a question: is the SSF a new challenge for a very ancient technology? In the last 20 years, there has been a resurgence of interest in many aspects of SSF, including the biochemistry, physical chemistry, SSF process engineering, and design of SSF bioreactors. However, because of the difficulties with process control and scaling-up, the development of SSF has been slow compared to Submerged Fermentation (SmF) [4].

SSF involves heterogeneous interactions of microbial biomass with moistened solid substrate. The microbial biomass inside the substrate matrix and on the substrate surface consumes substrate and secretes metabolites and enzymes. As there is no convective transport in the solid mass, concentration gradients are needed to supply the substrates and to remove the products. Gradients in the concentrations of substrates and products may cause local differences in metabolic activity. Similarly, gradients in the concentrations of inducers or repressors may affect enzyme production. These gradients are the most typical difference between SSF and SmF and can therefore be

assumed to contribute to the observed differences in gene expression, metabolism, product spectrum, and process efficiency between SSF and equivalent SmF processes [4].

Although SSF has currently yielded few new industrial applications e.g. pectolytic enzyme production it has a promising future, in particular for the valorization of agro/industrial byproducts, solid waste biodegradation, bioremediation of organic pollutants in soils, and reduction of atmospheric pollution by biofiltration. The main parameters to be measured and controlled in SSF processes are: temperature, homogeneous aeration, pH, and water bed content [5]. In [6] is clearly demonstrated that SSF, as long as the cultivation volume is kept to a liter scale, represents the superior technology with regard to process productivity, product quality and processing costs. However, SSF is difficult to scale up because of the build-up of gradients in temperature, pH, moisture, oxygen, substrate and inoculum.

SSF reproduces the natural microbiological processes like composting and ensiling. On one hand by utilizing the low cost agricultural residues SSF adds on to economic feasibility of the process and on other hand it solves the problem of its disposal which otherwise cause pollution [7]. Moreover, SSF presents certain singular challenges, including: sensors for the on-line measurement of key variables, such as water content and biomass, are expensive and difficult to use; a complex arrangement of four phases exists in SSF (biomass, solid, liquid, and gas), whose processes within and among them are not entirely understood; and limited capacity for dissipating metabolic heat, which generates hot spots inside the solid bed that harm the microorganism [8]. More about difficulties in measure some fundamental parameters during an SSF process (on-line or even off-line) can be viewed in [5, 9] and a considerable number of different types of reactors is shown in [3].

It is widely accepted that Cellulases secreted by fungi consist of three major components: 1,4-β-D-Glucan Glucanohydrolases (Endoglucanases, EGs, EC 3.2.1.4), 1, 4-β-D-Glucan Cellobiohydrolases (Exoglucanases, CBHs, EC 3.2.1.91) and β-D-Glucoside Glucohydrolases (β-Glucosidases, EC 3.2.1.21). The whole Cellulase activity is often represented by FPA including synergistic actions of the three parts. The single Endoglucanase activity is often represented by Carboxymethyl Cellulase (CMCase) activity [10].

The recent interest on biofuels, and more specifically on Second Generation Ethanol (SGE) has called a lot of attention on how to produce Cellulases and to ensure the economic viability of the enzymatic hydrolysis routes. This is a new challenge with respect to SSF process, even taking into account the lower cost of Cellulase production compared to SmF process [11].

In resume, SSF processes performed in bench scale appear to be superior when compared to Submerged Fermentation processes in several aspects, but the difficulties frequently arise when is tried a scale up experiment. The increased interest on the SGE processes has led to the search for more efficient SSF-based processes aiming to increase Cellulase production. Many different fungi species and solid substrates have been used and the results with regarding to the productivity reported. However it is difficult to compare the experimental results due to the absence of standard procedures. It is very known that proper aeration is an important condition during SSF processes and it would be useful a system that could be commanded in different set ups along the SSF process, measuring the main physical parameters, and reporting all data at the end of the process.

