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MULTIPLEX GAS SAMPLER FOR MONITORING RESPIROMETRY IN COLUMN-TYPE BIOREACTORS USED IN SOLID-STATE FERMENTATION

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ABSTRACT

A multiplex gas sampler for monitoring CO_2 and O_2 for column-type bioreactors used in solid-state fermentation was designed. The sampler mechanically couples up to 24 bioreactors to a central set of CO_2 and O_2 sensors. The user can set fermentation time, the number of bioreactors to be sampled by the sampling port, the sampling rate and the delay time between sampling (to guarantee the complete replacement of a gas sample). The user has the possibility to enable or disable sampling from bioreactor-ports. Due to its small size and weight, the gas sampler is portable. This device is also quite economical. This gas sampler was validated using two solid-state fermentation experiments. First, the CO_2 and O_2 measurements were confirmed to be highly reproducible. No significant differences were found for the fermentations of 19 experimental units (bioreactors) simultaneously run with Rhizopus sp. cultured at the same conditions. Second, the versatility of the gas sampler, operating simultaneously with several microorganisms cultured at different conditions, was demonstrated through the simultaneous monitoring of A. awamori and Rhizopus sp. solid-state fermentations, cultured at different temperatures and pH. By using the multiplex gas sampler, the above study was done in only one set of experiments rather than in five sets of experiments that would have been required by using other sampler, representing a great time saving.

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Introduction

Solid-state fermentation (SSF) is defined as the microbial culture developing on solid substrate, in the absence (or near absence) of free water; although the substrate must possess enough moisture to support growth and metabolism of microorganisms (14). SSF has been extensively used for the production of enzymes such as pectinases (1, 16), cellulases (4, 5) and lipases (6, 9, 20). SSF has several advantages over submerged fermentation, including higher productivity, low cost media, better oxygen transference, simplified technology and reduced energy and cost requirements (19).

A disadvantage of SSF is the marked absence of instrumentation for monitoring and control. The purpose of monitoring the fermentation is to understand the process evolution, and to detect perturbations that can reduce the yield and product quality. Current techniques for monitoring SSF include measuring temperature, humidity, water activity, pH, air flow, O_2 and CO_2 (2). In any microbial culture, it is highly desirable to quantify the biomass. However, this task is quite complex for filamentous fungi growing in SSF, because the biomass is tightly linked to the substrate or support. Thus, direct methods to quantify biomass concentration are not possible to be implemented in SSF. Respirometry (O_2 consumption and CO_2 production) represents the most convenient way for on-line monitoring of the microbial growth in SSF (8, 12, 26), and control algorithms have been designed for biomass growth based on O_2 and CO_2 measurements (22). Furthermore, enzymatic activity and respirometry can be correlated, which enables to estimate a suitable time to stop the fermentation, which is generally just before the desired enzyme activity is degraded by proteases (3, 10, 23, 24).

Monitoring CO_2 and O_2 in column-type bioreactors used in SSF still has some drawbacks. Monitoring different columns requires several gas sensors which must be manually installed in each column. In addition, the quantification of O_2 and CO_2 is typically performed with an expensive gas chromatograph. To solve these drawbacks, some interesting approaches have been developed to monitor microbial respirometry in SSF. Saucedo-Castañeda et al. (21) developed an on-line automated monitoring and control system for CO_2 and O_2 in SSF. Their system, based on electro-valves and coupled to a gas chromatograph, could monitor simultaneously eight different fermenters. A gas sensor system based in a commercial 16-position flow-through valve was used in SSF bioreactors (17, 18). Spier et al. (25) designed a data acquisition system for monitoring phytase production in SSF. This system was used to determine O_2, CO_2, temperature, volumetric flow rate and relative humidity. However, it could only be installed in one column.

The goal of the present study was to design a low cost, portable multiplex gas sampler for reliable quantification of CO_2 and O_2 from multiple column-type bioreactors used to monitor the microbial respirometry on SSF. A fermentation composed of 19 bioreactors containing solid medium inoculated with *Rhizopus* sp. under the same culture conditions was used to validate the multiplex gas sampler system. Additionally, the sampler was tested to monitor simultaneously two different SSF: a) *Aspergillus awamori* cultured to produce pectinases at 30 °C and pH of 4.0 and 5.5; and b) *Rhizopus* sp. cultured to produce lipases at 40 °C and pH of 4.0, 5.5 and 6.5. The designed sampler was able to monitor simultaneously the respirometry of 24 bioreactors using one or two microorganisms cultured on similar or different conditions in SSF.

Materials and Methods

Reagents and media

Triton X-100, Tween 80, *p*-nitrophenyl palmitate, potato dextrose agar (PDA) and potassium dihydrogen phosphate, were purchased from Sigma-Aldrich. Sugar cane bagasse used as a support for SSF was obtained from Ingenio Azucarero Melchor Ocampo (Autlán, Jalisco, Mexico). All other chemicals and solvents were of reagent or better quality and were obtained from local suppliers.

