Production of mycelial cell inoculum of *Pleurotus opuntiae* on natural support in solid state fermentation

S. ROUSSOS[1], E. BRESSON[1], G. SAUCEDO-CASTANEDA[2], P.MARTINEZ[3], J. GUINBERTEAU[4] AND J.M. OLIVIER[4]

1 ORSTOM, Laboratoire de Biotechnologie, B.P. 5045, 34032 Montpellier cedex, France.

2 Universidad Autónoma Metropolitana de Iztapalapa, Departamento de Biotecnología, A.P. 55-535, C.P. 09340 México D.F., Mexico.

3 Universidad Autónoma Tlaxcala, Departamento de Biotecnología, A.P. 55-535, C.P. 09340 México D.F., Mexico.

4 INRA, Station de Recherche sur les Champignons, BP 81, 33883 Villenave d'Ornon cedex, France.

SUMMARY

A new culture technique was developed for studying the physiology of *Pleurotus* mycelium in solid state fermentation (SSF) system. This method consists of utilization sugar cane bagasse, a by-product of sugar industry, as solid support for absorbing a liquid culture medium. Compared with classical techniques, it offers the advantage of better control of liquid medium composition as well as culture conditions. Fermenters were coupled with on-line gas analysis by using a gas chromatograph equipped with thermal conductivity detector. Solid state culture were aerated at a very low flow air rate (0.04 ml/g moist solids). Concentrations of CO_2 and O_2 in the exhaust air from fermenters were continuously analysed. By means of this technique, culture conditions were optimized respect to the ratio urea/ammonium sulphate, the particle size of bagasse and inoculation rate. Under best conditions, nearly 90% of substrate was consumed in 6 days. This culture technique offers, excellent practicability for the development of mycelium inocula for commercial exploitation of *Pleurotus* spp.

Keywords: Solid state fermentation, natural support, sugarcane bagasse, on-line gas analysis, gas chromatograph, mycelium, *Pleurotus opuntiae*, respirometry, physiology, endogenous pH control, inoculum production.

RESUME

Production de mycelium de *Pleurotus opuntiae* sur support naturel en fermentation en milieu solide.

ROUSSOS S., BRESSON E., SAUCEDO-CASTANEDA G., MARTINEZ P., GUINBERTEAU J. ET OLIVIER J.M.

Une nouvelle technique a été mise au point pour étudier la physiologie de croissance mycélienne de *Pleurotus opuntiae* cultivé en milieu solide. Cette méthode consiste à utiliser la bagasse de canne à sucre comme support solide pour y absorber une solution nutritive. Comparée à des techniques classiques, cette méthode offre des avantages pour un meilleur contrôle de la composition des solutions nutritives tout en respectant les conditions définies de fermentation en milieu solide. Les cultures en milieu solide ont été aérées avec un très faible débit d'air (0.04 ml/g de milieu humidifié). Les concentrations en CO_2 et en O_2 ont été analysées à partir des prélèments automatiques des effluents gazeux à la sortie des fermenteurs en utilisant un chromatographe de gaz équipé d'un détecteur à conductivité thermique. Par cette technique, les conditions de culture ont été optimisées en particulier le rapport urée/sulfate d'ammonium, la taille des particules du support et de l'inoculum. Sous ces conditions, environ 90% du substrat carboné a été consommé en 6 jours. Cette technique de culture offre des perspectives interessantes pour la production de mycélium de Pleurotes et peut être utilisée pour une exploitation commerciale.

Mots clés: Fermentation en milieu solide, support naturel, bagasse de canne à sucre, analyse en ligne, effluents gazeux, chromatographie en phase gazeuse, mycelium, *Pleurotus opuntiae*, respirometrie, physiologie, contrôle endogène du pH, production d'inoculum.

