Laboratory scale bioreactors for study of fungal physiology and metabolism in solid state fermentation system

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SUMMARY

Data on physiology, effect of various parameters on the growth as well as on the metabolism of the culture, monitoring and regulation of parameters are of critical importance in the design of a commercial process. The studies on these aspects at laboratory scale thus dictate the use of efficient laboratory scale bioreactors. A column bioreactor was developed at ORSTOM and the manufactured commercial units are, herewith, described along with various strategies for monitoring and control of different parameters. The design of the modified kneading machine is also reported for upgradation of wastes at a level of 120 L capacity. To overcome the lack of a system for inoculum development, a disk fermentor was developed, which was found to be highly efficient. A static fermenter of the capacity of 50 Kg substrate, named as Zymotis was developed for efficient control of the fermentation temperature and achievement of high productivities. All these bioreactors form one of the most important contribution of ORSTOM to solid state fermentations, based on the use of solid substrates/supports.

Keywords: Solid state fermentation, bioreactors, equipments, Zymotis, column bioreactor, kneading machine, disks fermentor, FMS 16-250.

RESUME

Bioreacteurs de laboratoire pour étudier la physiologie et le métabolisme de champignons cultivés en milieu solide.

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Les données de la physiologie, l'effet de différents paramètres sur la croissance aussi bien que sur le métabolisme de la culture, la supervision et la régulation des paramètres sont d'une importance critique dans la conception de procédés commerciaux. Les études concernant ces aspects à l'échelle du laboratoire impliquent donc l'utilisation des bioréacteurs de laboratoire efficaces. Le bioréacteur en colonne développé à l'ORSTOM et l'unité commerciale construite sont ici décrits, de même que les stratégies de contrôle et supervision de différents paramètres. La conception d'un pétrin de boulangerie modifié est également signalée pour la valorisation de déchets à une capacité de 120 litres. Afin de combler la manque de systèmes de production contrôlée d'inoculum, un fermenteur à disques fut développé, et se révéla d'une grande efficacité. Un fermenteur statique d'une capacité de 50 kg de substrat, nommé Zymotis, fut développé dans le but d'obtenir un contrôle efficace de la température de fermentation et d'atteindre des productivités élevées. Tous ces bioréacteurs forment l'une des plus importantes contributions de l'ORSTOM aux fermentations en milieu solide, basées sur l'utilisation de substrats/supports solides.

Mots clés : Fermentations en milieu solide, bioreacteurs, équipements, zymotis, bioreacteur de type colonne, pétrin de boulangerie, fermenteur à disques, FMS 16-250.

INTRODUCTION

Solid state fermentation offers various advantages in comparison with submerged ones, (Lambert and Meers, 1983). For example, aeration is facilitated through the spaces between the substrate. Substrate agitation, when necessary, is discontinued. The absence of a liquid phase and low substrate humidity levels permit a) reduction of the fermenter volume, b) reduction of the volume of liquid effluents from the process, c) reagents saving during metabolites recovery, d) reduction of bacteria contamination because of low humidity levels, and e) use of non sterile solid

substrate in some cases (Raimbault and Germon, 1976). Culture media are simple, and are mainly composed of agro-industrial residues. Culture growth conditions on solid state media are similar to those in the environment. For some fermentation, the solid support microflora is used as an inoculum (Perraud-Gaime, 1995). Direct use of the fermented substrate is possible in many solid state fermentations.

The main disadvantages are: a) high risks of temperature rise due to excessive metabolic heat generation, b) difficulty in parameter regulation, c) need for pretreatment of solid support/substrate, d) high loss of humidity in fermentations of long duration, e) necessity for high inoculation when natural microflora is not used, and f) the critical role of water and water activity.

Any industrial SSF application requires the understanding of some physiological, physical and chemical parameters, which are characteristic of the strain used and substrate employed. These include data on growth rate, optimum temperature and pH, gas exchange and gas requirement of the strain, water content and activity, nutrient content, etc. (Roussos *et al*, 1991a).