Fungal strains and inoculum preparation

Rhizopus sp. (8a strain) (Rh) and *Aspergillus awamori* (Aa) were maintained on PDA (Potato Dextrose Agar) slants at 4 °C. Two inoculums were prepared by culturing Rh and Aa in 250 ml Erlenmeyer flasks containing 50 ml of PDA at 40 °C and 30 °C, respectively, for one week. Spores were harvested with 50 ml of Tween 80 solution (0.01% w/v).

Solid media preparation and fermentation

Two impregnation media were prepared to stimulate lipases and pectinases production by Rh and Aa, respectively. The mineral composition for both media was (g/l): urea, 4; K_2HPO_4, 5; $MgSO_4$, 1. Olive oil (40 g/l) and apple pectin (10 g/l) were also added to the media as carbon source and enzyme inductor, respectively for Rh and Aa. The initial media pH was adjusted to 4.0, 5.5 and 6.5 for Rh, and 4.0 and 5.5 for Aa. The medium containing oil was emulsified using an Ultra-Turrax homogenizer (IKA) at 19,000 rpm for 10 min.

Sugar-cane bagasse was used as the SSF support. This agricultural residue was sieved to obtain particles ranging between 0.8 and 1.7 mm, washed three times with distilled water and then dried at 80 °C for two days. SSF was performed essentially as described by Raimbault and Alazard (15). For lipase production, the support was impregnated with a concentrated medium (1.5 times), in a ratio of 1 g per 2 ml, sterilized at 121 °C for 15 min. For pectinase production, 1 g of support was impregnated with 3 ml of medium. Inoculum was added to obtain a final concentration of 3×10^7 spores/g of dry matter and humidity was adjusted to 75%. Glass columns (2.5 cm × 30 cm) were packed with 12.5 g of solid culture media and incubated in a water bath set at 30 and 40 °C, for Aa and Rh, respectively. A continuous stream of water saturated air was injected into the bottom of each column at a rate of 18 ml/min.

Lipase activity measurement

Lipase was extracted from the fermented solid by adding 25 ml of 20 mM Tris-HCl buffer (pH 8) containing 0.5% Triton X-100 to each glass column and squeezing the solid ferment as described by Rodriguez et al. (20). Lipase activity was measured using *p*-nitrophenyl palmitate (p-NPP) as described by Kordel et al. (7), with some modifications. The reaction mixture was prepared by mixing one volume of a 10 mM solution of p-NPP in 2-methyl-2-butanol with 18 volumes of 50 mM Tris-HCl buffer (pH 8) and 0.5% of Triton X-100. 10 µL of enzyme extract at an appropriate dilution in 10 mM Tris-HCl (pH 8) were placed into the microplate wells and subsequently, 190 µL of the reaction mixture were incorporated and mixed (10). A control was prepared by adding 10 µL of 10 mM Tris-HCl (pH 8) instead of enzyme extract. The absorbance was measured at 415 nm and continuously monitored for 10 min using a Spectrophotometer XMark™ (Biorad). The reaction rate was calculated from the slope of the curve absorbance versus time, using a molar extinction coefficient of 12,750 $cm^{-1}M^{-1}$ for *p*-nitrophenol. One enzyme unit (U) was defined as the amount of enzyme releasing 1 µmol of *p*-nitrophenol per minute under the assay conditions. Lipase activity was expressed as U/g dry matter (DM). Assays were performed in triplicate and data represent the mean and standard deviation (lower than 20%).

Pectinase activity measurement

Pectinase was assayed by mixing 50 µl of enzyme extract with 150 µl of 0.5% apple pectin in a 0.1 M citrate phosphate buffer, pH 5.5. The reaction mixtures were incubated at 50 °C for 10 min. Pectinase activity was quantified by the release of reducing sugars using the dinitrosalicylic acid (DNS) method (11) and galacturonic acid as standard. Samples were measured in a microplate spectrophotometer at 540 nm. One unit (U) of activity was defined as the amount of enzyme releasing 1 µmol of product per minute under the assay conditions. Enzymatic activities extracted from the fermented solid were expressed in U/ml. Pectinase activity was expressed as U/g dry matter (DM). Assays were performed in triplicate and data represent the mean and standard deviation (lower than 20%).

Monitoring equipment description

Fig. 1 illustrates the monitoring system. The water bath (50 cm × 50 cm × 40 cm) had a capacity to hold up to 30 glass column-type bioreactors (2.5 × 30 cm). An air humidifier was connected to the bottom of each column. The temperature in the water bath was controlled with a Thermoregulator Feed Electric Model TEPS-1. Air flow rates were measured with an Agilent Model 5067–0223 flow meter. The outlet air from the column bioreactors was dried using a filter to avoid damage to the electronic sampler components (21). The dry air from the outlet of up to 24 columns was connected to

the sampler through hoses. One CO_2 sensor and one O_2 gas sensor (Pasco, CI-6561 and CI- 6562, respectively) were placed at the end of a gas collector tube. Both gas sensors were connected to the Pasco ScienceWorkshop 750 interface. A PC was used to collect data from the gas analyzers via a USB port. The sampler operation was controlled by means of an USB-1024LS control card (Measuring Computing).