INTRODUCTION

Production of mushrooms (*Agaricus, Pleurotus, Lentinus, Auricularia*, etc) has witnessed significant increase throughout the world, the increase being from $2182x10^3$ to $3763x10^3$ tonnes/annum in the last ten years (Chang and Miles, 1991). *Pleurotus* is cultivated extensively in tropical areas and the increase in its production is highest in last ten years (169 to 909 tonnes/annum) among the different cultivated mushrooms. Even, the yield of *Pleurotus* is also higher.

Latin American countries, like Mexico, produce mainly *Agaricus*, but the production of *Pleurotus* is also on increase (Martinez-Carrera *et al.* 1989). The inoculum, popularly referred as spawn, for mushroom production is generally of the mycelial inoculum in the substrate used for mushroom cultivation (Fritsche, 1981; Yang and Jong, 1987). Straw of different agro-industrial residues can also be used for spawn production, but it poses problems in uniform distribution of the inoculum. Moreover, the cereal grains are costly and appropriate grains, such as those of wheat or sorghum, are not sufficiently available for spawn production, as these are consumed as stable food in many countries (Goltapeh and Kapoor, 1987). In addition, these are not produced in a number of tropical countries.

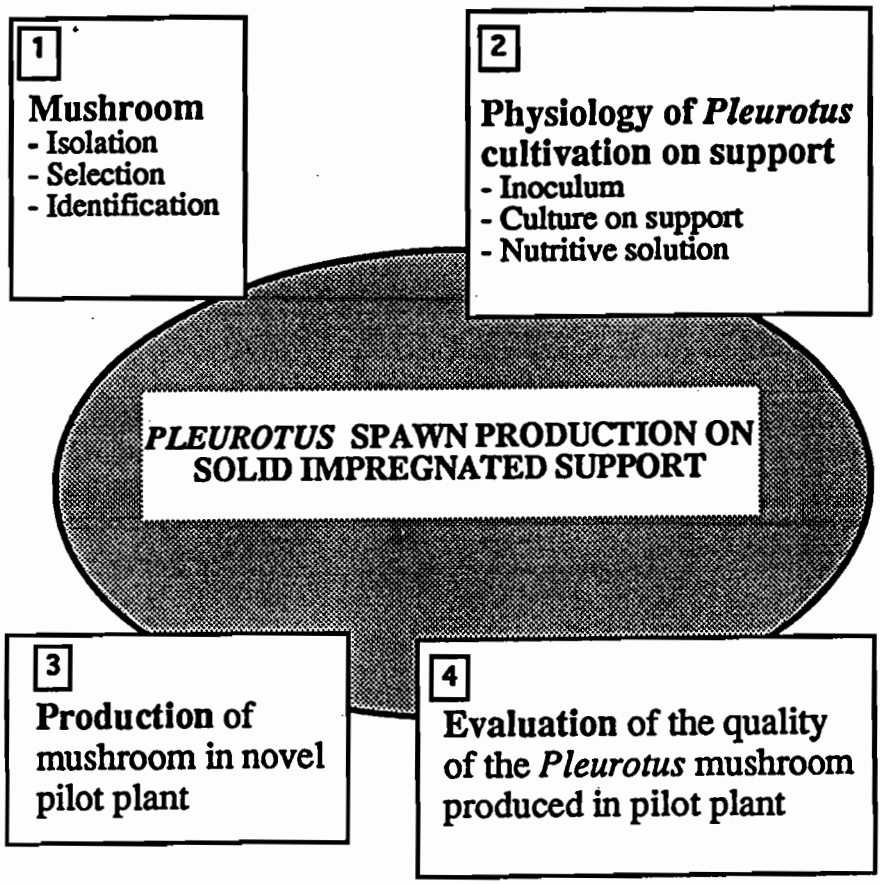
In their programme to initiate large scale production of *Pleurotus* Mexican industries faced problems in economic production of spawns of *Pleurotus* on wheat grains at industrial scale. Hence a need was felt for developing a simpler, cost-efficient and newer solid state fermentation process, based on substrate other than wheat or cereal grains (Roussos, *et al.* 1990). Sugar cane pith bagasse has been successfully used in ORSTOM, Montpellier, France, as an inert support in solid state fermentation for production of various fungal and yeast metabolites (Oriol *et al*, 1987; Trejo-Hernandez *et al.* 1993; Saucedo-Castañeda *et al*, 1993) as well as biomass (Saucedo-Castañeda *et al.* 1992). Its use for spawn production, after absorption of nutritive medium, may also prove potential and provide advantage of economy.