Based on the above description it is proposed in this work an automated system that performs SSF processes. By automatically measuring and controlling some SSF parameters such as air humidity, air flow, and air temperature it is possible to analyze the enzyme production under different fungal cultivation conditions. The system flexibility permits to impose different values of air flow, temperature, and humidity set points. Furthermore the substrates are prepared with initial moisture previously chosen. Enzyme production can be extensively analyzed but it is shown data with respect to the Cellulase production, specifically the Endoglucanase activity.

Experimental Set up

Basically SSF processes need a conditioned clean air forced into the substrate. Controlling the parameters of the resulting air flow that feeds an SSF process is the first step to do it. This means

to keep desirable values of flow, humidity, and temperature according to the operator. One precise way to do this is to balance the ratio of dry air and wet air being the sum of both air flows equal to the desired final value. All air lines must be kept under the same temperature, including the air dryer and wetter. This is sketched in Figure 1. CF1, and CF2 are flow controllers fed by one line of wet air, and another of dry air. Both controllers have outputs (M1, and M2) indicating the measurement of the flow value, and inputs (C1, and C2) that receive control signals. The sum of both flows resulting from the commanded signals gives the desired flow and humidity chosen by the operator. At the input of the Reactor there is a combined sensor of temperature and relative humidity, RHT1, where are read the values M1, and M2. The control loop is closed with the help of a personal computer, with a signal acquisition board, and a software component that translates the signals to a graphical interface calculating the values and delivering the output signals (C1, and C2). In resume, the system reads the signals M1, M2, M3, calculates the ratio of both flows and delivers the signals C1, and C2. The signal M4 is used to monitor the resulting air temperature.

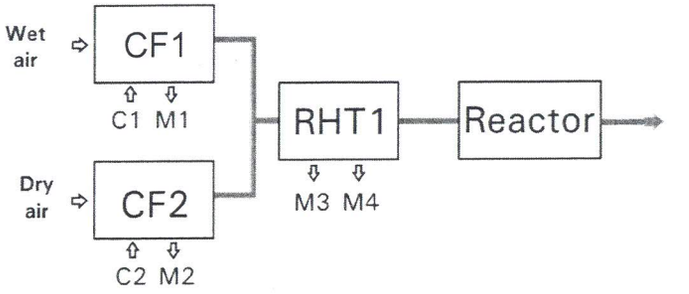

Figure 1. Diagram of the air lines that feed the reactor.

The software that permits the integration between the computer, acquisition board, and sensors was programmed taking into account the error between the desired set point and the actual measurement obtained in M1, M2, and M3. This is a classical closed loop control configuration. Commercial devices can be used in this system, like mass flow controllers, relative humidity, and temperature sensors. It was also used a signal acquisition board with 4 analog outputs, and 16 analog inputs with 16 bits of resolution each one. I was used the software LabView® (National Instruments) and the control law applied to the loop was the classical PI (Proportional plus Integral) controller, easily implemented. The SSF reactor recipient is made of one or more small columns filled with approximately 16g of substrate each one. The conditioned air flow is forced in the bottom side of the column. At the top of the column was installed a CO_2 sensor whose output is connected to another input channel of the acquisition board. This signal is useful to analyze the biological activity by means of cell respiration.

The microorganism used in this study was a strain of *A. niger* from Embrapa Food Technology collection, Rio de Janeiro, Brazil. The culture was kept on dry sand under freezing conditions (-18 °C). Microorganism activation was carried out in a basic medium agar slants incubated for 7 days at 32 °C. After this period, conidia were harvested by adding 10 mL of 0.1% Tween-80 to the slant [12].