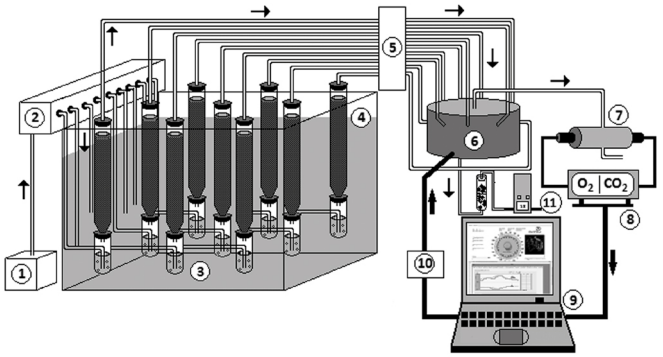

Fig. 1. On-line monitoring system for CO_2 and O_2:1) air pump, 2) air distribution system, 3) temperature controlled water bath, 4) humidifiers and column bioreactors, 5) drying filters, 6) multiplex gas sampler, 7) gas collector tube with sensors of CO_2 and O_2 at each end, 8) gas analyzer interface, 9) computer, 10) control card and 11) digital flowmeter.

Software

A program was designed using Labview™ 8.2 to control the gas sampler and to display the respirometry values in the PC screen. Pasco™ software drivers enabled the interface between the Pasco™ ScienceWorkshop 750 and Labview™ 8.2. Statgraphic™ Plus version 5.1 was used for statistic analysis.

Sampler operation validation

To validate the performance of the multiplex gas sampler system and to confirm its reproducibility on the CO_2 and O_2 measurements during a fungal culture, 19 column-type bioreactors containing solid medium inoculated with Rh were cultured at the same conditions (pH 6.5 and 40 °C) in SSF. Two additional columns containing the same solid medium but non-inoculated were used as controls. The fermentation was monitored for 18 h and then stopped. No columns were removed during the experiment. The content of each column was used for further lipases activity assays. In order to guarantee sufficient gas displacement in the collector tube, the delay time was set to 8 min, and then two samples were taken every 15 s by the sampling ports.

Sampler performance evaluation

Several fermentations using two different fungal strains, cultured simultaneously at different conditions in the same experiment, were conducted to evaluate the performance of the gas sampler. 1) In a water bath set at 30 °C, Aa was cultured to produce pectinases at pH 4.0 and 5.5. 2) In a water bath set at 40 °C, Rh was cultured to produce lipases at pH 4.0, 5.5 and 6.5. Additionally, one column without inoculum was used as a control for each type of fungal fermentation. The fermentations were monitored for 55 h and then stopped. No columns were removed during the experiment. The content of each column was used to assay enzyme activity. Each experimental unit (culture condition and fungal strain) was performed in triplicate. In order to guarantee sufficient gas displacement in the collector tube, the delay time was set to 8 min, and then two samples were taken every 15 s by the sampling ports.

Results and Discussion

Gas sampler description

The O_2 and CO_2 gas sampler consisted of a stainless steel cylinder (20 cm of diameter, 14 cm of height and 4.35 kg of weight) divided in two sections. Electronic circuits for driving the stepper motors were located in the bottom half. The rotating sampling arm was placed in the center of the upper half. Twenty-four sampling ports (8 mm of diameter) were positioned around the cylinder (**Fig. 2**). A first stepper motor located in the center of the cylinder moves the sampling arm clockwise from an initial position to a desired position. When the sampling arm reaches the desired sampling port, a second stepper motor couples the sampling arm to the sampling port. An o-ring in the tip of the sampling arm guarantees a tight coupling with the port. The sampling arm remains in the sampling port until the sample(s) is acquired, and then the sampling arm is decoupled and positioned in the next sampling port. The sampling process proceeds until the last gas sample has been obtained, then the sampling arm returns to the initial position, and the cycle process repeats again until the fermentation ends. If the gas sampler is in a sampling cycle when the fermentation end time is reached, the sampler will complete the cycle unless the user stops the program. The flow diagram of the process is shown in **Fig. 3**.

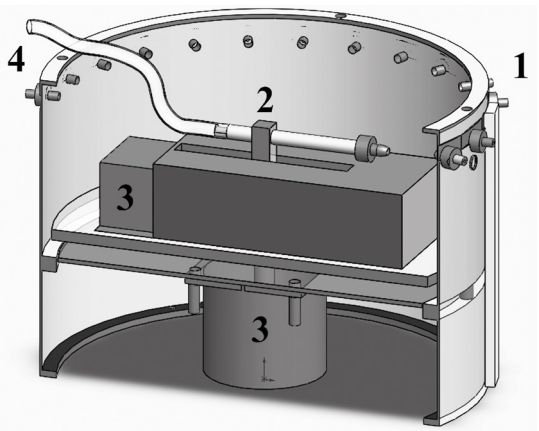

Fig. 2. Gas sampler transversal cut, 1) sample ports, 2) sample arm, 3) stepper motors and 4) sampled gas output.