A team was, therefore, formulated involving l'Institut Français de Recherche Scientifique pour le Développement en Coopération (ORSTOM), Montpellier, France; Institut National de la Recherche Agronomique (INRA), Bordeaux, France; and Universidad Autonoma de Tlaxcala (UAT), Tlaxcala, México, with a goal of the production of *Pleurotus* spawn on inert natural solid support impregnated with nutritive medium. The work was carried out in four stages, as shown in Fig. 1.

The aim of the work, reported in the present paper, was to use chemically defined synthetic medium, absorb it on inert support and study the utilization of soluble sugars, pH evolution and support colonization during the mycelial growth in column fermenter, as well as under the influence of specific physico-chemical factors. In addition, attempt has been made to relate on-line measurement of oxygen utilization and carbon dioxide evolution with physiological state of the culture.

It is emphasized that a lot of work has been carried out on production of spawn in liquid and solid culture for use in the production of *Pleurotus*. Most of it concerns with delignification (Sannia *et al.* 1991; Valsameda *et al.* 1991; Martinez *et al.* 1993), bioconversion of lignocellulosic agro-residues (Martinez-Carrera *et al.* 1985; 1986; Guzman and Martinez-Carrera, 1986; Bisaria *et al.* 1987; Gupta and Langar,1988) and concomitant formation of enzymes (Kannan and Oblisami 1990;) during the growth of the spawn on cereal grains (Aslam Azizi *et al.* 1990, Guillen F.

et al. 1990). In all these cases, molasses, starchy substrates or cereal grains were used as substrate in polyethylene bags, bottles or Petri dishes. The column fermenter was never used earlier, nor the medium was aerated during the cultivation. Similarly, no work has been carried out yet on the physiology and metabolism of *Pleurotus* mycelial cell growth in solid state fermentation system.



Organisms participants :

1- UAT, INRA Bordeaux ; 2- ORSTOM Montpellier
3 - UAT, Mexico 4- INRA, Bordeaux

Fig. 1 : Stages of the collaborative projet.

MATERIALS AND METHODS

MICROORGANISMS

A total of 6 strains each of *Pleurotus* were isolated at the Universidad Autonoma de Tlaxcala, Mexico, and INRA, Bordeaux, France. These were subjected to preliminary sceening and *P. opuntiae* C73, the isolate from Tlaxcala, Mexico, was selected for further studies. It was maintained on potato dextrose agar plates at 4°C and subcultured every two months.

INOCULUM DEVELOPMENT

Two different media, potato dextrose agar and sugar cane pith bagasse medium, were used for inoculum development for use in physiological studies. The inoculum in each case was developed first in Petri dishes, using above media. The plates were incubated at 25°C in the absence of light and without any aeration for 5 days. This served as a freshly grown culture for futher development of inoculum in culture bottles of 250 ml total capacity in case of potato dextrose agar or column fermenter of 100 ml total capacity in case of bagasse medium.

The sugar cane pith bagasse was washed repeatedly to remove all the nutritive substances, sieved to obtain a particle size of 0.8 to 2.0 mm length, dried and sterilized as per the procedure of Saucedo-Castañeda *et al* (1992). Such dried bagasse was used throughout this study to eliminate any influence of the variation in the composition of sugar cane pith bagasse and its processing method. It was hydrated to 70% initial moisture content with a nutritive solution containing (g/L) soluble starch 70.0, malt extract 10.0, urea 2.9, ammonium sulphate 3.5, KH_2PO_4 4.0 and distilled water 1000. The moist bagasse medium was autoclaved at 121°C for 30 min, cooled to 25°C, inoculated and 40 g moist medium was charged in column fermenter.