All these parameters have to be considered when designing SSF equipment (Aidoo *et al*, 1982; Moo Young *et al*, 1983; Lonsane *et al*, 1985, 1992; Hesseltine, 1987). Futhermore, the monitoring and controlling of these parameters need to be studied carefully (Saucedo-Castañeda *et al*, 1994).

Some other important parameters, such as inoculum development, in addition to process and equipment scale-up, have to be taken into consideration while studying SSF. (Roussos *et al*, 1991a).

Considering all aspects mentioned above, bioreactors for different purposes have been developed in ORSTOM. These are described in the present paper.

COLUMN BIOREACTOR

The column bioreactor is the first equipment developed by ORSTOM in the years 1975-1980 to study solid state fermentation. Its principle has been patented in 1976 (Raimbault and Germon, 1976). It is composed of a small glass column of 4 cm in diameter and 20 cm in length, having an effective reaction volume of 250 mL. The column is filled up with substrate, as shown in Fig. 1 and placed in a thermoregulated water bath. Several columns can be placed within the same water bath.

Aeration is provided by using a compressor. Air passes through three humidifiers, the second one acting as an air distributor. Air saturation is further assured in the third humidifier which is composed of a small bubbler, positionned at the bottom of a vat, filled with water and placed immediatly below the fermentation column

(Fig. 1). The third humidifier and the fermentation column are submerged in the water bath, thus allowing a good substrate thermoregulation.

The column bioreactor has been widely employed by many researchers (Raimbault and Alazard, 1980; Huerta, 1984; Roussos, 1985; Trejo *et al*, 1992: Oriol, 1987; Dufour, 1990; Saucedo-Castañeda, 1991; Saucedo-Castañeda *et al*, 1992 a,b; Soccol *et al*, 1994). It should shortly be replaced by the recent bioreactor developed by ORSTOM, in collaboration with Gauthier Agro-industries, using the same principle, as described in the last section of the paper.

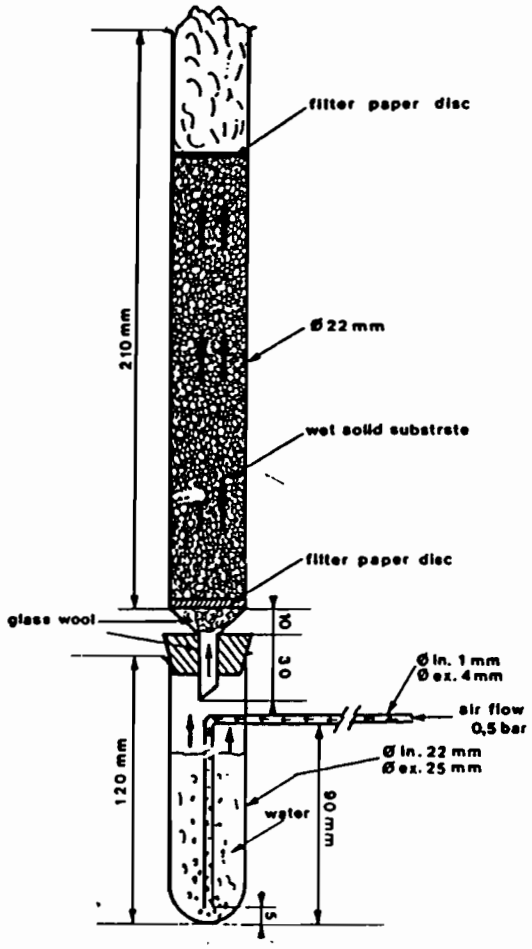

Fig. 1. Column bioreactor

The micro-organisms grown and studied using the column bioreactor, with or without the gas analysis system, have been filamentous fungi (*Trichoderma*,

Rhizopus, Aspergillus, Penicillium), yeasts and mushroom mycelia (*Pleurotus, Agaricus*). The metabolites obtained include enzymes (cellulases, amylases, proteases, lipases), probiotic substances prepared from filamentous fungi, biopesticides, alkaloids, antibiotics, phytohormones, etc. (Table 1).

Table 1. Aplication fields of columns bioreactors in various solid state fermentations.