The sampled gas is conducted to a collector tube where the CO_2 and O_2 sensors are placed. The gas collector tube was designed to allow enough air to accumulate and stabilize before

taking a steady CO_2 and O_2 measurement, so the residual air in the cylinder is totally displaced by the air coming from the next bioreactor to be sampled. The minimum time required to displace the residual gases (O_2 and CO_2) depends on the air flow used for each fermentation condition, usually it is about eight minutes for fermentations with aeration flow rates ranging from 18 to 60 ml/min. Therefore, the delay time is set to guarantee that the gas has been stabilized in the collector tube.

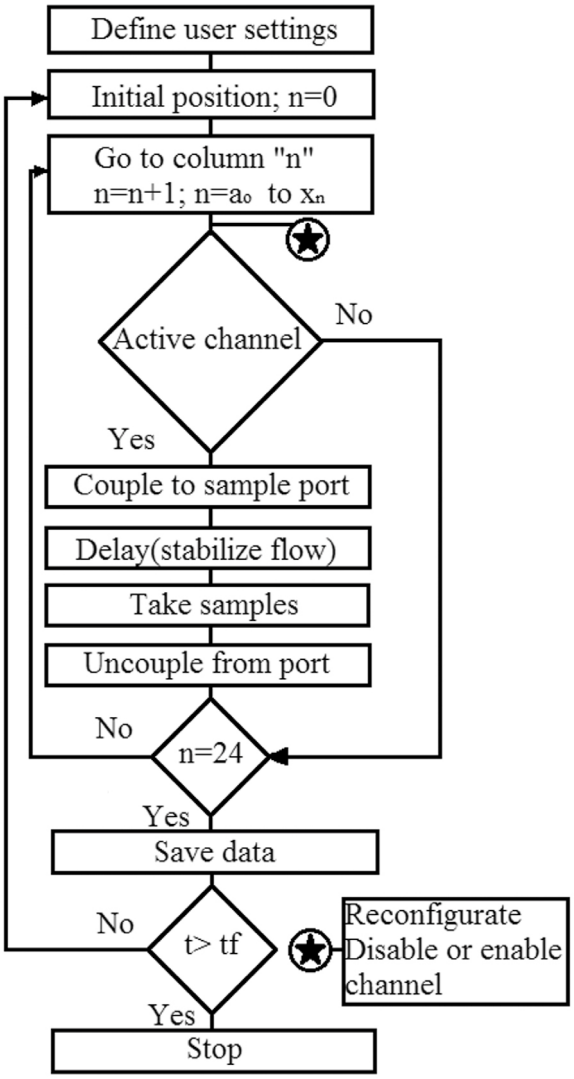

Fig. 3. Algorithm to control the multiplex gas sampler.

Gas sampler software interface description

(**Fig. 4**) shows the software interface, developed in Labview™ 8.2, used to control the gas sampler and to display the O_2 and CO_2 values from each sample port. The program has a simple graphical interface, which allows the user to set the fermentation time, the delay time (to guarantee the air displacement in the gas collector tube and a steady gas concentration), the sampling time and the number of samples to be taken in the sampling port. In the main window, it is also possible to enable or disable monitoring of specific columns. If a column is removed from the fermentation system for further analysis (e.g. enzyme activity), the associated port can be disabled in order to skip this port for further gas assays. In a second window, a specific respirometry from any of the 24 columns can be selected and displayed. At the end of each complete sampling cycle, the data are saved as a text file that can be imported into spreadsheet software. The minimum time for a complete sampling cycle is 15 min, which is achieved when the delay time is zero and a single sample is acquired per sampling port. This time will be longer depending on the delay time and the number of samples acquired from each sampling port.

Experimental gas sampler validation

To validate the performance and reliability of the gas sampler, a SSF with *Rhizopus* sp. (Rh) was carried out. Rh shows a fast metabolism, producing maximal biomass and lipases in only 12 h (20). The experiment was run with 19 inoculated columns and two non-inoculated columns (controls) at the same culture conditions. The results showed that the respirometry for all the sampled columns was homogenous. The outlet gas from each column was repetitively sampled every 8 min for 17 h. Eight minutes was calculated as sufficient time to displace at least 90% of the gases from the gas collector tube by the new air to be sampled (**Fig. 5**). Therefore, to guarantee complete gas displacement in the collector tube, the delay time was set at 8.5 min. After each delay time, two samples were acquired every 15 s from the sampling port.