In case of potato dextrose agar medium, the autoclaving was at 121°C for 15 min in the culture bottle itself. It was used in such a way that the flat surface of the 1 L solidified medium occupies about 50% of the total legth of 8O cm of the bottle.

The inoculation of these two media was done by using 1 cm^2 units of the mycelial mat from Petri dishes. In each case, three such units were used and these were placed at equal distance from each other, one unit being at the bottom side of the medium, while another at the top side of the medium. The growth of the mycelial cells was allowed to takes place at 25°C under darkness and without any aeration for 5 days. The mycelial mat formed on potato dextrose agar served as source of final inoculum

in case of culture bottle. The mycelial cells grown in the column fermenter were mixed thoroughly with the bagasse medium for use as inoculum. This latter inoculum is referred as activated bagasse hereafter.

Comparison of Inoculum Grown on Potato Dextrose Agar in Culture Bottles and Bagasse Grown Column Fermenter

The inocula grown as above in two media were tested for comparative performance in column fermenter, using sugar cane pith bagasse, after impregnation with nutritive solution. In case of inoculum from potato dextrose agar, three inoculum units were used to inoculate the medium in the column. Thoroughly mixed activated bagasse was used at 10% (w/w) level, based on the moist weight of the inoculum as well as the fresh bagasse medium. In the latter case, the inoculum was mixed thorougly with the fresh medium and the inoculated medium was charged in the column. In case of inoculum from potato dextrose agar, the fresh bagasse medium was charged in the column and the inoculum units were placed appropriately during the charging procedure. The inoculation was done in laminar air flow unit in all the cases.

The column fermenter unit, consisting of 24 columns each of 100 ml total capacity and as developed by Raimbault and Alazard (1980) was adopted for all studies on growth and physiology of *P. opuntiae*. The fermentation was carried out at 25°C for 74 h in darkness at the aeration rate of 0.15 ml air/min/g dry matter. Samples were removed at specific intervals. The contents of each column served as a sample. The growth of the mycelial cells is expressed as the velocity of the growth/100 g bagasse support medium. The cultures were also observed every day macroscopically to note the mycelial growth, especially the compactness of the substrate due to the mycelial growth.

Effect of Light on Growth and Metabolism

The column fermenters were wrapped in aluminium foil for fermentation in darkness. In case of fermentation under light, the natural light cycle was used, i.e. the fermentation was no light in the night. All other conditions were as described above.

Effect of Ratio of Urea and Ammonium Nitrogen

Three different ratios were used. In medium A, the ratio of urea to ammonium nitrogen was 1:3, while media B and C contained 1:2 and 3:1 ratios (Table 1). All other conditions were same as specified earlier. In all the case the C/N ratio was maintained constant at 14 (Khanna and Garcha, 1985).

Table 1 : Composition of the media containing different ratios of urea and ammonium nitrogen.

Composition	Medium		
	A	**B**	**C**
Sugar cane pith bagasse	80	80	80
Soluble starch	20	20	20
Malt extract	2.9	2.9	2.9
Nutritive solution (g/100 g starch)			
Urea	2.4	6.0	8.4
$(NH_4)_2SO_4$	9.7	6.1	3.7
KH_2PO_4	5.0	5.0	5.0
Distilled water	1000	1000	1000
pH	6.1	6.1	6.2

SAMPLE TREATMENT AND ANALYSES

Dry matter content was determined in each sample by drying it at 105°C overnight and the data are expressed as % dry matter loss. These results were also used to calculate moisture content of the fermentation medium. For measurement of total soluble sugar and pH of the medium, 100 ml distilled water was added to 5 g sample, mixed for 2 min in Ultraturrax and the clear filtrate was subjected to analyses. Total soluble sugars were estimated by the method of Dubois *et al.* (1956) while pH of the clear filtrate was measured using pH meter. The exhaust gas from each column was subjected to on-line analysis of carbon dioxide during whole of the fermentation period, as per the methodology of Saucedo-Castañeda *et al.* (1993).