Application fields	End products	Microorganisms	References
Fermented food	Koji	*Aspergillus oryzae*	Raimbault, 1980
	Pozol	*Lactobacillus* ssp and yeasts	Saucedo-Castañeda, 1987
Protein enriched	Feed	*Aspergillus terreus*	Gonzales-Blanco *et al*, 1990
Enzyme production	Amylases	*Aspergillus*	Oriol, 1987
	Lipases	*Rhyzopus delemar*	Martinez-Cruz *et al*, 1993
	Cellulases	*Trichoderma*	Roussos, 1985
	Pectinases	*Aspergillus*	Dufour, 1990
Secondary metabolites	Aroma	*Ceratocystis fibriata* *Trichoderma*	Christen and Raimbault, 1991 *et al*, 1995
	Penicillin	*Penicillium*	Barrios-Gonzales *et al*, 1988
	Aflatoxin	*Aspergillus flavus*	Barrios-Gonzales *et al*, 1990
Organic acids	Citric acid	*Aspergillus niger*	Gutierrez-Rojas *et al*, 1995
	Gallic acid	*Aspergillus niger*	Raimbault, 1980
	Lactic acid	*Rhyzopus oryzae*	Soccol *et al*, 1994
Alcohol production	Ethanol	*Schwanniomyces castellii*	Saucedo-Castaneda, 1991
Spores production	Insecticide	*Trichoderma harzianum*	Roussos, 1985
Ensilage	Ensiled substrate	*Lactobacillus* spp	Saucedo-Castaneda, 1990
		Mixed cultures	Perraud-Gaime, 1995
Higher mushrooms	Spawn	*Pleurotus spp*	Roussos, *et al*, 1996
		Morchella	Kabbaj *et al*, 1996

KNEADING MACHINE

A kneading machine, used in the bakery for dough preparation, has been adapted in 1979 by ORSTOM and IRCHA, for the study of SSF in large volumes (Meyer and Deschamps, 1979; Deschamps *et al,* 1980). It is composed of a cylindrical open bowl of 1,200 L capacity with a rounded base, freely operating around a vertical axis through a speed regulated motor. It is equiped with a 3 fingers stirrer forming an angle of 30° with the bowl axis (Fig. 2). The bowl has got a double jacket on the bottom side, allowing an air flux through a serie of holes, made in its rounded base. Temperature, pH and humidity are measured and controlled. Temperature and humidity saturation adjustments are obtained by a continuous mixing of the substrate and a simultaneous vaporisation of water mist on substrate surface. This combined operation avoids during of the substrate surface, pH adjustment is implemented by means of addition of a urea solution addition when vaporizing the substrate.

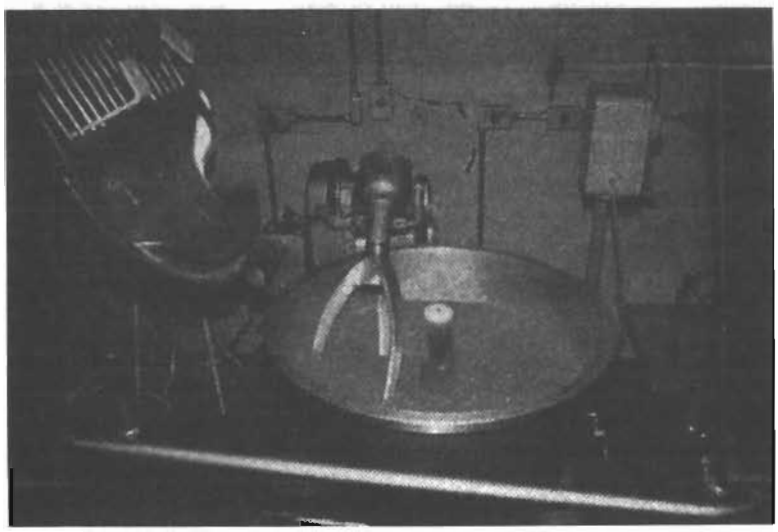

Fig. 2. Kneading machine, implemented by IRCHA for fungal cultivation on solid substrate.