Nineteen graphs of respirometric data were obtained from the Rh SSF, representing the consumption of O_2 and the production CO_2 for each sampled column. In order to show the assays' reproducibility, a graph representing the full dataset of all respirometries for the 19 columns was created (**Fig. 6**). The total sampling cycle for all 19 columns was 3.6 h, so an average of six samples per column was taken every 24 h. None of the inoculated columns (controls) showed any variations in CO_2 or O_2 concentrations in the outlet air samples (data not shown). Consumption of O_2 and production of CO_2 started after 4 h of fermentation. The gases data from the sampled columns showed a typical respirometry profile of Rh (10, 20). In addition, maximal CO_2 and minimal O_2 concentrations were observed at 8 h of culture.

Lipase activity was determined at the end of fermentation for the 19 columns and analysis of the variance was carried out, at significance level of 0.05, F ratio of 1.92 and a *p*-value of 0.0834. This statistic analysis revealed that no significant differences were found among the 19 columns used for the Rh SSF. Global lipase production in this work was 1.057 ± 0.0094 U/g of Dry Matter (DM).

In a typical experiment using SSF, each system (water bath) contains around 10 to 24 bioreactors (columns), and only a couple of columns are monitored to track the respirometry

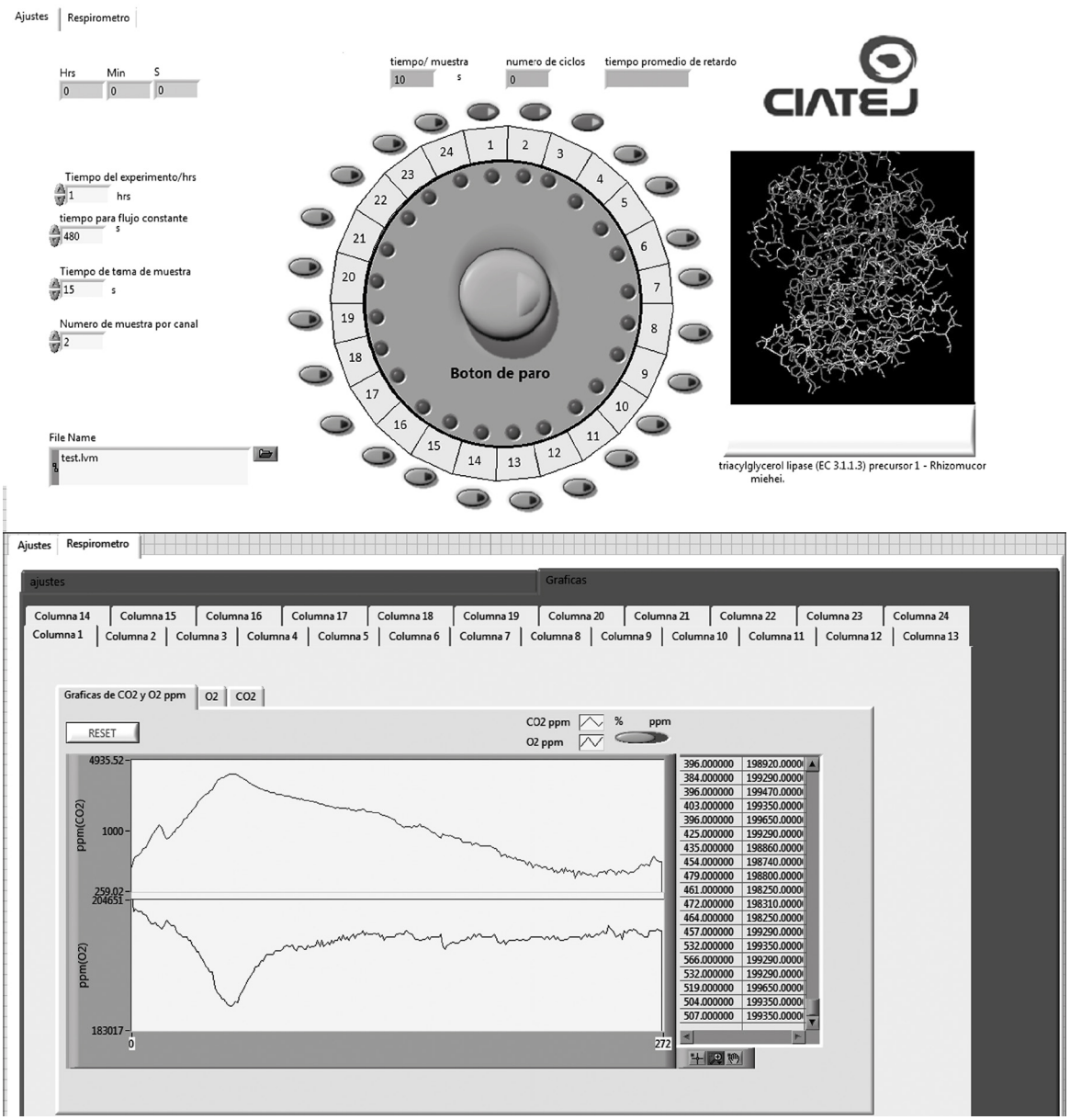

Fig. 4. Software interface used to control the gas sampler and to display the O_2 and CO_2 values from each sample port.

of the process, with the expectation that these columns would be representative of the entire process: However, this premise has never been demonstrated. Our results of Rh SSF proved this supposition, in spite of the experimental variations due to the solid support nature, such as: i) polymeric heterogeneity of sugar cane bagasse components, affecting humidity and distribution of the impregnation medium; ii) differences in particles size, affecting the intraparticle spaces and aeration, iii) heterogenic distribution of inoculums (13).