RESULTS AND DISCUSSION

COMPARISON OF INOCULA

In solid state fermentation, the size of inoculum is often given as number of spores/cells per g moist medium or initial dry matter content of the medium (Raimbault and Alazard, 1980; Oriol *et al*, 1987; Roussos *et al*, 1991; Soccol *et al*,

1994). In case of *Pleurotus*, this strategy can not be used. In another work (Martinez-Ortiz and Roussos, 1991), it was been clearly demonstrated that the apical growth of *Pleurotus* is linear. It is therefore, necessary to augment the inoculum units to obtain a rapid and homogeneous colonization of the substrate. For this reason, three units of inoculum were used in case of potato dextrose agar medium, while 10% of the fermented bagasse support medium was used in column fermenter.

Table 2 : Comparative performance of the inoculum developed on potato dextrose agar and on bagasse support medium.

Inoculum source	Fementation time (h)	pH of the medium	Consumption of soluble sugar (%)	content of the medium (%)	Loss of dry matter (%)
PDA*	0	6.1	0.0	77.6	0.0
	70		30.0	76.3	12.0
	80	6.2	43.0	77.8	19.5
	103	6.2	72.0	78.3	22.0
	143	6.5	82.0	79.3	28.7
BSM**	0	6.2	0.0	76.7	0.0
	20	5.9	5.2	76.4	2.0
	42	5.1	32.0	78.0	9.4
	50	5.0	43.0	78.0	12.0
	74	4.8	62.0	78.0	27.0

PDA*: from Potato dextrose agar ; BSM** : from Bagasse support medium

The data on the comparative performances of these two types of inocula are presented in Table 2. The data indicated that the start of the growth of *P. opuntiae* C73 is directly related to the source of inoculum. Another industrially and economically important observation is the significant reduction in the lag phase of the culture, with the use of inoculum of activated bagasse. On the contrary, the start of the growth of the mycelial cells is very long and the growth is very slow in case of the use of inoculum from potato dextrose agar (PDA). This prolongs the process time in case of inoculm from PDA, though the velocity of the production of carbon dioxide is higher (Fig. 2). The results demonstrate that the total carbon dioxide production is affected by the source of the inoculum. In case of activated bagasse inoculum, the rate of total dioxide production is 30 ml/g initial dry matter (DM) after 74 h, as

against that of 151 ml/g in case of the inoculum from PDA. Moreover, the total production of carbon dioxide after 140 h in case of inoculum from PDA is comparatively much higher , through its production is slower.