This kneading machine has been used to enrich various amylaceous substrates (cassava, potato, banana) with proteins for animal feed production (Senez *et al,* 1980; Deschamps *et al,* 1980). This type of equipment has also been used for the fermentation of wheat straw (Deschamps *et al,* 1985). An adaptation of this

equipment principle has been made by the researchers of INRA-Dijon (Durand, 1983) for the protein enrichment of beet root pulp.

COMPUTERISED SYSTEM FOR FOLLOW-UP AND MONITORING OF SSF

In order to follow-up and control the fermentation parameters, when using solid state bioreactors, Saucedo-Castañeda *et al*, 1992a, b, have developed a system, which has the ability to continuously monitor the concentration of CO_2 and O_2 in the reactors exhaust air. The system automatically modifies the air flow rate in order to maintain the levels of CO_2 and O_2 at constant values in the gaseous effluents.

For that, air is analysed through classical GC or infrared gas analysers and data are processed using a computer. A program controls air valves for air rate managing.

Such an apparatus can not function in conditions of humidity. Therefore, a sample of the exit gas passes through a chilled water condenser, which separates moisture from gas and then, the gas is directed through a silica gel drying column. Dried air is then analysed. The process operates continuously for each individual column bioreactor through an automatic multiple ways valve, which selects the different outlet of gases to be analysed. Eight to sixteen columns can be analysed, along the fermentation process.

The prototype schematic representation is given in Fig. 3. It consists of a chilled water glass condenser for moisture separation (items No.14 and 15), a silica gel column for gases drying (item No.16), the gases analysers for CO_2 and O_2 (item No.18) with their injection valves (item No.17) and the computer unit with its accessories (item 13).

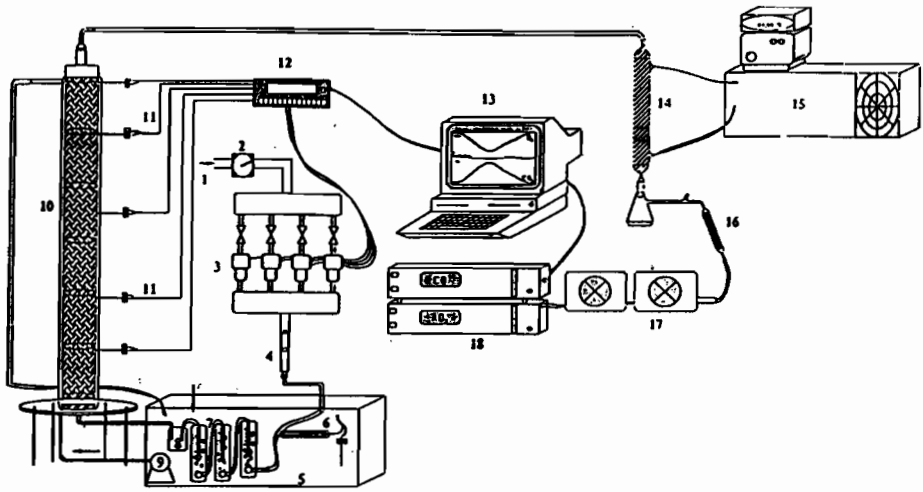

Fig. 3. Diagramatic sketch of the system developed for monitoring and control of exhaust gases in aerobic solid state cultures.

DISKS FERMENTOR

In 1985, ORSTOM researchers adapted a disks fermentor, in order to use it for the study of *Trichoderma harzianum* conidiogenesis. The aim was to develop an efficient technique of spore production, which is not yet available for the industrial applications of solid state fermentation. The micro-organism *Trichoderma harzianum*, was chosen, because of its large potencial in industrial applications: cellulases (Roussos *et al*, 1991b), biopesticides (Elad *et al*, 1993), cassava flour protein enrichment (Muindi and Hansen, 1981) and flavour compounds (Okuda *et al*, 1982). The equipment has been patented in 1985 (Raimbault and Roussos, 1985).