Evaluation of the gas sampler performance using two different fungal strains cultured in SSF

A second experiment was conducted in order to test the gas sampler scope using simultaneously two fungi cultured by SSF under several operational conditions. Rh (a thermotolerant fungus) was cultured to produce lipases at 40 °C and pH of 4.0, 5.5 and 6.5. *Aspergillus awamori* (Aa, a mesophile fungus) was cultured to produce pectinases at 30 °C and pH of 4.0 and 5.5. In order to guarantee sufficient gas displacement in the collector tube, the delay time was set to 8 min and then two samples were taken every 15 s by the sampling ports.

Respirometries for Rh and Aa fermentations are shown respectively in (**Fig. 7**) and (**Fig. 8**)**.** The results showed that the gas sampler was able to detect respirometry differences for the distinct experimental conditions that were tested. In fact, different profiles in CO_2 production and O_2 consumption were observed during the Rh and Aa fermentations. For both

fungi, maximal CO_2 production and O_2 consumption increased as pH did. CO_2 production reached a maximum at 8 h and 13 h, for Rh and Aa, respectively, revealing a faster metabolism for Rh. At the end of the Rh and Aa fermentations (55 h), lipase and pectinase activities were measured for all the tested experimental conditions (**Table 1**). Remarkably, as shown by respirometry analysis, enzymatic activities also increased as pH increased for both fungal cultures. These experiments also confirmed the importance to maintain the air flow at least to 18.0 ml/min in order to displace satisfactorily the gas from the previous sample.

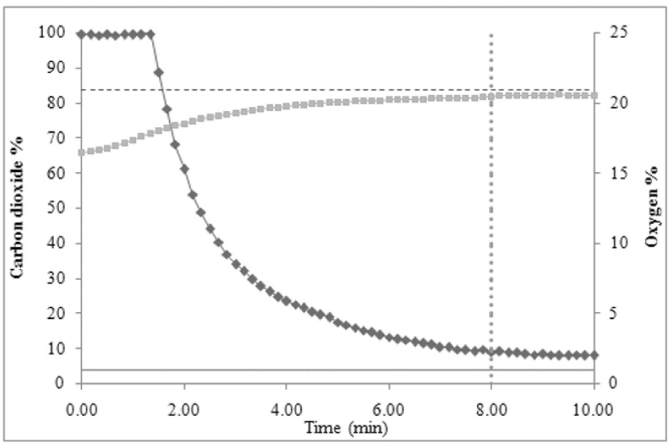

Fig. 5. CO_2 and O_2 displacement in the collector tube at inlet flow rate of 18 ml/min: CO_2 production (◆), O_2 consumption (■).

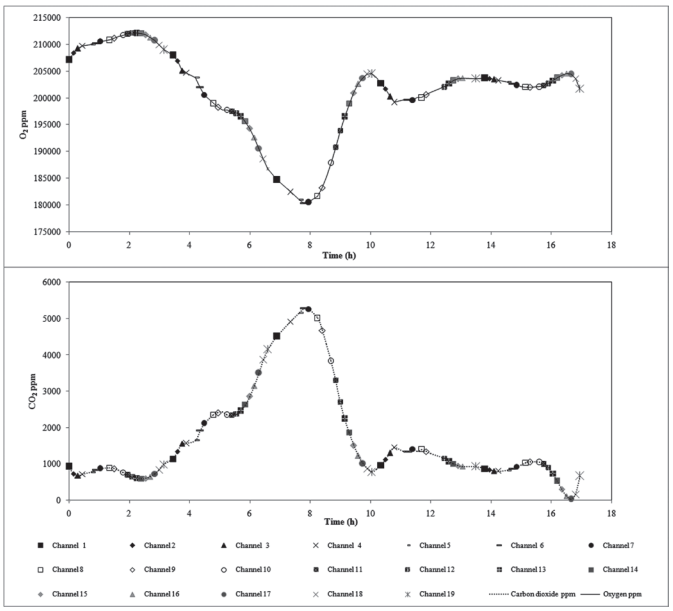

Fig. 6. Smoothed respirometric profile of *Rhizopus* sp. for 19 columns of SSF, performed at the same culture conditions: O_2 consumption (**a**) and CO_2 production (**b**).