Fermentation experiments were carried out at 32 °C for 70 h using wheat bran as solid substrate. These conditions were selected based on previous studies (unpublished data). Substrate moisture can be adjusted with ammonium sulfate solution being balanced according to each experimental condition. After each fermentation period, the solid medium was transferred to Erlenmeyer flasks and the enzymes were extracted by adding 0.2 mol/L sodium acetate buffer at pH 4.5 into each flask. The suspension was stirred at 120 rpm for 1 h at 32 °C and the enzymatic solution was recovered by filtration. The recovered enzyme extracts were stored at -18 °C for further analysis. Endoglucanase activity was measured using an assay based on the Ghose methodology. Briefly, a volume of the appropriately diluted enzyme extract was incubated at

50 °C for 10 min with a 1% CMC (Sigma, USA) solution prepared in 0.050 mol/L citrate buffer at pH 4.8 as substrate. One unit of Endoglucanase activity corresponds to 1 μmol of Glucose released per minute at pH 4.8 and 50 °C.

Results

First experiments were chosen to know the automatic system capability to keep the desirable conditions of air flow rate, relative humidity, and temperature. Figure 2 shows the results due to two different set points of relative humidity: 70% and 40%. In each set point it was chosen arbitraries lower values as starting points. Both air lines were immersed in water bath with controlled temperature. It can be seen in the figure that except by an overshoot of 2 % in the relative humidity M3, the responses are precise and stable along the time. In order to register the dependence of the resulting air temperature due to the variations existing in the inlet air temperature delivered by the air compressor it was used another analog channel and one more temperature sensor. That variation is seen in Figure 2 as Tin, while M4 returns values around 35 °C ±1°C. The total air flow was chosen to be equal to 500 ml/min. and was kept constantly inside the error range of each flow mass controller (± 1.5 %). The resulting air flow could be equally divided in 16 outputs to feed one or more column reactors.

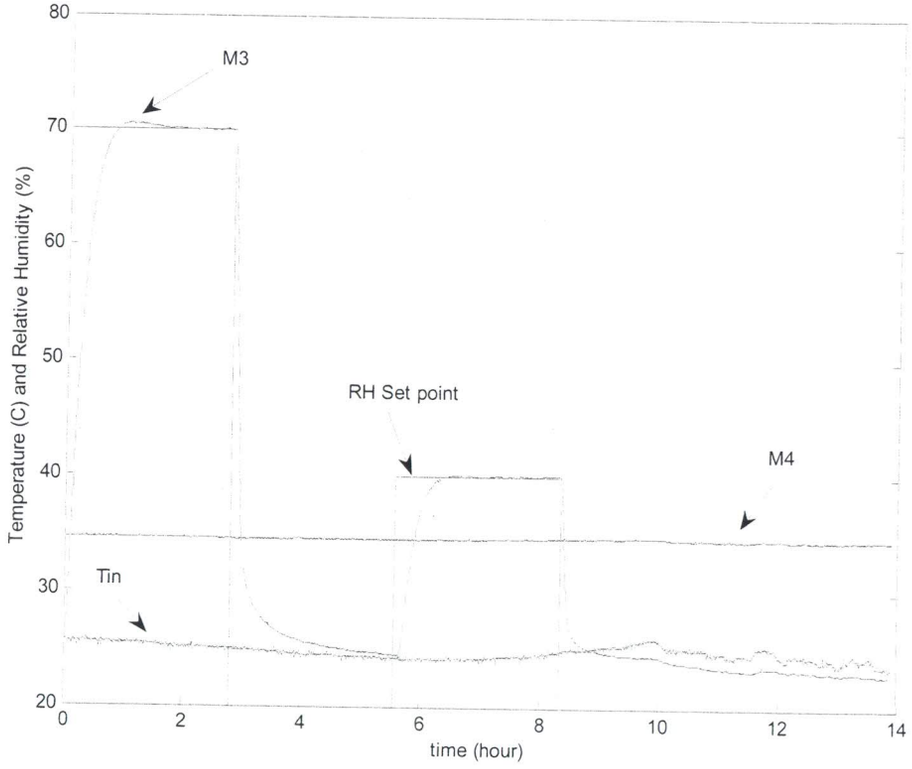

Figure 2. Relative humidity response due to two different set points (70% and 40%) and dependence of the air temperature M4 in relation to the compressor air temperature Tin.