Fig. 4 shows a schematic representation of the disks fermentor. The equipment is composed of a cylindrical glass column of 800 mm in length and 92 mm in internal diameter, with flanged open ends, which can be closed by means of stainless steel plates, "O" rings and winged nuts for asceptic operation. It offers a total capacity of 5 L. Inlet and outlet ports are provided with the column for gas or liquid flux. The column is equipped with 35 disks of 50 mm in diameter, each disk being composed

of 2 steel gratings of 2 mm thickness and having a mesh of 4 mm, thereby giving a working area of 4,960 cm^2. Spacing between the disks is 10 mm. The gratings are fixed on a central shaft, which is connected to a stirring motor. The disks fermentor is positioned horizontally, with the help of appropriate stands for its operation. The cylinder is first filled with an adequate volume of a solution of nutrients as well as agar and sterilized by autoclaving. When cooled to about 45°C, the still liquid medium is inoculated with spores and then allowed to cool down 25-29°C, under slow rotation (30 rpm). This permits the mixed inoculated agar medium to homogeneously solidify on the disks interspace. Before the complete solidification of the agar, rotation is stopped and excess of inoculated medium is drained off from the fermenter (Roussos *et al*, 1991a).

Air is supplied from a compressor and is difated and filtered. It passes through a water bath to get humidified. The disks fermentor, also called sporulator, was used at room temperature, but a temperature control may be obtained by submerging the fermentor in a water bath. The equipment is nevertheless limited to a small production of spores (Roussos *et al*, 1991).

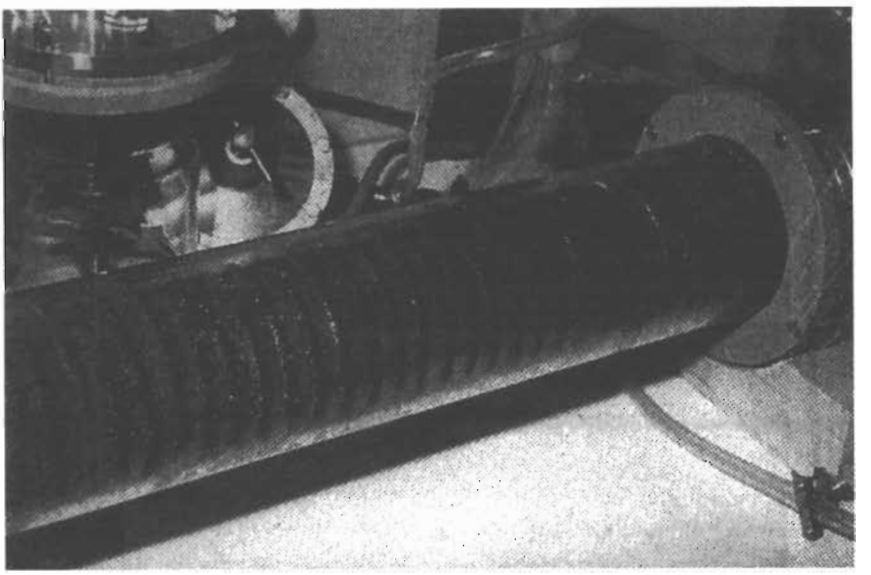

Fig. 4. Schematic representation of disks fermentor.

ZYMOTIS FERMENTOR

As the process of spore production at large scale is still not achieved, the ORSTOM group of researchers worked on the design of a new fermenter, taking into account its scale-up potential for industrial purposes. The main problem of solid state fermentation at large scale is the metabolic heat elimination. As the fermenting solid substrate forms a mycelial coat, which restricts air transfer, it seemed almost impossible to envisage large scale SSF production, with high productivity in static conditions. Large scale SSF production was, therefore, mainly carried out, using agitated systems. The equipment developed presents a very efficient cooling system, that solves the metabolic heat problem and allows very high productivity.

Zymotis is the so called new fermentor (the name comes from the Greeek term "zymotiras", which means the fermentor). It is a large scale static solid state fermentor, which offers efficient control of various parameters, such as aeration, temperature and substrate moisture. The equipment has been patented in 1985 (Prébois *et al*, 1985) and its use has been published (Roussos *et al*, 1993).

The fermentation vessel is a rectangular box of 5,000 mm in length, 4,000 mm in width and 6,500 mm in height, made-up of acrylic material and giving a working capacity of 100 L. An acrylic dome-shaped cover fits closed on the top side of the unit, avoiding contamination risks from outside to inside and vice versa. It can, thus, be used even for the production of hazardous micro-organisms or molecules.