Saucedo-Castañeda et al. (21) designed an O_2 and CO_2 sampler using solenoid valves which was capable of monitoring up to eight columns in SSF. Richard and Walker (18) used an O_2 and CO_2 sampler based on two commercial 16-position flow-through valves for microfluids to sequentially sample 36 micro-reactors for solid-state biodegradation. It is worth noting that the gas sampler proposed in our work has some advantages compared to previous designs: a) no gas chromatograph, solenoid or other expensive valves are required, b) the multiplex gas sampler has 24 sample ports, c) the sampling ports can be enabled or disabled as columns are added or retired from the fermentation system, and d) the sampler was designed and built in our laboratory with commercial low cost mechanical and electronic components. Spier et al. (25) designed a data acquisition system for monitoring phytase production in SSF. Their system was able to monitor O_2, CO_2, temperature, volumetric flow rate and relative humidity; however, it could only be used for one column, limiting its use in the optimization of fermentative processes.

TABLE 1

Enzyme activities for *Rhizopus* sp. (Rh) and *A. awamori* (Aa) cultured on SSF at 40 and 30ºC, respectively, and different pH*

pH	Rh lipase activity (U/g DM)	Aa pectinase activity (U/g DM)
4.0	0.3692±0.07	0.0767 ± 0.006
5.5	0.4612±0.09	0.1929 ± 0.007
6.5	0.5016±0.06	no tested pH

* These experiments were carried out to test the multiplex gas sampler performance, analyzing fungal respirometry.

Parameters such as O_2 consumption, CO_2 production and the respiratory quotient are important tools to study microbial physiology and metabolism. Consequently, our proposed sampler may be of importance to estimate microbial growth and metabolites production (e.g. enzymes), mainly for SSF processes. It is important to remark that air humidity should be avoided, since water could damage the multiplex sampler electronic parts or perturb the flow meter. This problem was solved by passing the air samples through a silica filter as reported by Saucedo-Castañeda et al. (21). The gas holder tube is currently the bottleneck of the sampler, since a delay time of at least 8 min is required between samples to guarantee the complete replacement of a gas sample and consequently, accurate O_2 and CO_2 measurement. In addition, since all disabled columns taken out of the fermenter will be skipped on the next sampling cycle, the time length of the next sampling cycle will be reduced, increasing the number of samples taken per cycle.

Remarkably, the multiplex gas sampler allowed the simultaneous study of multiple fermentations performed with two fungal strains cultured at different conditions in only one experiment. If a commercial equipment to measure respirometry had been used for the above experiment, instead of the multiplex sampler, five experiments would have been required, consuming a lot of time. In addition, no gas chromatography was required. Due to its size and weight, the gas sampler is portable and easily transportable. This device also emits a low noise level compared to solenoid valves.