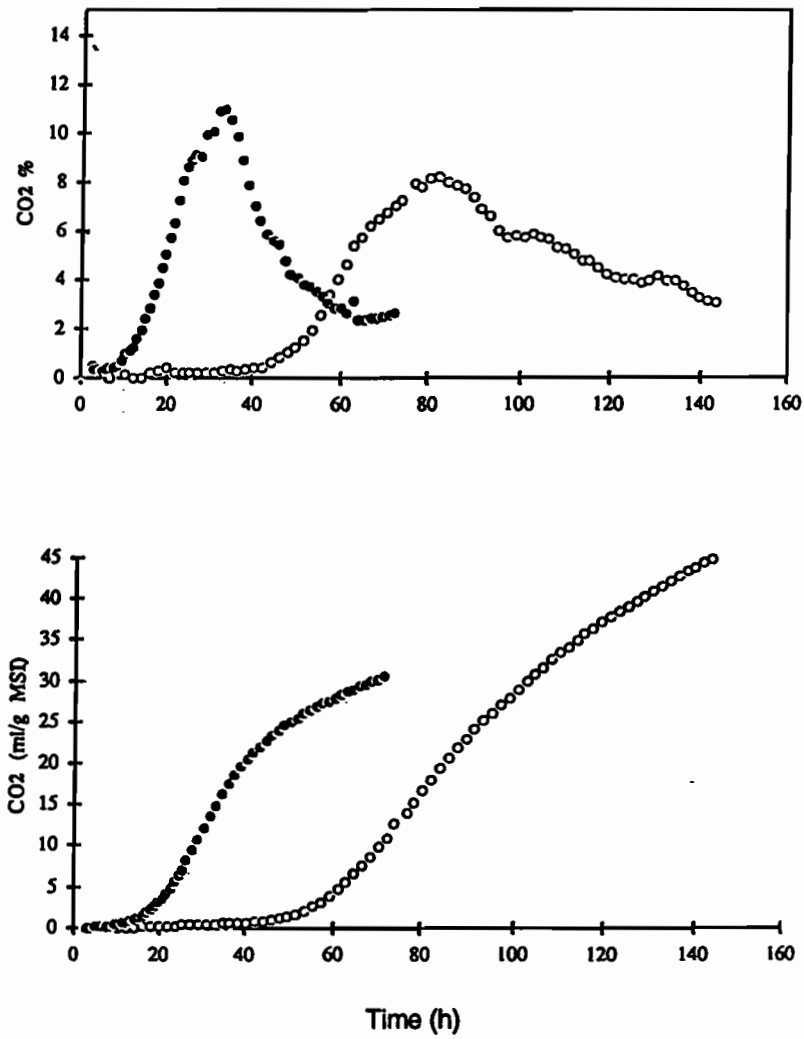


Fig. 2 : Influence of the source of inoculum on evolution of carbon dioxide during growth of *P. opuntiae* C73 in bagasse support medium in column fermenter. MSI = Initial Dry Matter. Inoculum from bagasse support medium (●), and from Potato Dextrose Agar (○).

In both cases, the moisture content of the medium during the fermentation is fairly constant and varies between 76.3 to 79.3%. At the end of both the fermentation (143 h in case of inoculum from PDA and 73 h in case of activated bagasse inoculum), the solubles sugars consumption was 82% in the former case, as against that of 62% in the latter case. In addition to this difference, another critically important difference was observed with respect to the change in the pH of the medium during the course of fermentation. In case of inoculum from PDA, the pH of the medium remained stable and varied between 6.1 to 6.5 during the course of 143 h fermentation. However, the pH of the medium was reduced rapidly from initial value of 6.2 to that of 4.8 by the end of 74 h fermentation. It is clearly apparent that this reduction in the pH of the medium stopped the respiration of the culture and, consequently, the consumption of the carbon substrate (solubles sugars).

In addition, the % dry matter loss is more rapid and higher in case of activated bagasse inoculum. The value at 74 h fermentation was 27%, as against that of 12% at 70 h in case of inoculum from potato dextrose agar. It is of interest to note that the % dry matter loss is nearly same at 74 and 143 h in case of activated bagasse and potato dextrose agar inocula, respectively.

On the whole, the above data indicated that the best source of inoculum is the activated bagasse. With its use, the number of inoculum points in the inoculated media are numerous and consequently very homogeneous growth is achieved and the start of the growth is also rapid. In addition, the utilization of soluble sugars is rapid, but it leads to the acidification of the medium. This problem can be overcome by using strongly buffered medium and with initial pH near to neutrality for favoring mycelial growth.

EFFECT ON LIGHT ON MYCELIAL GROWTH

Literature survey indicated the effect of light on the growth of *Pleurotus*. For example, Olivier *et al* (1991) have reported that the exposure of the culture to light during mycelial development accelerates the fructification of *Pleurotus* by reducing the lag phase and consequently, the time of cultivation. Hence, effect of light during mycelial growth of *P. opuntiae* C73 was studied.