Substrate temperature is controlled by water circulation through a total of 10 stainless steel heat exchanger plates, which are provided along the depth of the fermentor vessel and assembled in parallel to each others (Fig. 5), all of them fitted tightly in the fermentor. This makes nine rectangular compartments in the fermenation vessel (Fig. 6). The compartment length can, therefore, be modified by suitably shifting the location of the heat exchanger plates so as to study the effect of substrate thickness, as a function of various growth and production parameters. It is also possible to remove some of the heat exchanger plates, as their water inlet and outlet are individually connected from outside the fermenter.

Aeration is provided, using the same system as that for the disks fermentor. Air is of the same quality (de-oiled, filtered and humidified). It is distributed in each compartment by using nine inlet tubes. For a more precise work, each air inlet can be equipped with its own air control valve, rotameter and humidifier. The system of control and monitoring, developed by ORSTOM (Saucedo-Castañeda *et al*, 1992), can also be used with the Zymotis fermenter.

As the material used for the building of the Zymotis fermentor can not be steamed or autoclaved, it is not possible to work in strictly sterile conditions, but good conditions of sterility can be obtained by cleaning the apparatus with an alcoholic

solution. It is also envisaged to replace acrylic sheets by stainless steel at industrial scale, to obtain a stream resistant equipment, that would be sterilizable.

This unit is of potential promise for industrial exploitation of SSF, as its scale-up is easy. Humidity saturation, temperature and aeration control can be monitored. Futhermore, microorganism growth and metabolite production, with high productivity, can be obtained, using the exhaust gas monitoring system developed by ORSTOM, thereby, making Zymotis an automated high productivity fermenter.

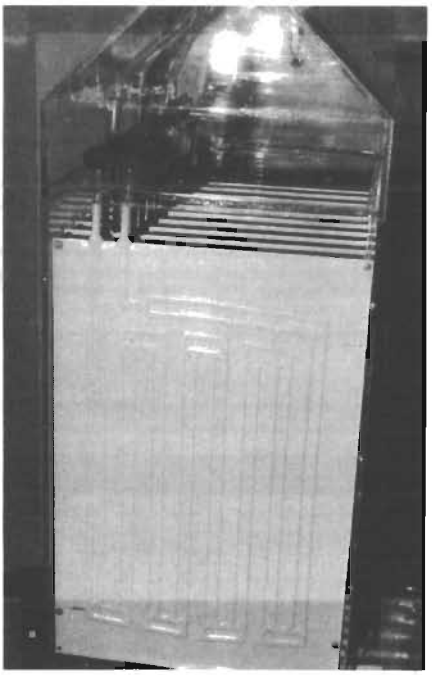

Fig. 5. Zymotis heat exchanger plate showing the tubular pipes for water circulation.

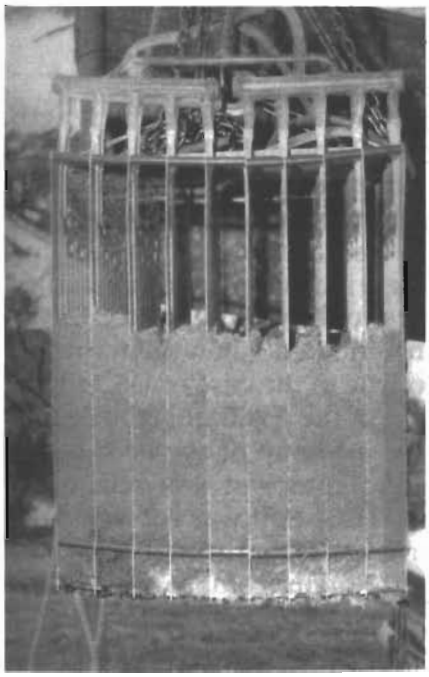

Fig. 6. View showing the rectangular compartments (9 Nos.) of the Zymotis.

MULTIPLE COLUMNS FERMENTOR: FMS 16 - 250

After having worked on SSF process and equipment design, ORSTOM retained 3 systems. The first is the column fermentor, which allows the study of microorganisms, processes or production parameters at a laboratory scale. The second one is the Zymotis fermenter, which allows the production and study of micro-organisms or metabolites at large scale. The third one is the system of control and monitoring of these fermenters.