The behavior of the automated system showed robust characteristics along the time with respect to the stability and performance. Different set point values could be imposed and kept during several hours. The next step was to apply the resulting conditioned air into the fermentation reactor. It was performed a repetition experiment. Two flasks were equally prepared: same substrate weight

(16 g), initial moisture (70%), and inoculum concentration. Both of them were blown with the same air flow condition: 24 mL/minute, with 70 % of humidity. In Figure 3 is shown the resulting CO_2 concentration measured at the output of each flask indicating that the biological activity is very similar concerning to the microorganism respiration. Endoglucanase activity values were found to be 53.5 U/g of substrate in the case of the lower curve in the figure, and 56.3 U/g of substrate in the case of the upper curve. The relationship between the produced CO_2 mass and the substrate mass was equal to 0.088 g of CO_2/g of substrate in the case of the lower curve, and 0.099 g of CO_2 / g of substrate in the case of the upper curve in the figure.

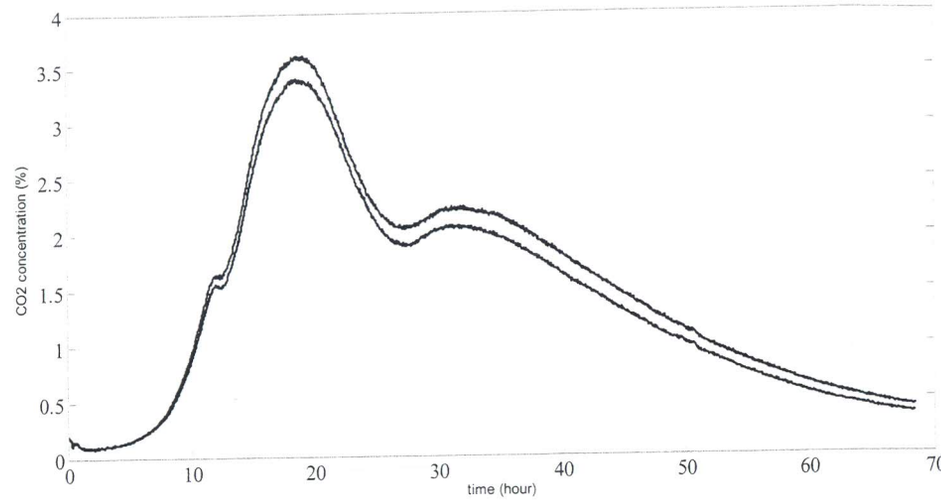

Figure 3. CO_2 concentration measured at the output of 2 flasks during the fermentation experiment. Both flasks were prepared with the same initial condition and were blown with air flow equal to 24 mL/minute, with 70% of relative humidity.

Another experimental test was made to investigate the Endoglucanase activity values when it is imposed different conditions of air flow and initial substrate moisture. In the Figure 4 is shown the responses of the CO_2 concentration measured at the flask output during two fermentation experiments performed at the same time. Both flasks were prepared with the same substrate weight (16 g), initial substrate moisture (80%), and inoculum concentration but the air flow blown in one flask was equal to 12 mL/min, and in the other was equal to 36 mL/min, and relative humidity equal to 60%. It can be seen in the figure that higher flow rate caused lower biological activity (curve 2) and vice versa (curve 1). As expected the Endoglucanase activity values were found to be 66.5 U/g of substrate in the case of the curve 1 and, and 54.2 U/g of substrate in the case curve 2.

The experimental conditions could be varied with respect to the aeration in different assays as is shown in Table 1. In each fermentation was measured the Endoglucanase activity and the produced CO_2 mass regarding to the substrate mass. Columns 1 and 2 in the table show the relative humidity of air, and the initial substrate moisture, respectively. All the fermentations started with the same inoculum concentration. It can be seen that initial substrate moisture can be relevant when compared with the same air flow rate despite of the similar CO_2 production (experiments 2 and 4). In all the cases an excessive air flow rate caused a lower Endoglucanase activity production. All experiments showed a CO_2 concentration peak around 20 hours of fermentation indicating similar behavior related to the curves showed in Figures 3 and 4. It also can be seen the same behavior commented above: lower aeration, higher Endoglucanase production, and vice versa.