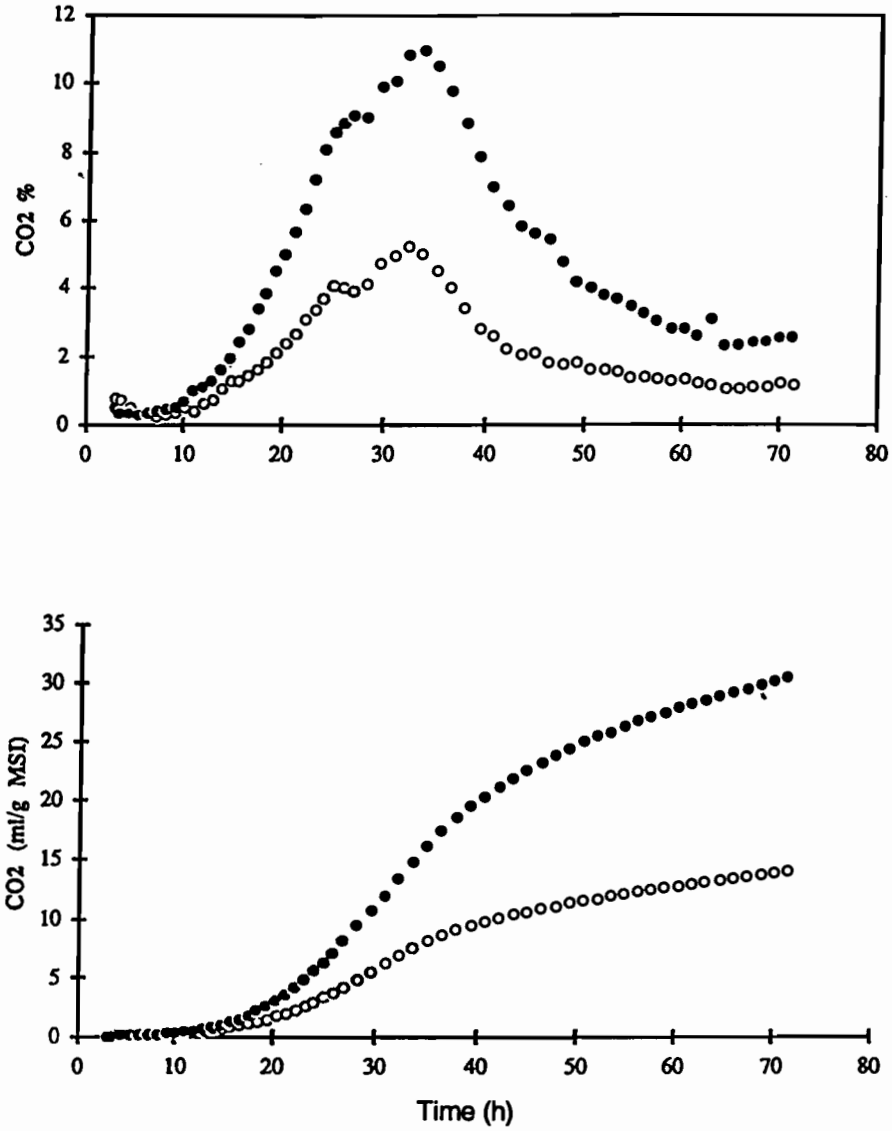


Fig. 3 : Influence of light (○) and darkness (●) on the evolution of carbon dioxide during the growth of *P. opuntiae* C73 on bagasse support medium in column fermenter. MSI = Initial Dry Matter.

The results demonstrate that the exposure of the culture to light acts negatively on the growth of the mycelium and the consumption of soluble sugars (Table 3). In particular, the maximum velocity of carbon dioxide production was 2 times lower in case of exposure to light, as compared to the cultivation in darkness (Fig. 3). The data, thus, confirm that the exposure of the culture to light during mycelial development is an important factor to control the growth and metabolism of *P.opuntiae* C73. The velocity of apical growth, the biomass formation, % loss of dry matter and consumption of soluble sugars decreased in the absence of light. However, the changes in pH and moisture content of the medium were nearly same in both the cases.

Table 3 : Influence of light and darkness on the growth of mycelium in column fermenter.