In order to continue research on SSF processes, which is widely carried out at laboratory scale, ORSTOM desired to obtain a standard SSF equipment, consisting of 16 columns, based on the column principle, and designed for a distribution in the world trade market.

In 1993, Gauthier Agro-industries Ltd obtained licence for the design and manufacturing of the commercial equipment. Some modifications and implementations of the laboratory system took place.

For each laboratory system developed by ORSTOM, Gauthier Agro-industries engineers had selected some principles: fermentation columns made-up of glassware, temperature regulation by subimmersion of the column in a water bath, precise regulation of air flux and possibility of continuous analysis of gaseous effluent. Some others had to be improved, i.e., air humidity saturation, air partition, independent air flow setup on each column, continuous air flow measurement, preservation of substrate humidity along the fermentation, commodity of use in laboratory environment, and commercial design in accordance with ergonomy standards.

The working principle of the apparatus, named FMS 16 - 250, is presented in Fig.7.

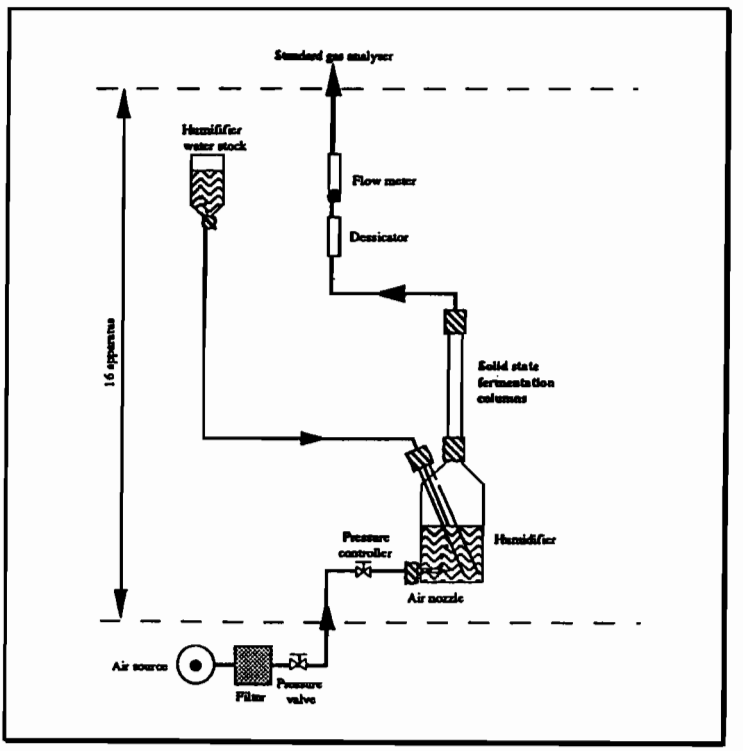

Fig.7. Working principle of FMS 16 - 250.

Air, obtained from a compressor (which can be supplied with the equipment), is difated and filtered through a submicron filter of 0.3 µm pore size. Its pressure is set by passing through a pressure regulator, equipped with a manometer. It is then distributed to feed 16 independant bioreactors of 250 mL each. A pressure setting valve is installed at each bioreactor inlet, allowing a precise setting of air flow below 4 L/h. The loss in pressure on the general circuit, when setting the air flow on a column inlet, is compensated by a high pressure in the upstream circuit, thus allowing the precise regulation of the air flow of a column, without modification of the air flow of another one.

On the bottom section, the bioreactor is composed of a glass humidifier with an air nozzle and water feeding, while the glass fermentation column is on the top part. The air nozzle allows to obtain very thin air bubbles, thus facilitating air humidification. The level of water in the humidifier is maintained at a constant value, through a water reservoir placed on the upper compartment of the FMS 16-250. A glass tube protects the water feeding from being in contact with the air bubbles. It avoids air loss via the feeding system, which otherwise would lead to an over feeding of the humidifier, because of express pressure in the water reservoir. The column (40 mm diameter and 200 mm length), is screwed on the humidifier, using a tight system containing a metallic grid, thereby retaining the solid state support in the column. Tightness is assured by a system of gasket and a positionner.