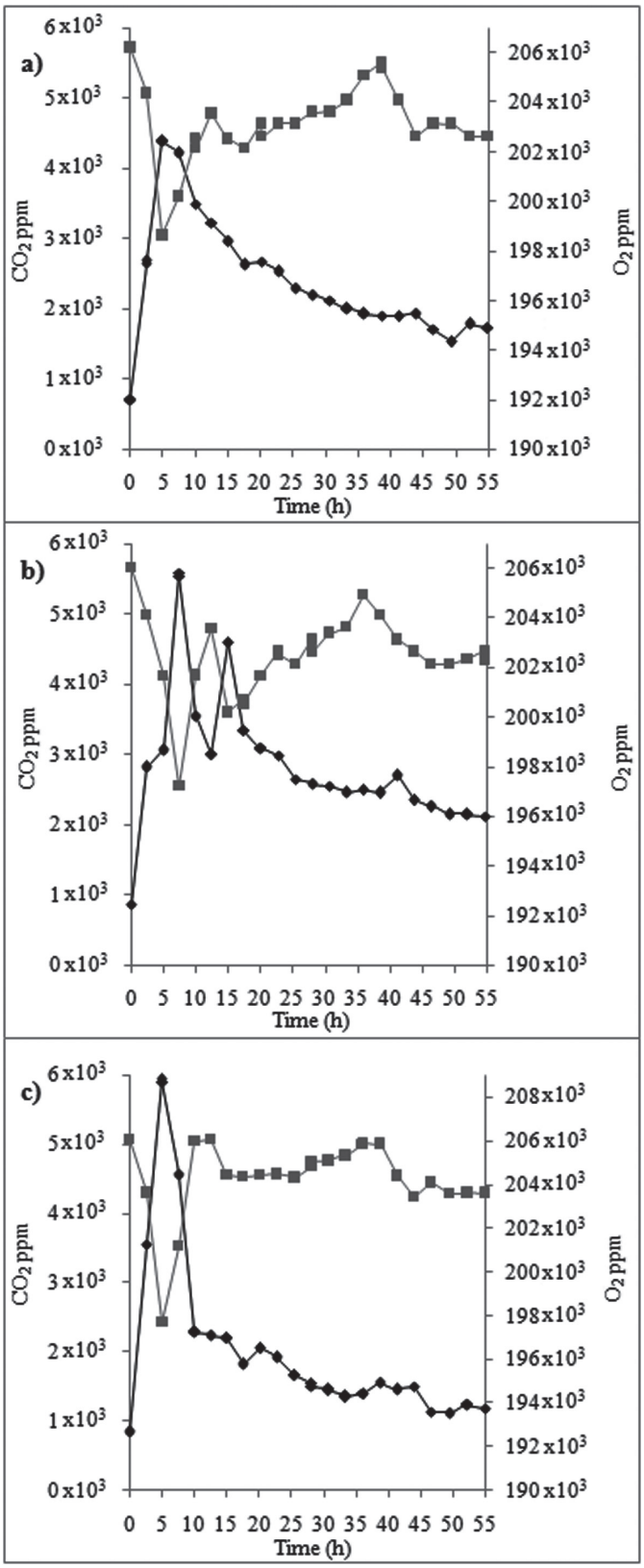

Fig. 7. Simultaneous monitoring of the respirometry of *Rhizopus* sp. (a lipase producer thermotolerant fungus) cultured by SSF at 40 ºC and pH 4.0 (**a**), 5.5 (**b**) and 6.5 (**c**); CO_2 production (◆) and O_2 consumption (■).

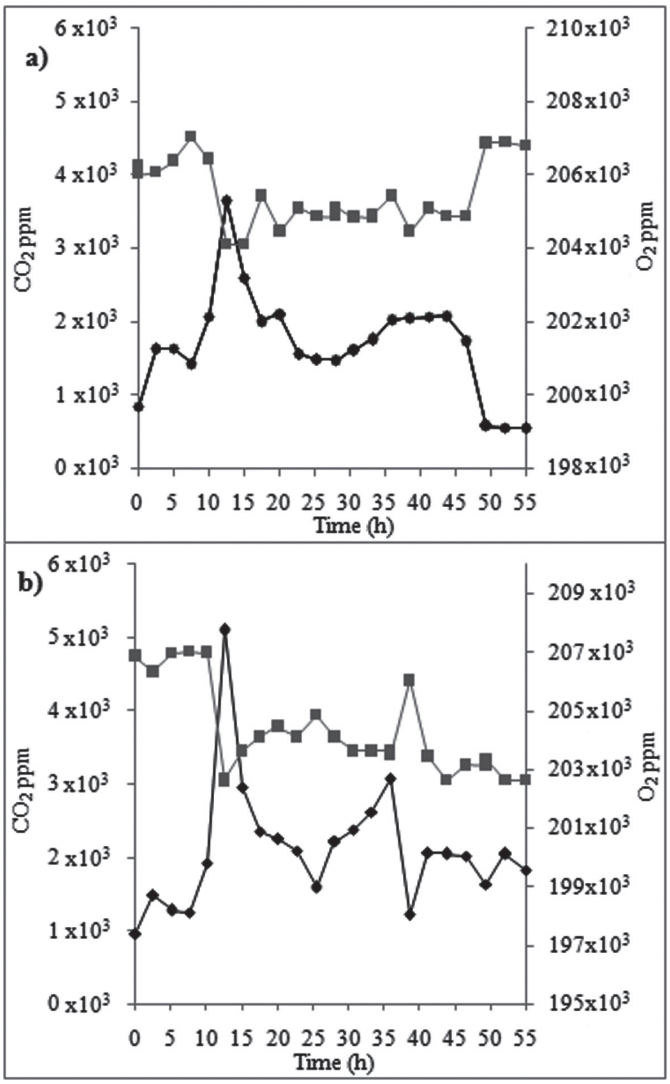

Fig. 8. Simultaneous monitoring of the respirometry of *A. awamori* (a pectinase producer mesophilic fungus) cultured by SSF at 30 ºC and pH 4.0 (**a**) and 5.5 (**b**); CO_2 production (◆) and O_2 consumption (■).

Conclusions

In this work, a new sampler for monitoring O_2 and CO_2 for column-type bioreactors used in SSF was designed and validated. The O_2 and CO_2 measurements were highly reproducible and no significant differences were found for the fermentations of 19 experimental units (bioreactors) simultaneously run with *Rhizopus* sp. cultured at the same conditions. Furthermore, the potential utility of the multiplex gas sampler was demonstrated through the simultaneous monitoring of the respirometry of *A. awamori* and *Rhizopus* sp. cultured on SSF at different temperatures and pH. Using the multiplex sampler, the above study was performed in only one set of experiments compared to five sets of experiments required by other samplers, representing a great time saving. Due to its size and weight, the gas sampler is portable and easily transportable. Further improvements will focus on the gas collector tube to reduce the delay time and the air inlet to guarantee a steady flow. The multiplex gas sampler can

be used to monitor respirometry simultaneously for several microorganisms cultured at different conditions, facilitating the optimization of diverse SSF processes, operating at different culture conditions. This enables the screening of microorganisms capable to growth on specific substrates and potentially producers of metabolites of interest.

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