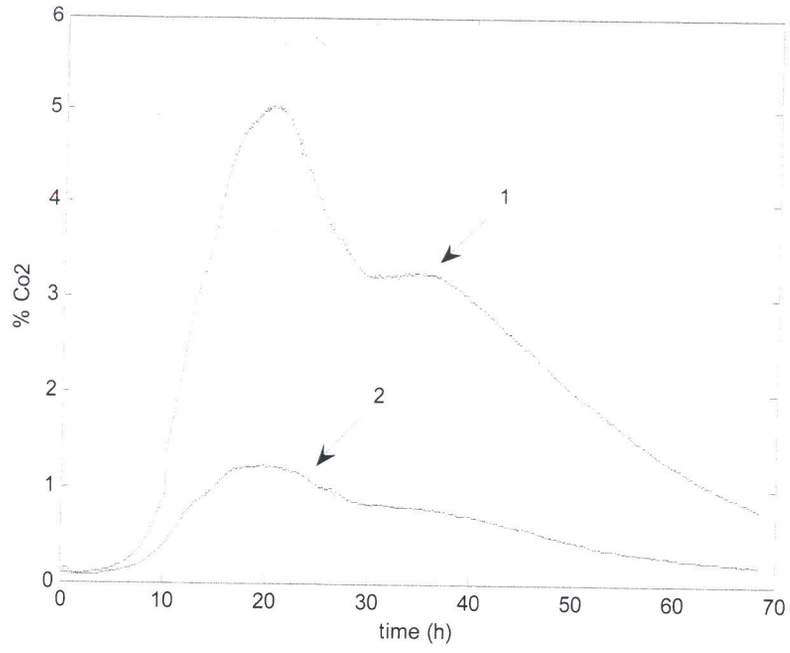

Figure 4. Responses of CO_2 concentration. Curve1: air flow rate of 12 mL/min. Curve 2: air flow rate of 36 mL/min. Both experiments were initiated with inlet air relative humidity of 60% and initial substrate moisture of 80%.

Table 1. Fermentation experiments under different physical conditions.

Experiment	Air relative humidity (%)	Initial substrate moisture (%)	Air flow rate (mL/min.)	Endoglucanase activity (U/g substrate)	CO_2 mass/ substrate mass (g/g)
1	60	60	36	43.6	0.128
2	80	80	12	66.0	0.171
3	80	80	36	58.4	0.164
4	80	60	12	44.0	0.169

CONCLUSIONS

It was shown in this work that a not so complex system can be useful for developing strategies with respect to Cellulase production process characterizations. The automated system presents enough stability and performance robustness along several hours, keeping the set points adjusted according to the operator. It could be seen that results on a simple fermentation experiment show a reasonable similarity in a repetition test. In physical systems this can be trivial but the same cannot be said about biological systems interacting with physical environment.

Results showed accordance with the expected: the higher biological activity, the higher respiration. But this do not necessarily means higher Cellulase production. This condition needs to be previously investigated during the microorganism screenings indicating which microorganisms and substrates are suitable to produce it. Another interesting result is the possible dependence

between the initial substrate moisture and the Endoglucanase activity. Substrate moisture is an uncontrollable system state and it cannot be observed during the process.

The automated system is flexible sufficiently to be expanded by adding more sensors and column reactors, for instance to perform growing kinetic, measuring CO_2 concentration in the output of each column. Future works are being planned to know how the Cellulase production depends on the air flow variation (flow rate, temperature, and relative humidity).

The good understanding of each experimental result, relating to the Cellulase production, can be useful for the scale-up strategy if is thought that each bad or good response can be a volumetric part of a large reactor. Another promising application is the investigation of extreme conditions with regard to the biological activity during fermentation showing how the behavior of the microorganisms in stress condition is. Future works will focus a larger set of experiments aiming for the optimal production result.

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