Each fermentation column outlet is connected to a chilled water condenser, then to a silica gel dessicator and finally to an air flowmeter. The condenser and dessicator remove the excess humidity, allowing a correct use of the flowmeter and the analysis of gaseous effluents by a standard apparatus (GC, IR).

The FMS 16 - 250 apparatus is composed of a metallic painted frame, with 4 stands to support a thermostated water bath (1,650 mm in length, 300 mm in width and 450 mm in height), whose temperature is regulated. The bioreactors (humidifier + column) are submerged in that water bath, so their temperature could be maintained at a constant value. Temperature can be regulated between ambient and 50°C in the standard model, but work below the ambient temperature can be envisaged by installing a chiller on the thermoregulation system. A submerged pump assures the water homogenisation in the bath and can also be used for the bath draining. The bioreactors are arranged in the water bath in 2 rows, each consisting of 8 column bioreactors.

The top of the frame offers 2 compartments, one of which is closed and where the electrical command components are located. The second one, which is illuminated, is equipped with a door and contains the water reservoirs and the systems of gaseous effluents drying. The flowmeters are placed on the door at the height of the eye, thereby facilitating reading and precise adjustment.

The air treatment system is located on the left side of the frame, while the dried gaseous effluent outlets are located on the right side of the frame. The pressure setting valves are located on the front side of the frame, just above the bioreactors at the height of the hand for easy operation. Each system is perfectly referred to the frame and panel. The air and water inlets and outlets are all well equipped with rapid connectors, to facilitate handling. An effort has been made to hide the tubings inside the frame for a better appearance.

Each column can be considered as a single bioreactor. It is independently aerated and humidified. Its temperature is regulated. It is capable of receiving any type of solid state support/substrate. Its air flow is continuously measured after dehumidifying/drying. Gaseous effluent outlets are also equipped with rapid connectors, to facilitate their connection to the standard gas analysis systems. A photograph of the FMS 16 - 250 is given in Fig. 8.

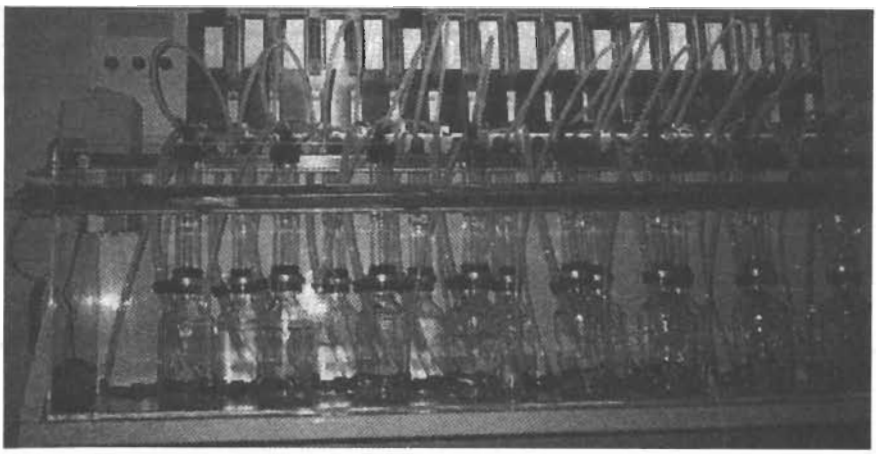

Fig. 8. Photograph of the FMS 16 - 250.

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Laboratory scale bioreactors for study of fungal physiology and metabolism in solid state fermentation system.

In : Roussos Sevastianos (ed.), Lonsane B.K. (ed.), Raimbault Maurice (ed.), Viniegra-Gonzalez G. (ed.) Advances in solid state fermentation : proceedings of the 2nd international symposium on solid state fermentation. Dordrecht : Kluwer, 93-111.

FMS-95 : Solid State Fermentation : International Symposium, 2., Montpellier (FRA), 1997/02/27-28. ISBN 0-7923-4732-3