Attribute	Fermentation time (h)	pH of the medium	Consuption of soluble starch (%)	Moisture content of the medium (%)	Loss in dry matter (%)
Natural light	0	6.2	0.0	76.7	0.0
	20	6.0	0.4	77.8	7.3
	42	5.1	35.3	77.4	6.6
	50	5.0	42.5	78.3	10.5
	74	4.8	57.5	78.2	11.3
Darkness	0	6.2	0.0	76.7	0.0
	20	5.9	5.2	76.4	1.8
	42	5.1	32.0	78.1	9.2
	50	5.0	43.3	78.0	12.0
	74	4.8	62.0	78.0	26.7

It is of interest to note the profile of carbon dioxide production between 24 to 30 h in Fig. 2. At above 24 h, the carbon dioxide % in the exhaust air became stationary for few hours and then again sturted rising, but also for few hours, before declining in the further period of fermentation. It probably indicates the change in the metabolic pattern of the culture during these periods. This is also corroborated by a very large increase in the consumption of soluble sugars between 20 and 42 h (Table 3).

EFFECT OF RATIO OF UREA AND AMMONIUM NITROGEN ON THE pH CHANGES IN THE MEDIUM

Data in Tables 2 and 3 and Figs. 2 and 3 indicate that the acidification of the medium during the growth of mycelial cells results in arresting the growth and metabolism of *P. opuntiae* C73. By overcoming this problem, it may be possible to continue the growth of the culture beyond 74 h.

To avoid changes in the pH of the medium during mycelial growth in bagasse support medium and also to prevent diminution of the growth, an endogenous strategy was formulated and verified experimentally. It involves the use of different ratios of urea and ammonium nitrogen, without changing the C/N ratio of the medium (Table 1). This can prevent the changes in pH of the medium, as these will be no H^+ ions liberation during the assimilation of urea, in contrary to that in case of ammonium nitrogen. Thus, the use of more of urea nitrogen can prevent the acidification of the medium during mycelial growth.

Media A, B and C, containing three different ratios of urea and ammonium nitrogen (Table 1) as described by Saucedo-Castañeda *et al* (1992), were evaluated. Literature survey indicated the positive effect of CO_2 on the growth of *Pleurotus* (Zadrazil, 1975) and hence the rate of air flow was reduced by 10 times (10^{-2} in place of 10^{-1} ml of carbon dioxide/g initial dry matter).

The results showed that the pH values changed negligibly up to 70 h fermentation in all the three media. Thereafter, the medium A was progressively and gradually acidified, but no such change took place in media B and C, in spite of better soluble sugar utilization to a tune of more than 80%. The lower consumption of dry matter in medium A confirm that *P. opuntiae* C73 does not normally grow in medium whose pH is less than 6.0.

Data on the analysis of carbon dioxide in exhaust gases indicated different phases of mycelial growth in media B and C (Fig. 4). Total carbon dioxide production was nearly equal in these two media. The respirometric data (Fig. 4) also demonstrated very poor growth in media A.

The results (Table 4 and Fig. 4) confirm that *P. opuntiae* C73 has a optimum pH around neutrality for growth. This was also confirmed by the results in media B and C, which have a strong buffering action. The balance between these two sources of nitrogen (urea and ammonium sulphate) or a little excess of urea, permitted to maintain the pH value of the medium at near neutrality.

Table 4 : Effect of different ratios of urea and ammonium nitrogen on the growth and metabolism of *P. opuntiae* C23 in bagasse support medium in column fermenter.

Parameters Evolution	Fermentation time (h)	Culture media		
		A	B	C
pH of the medium	0	6.02	6.06	6.14
		5.98	6.10	6.17
	80	5.68	6.24	5.99
	103	5.76	6.24	6.68
	143	5.56	6.50	6.78
Consumption of soluble sugars (%)	0	0.0	0.0	0.0
	70	16.5	30.5	50.9
	80	31.0	42.7	52.4
	103	11.4	71.8	74.0
	143	39.0	81.7	85.7
Loss of dry matter (%)	0	0.0	0.0	0.0
	70	3.7	11.9	13.4
	80	10.6	19.5	21.8
	103	9.8	21.9	25.0
	143	13.7	28.7	25.6
Moisture content of the medium (%)	0	78.1	77.6	78.7
	70	77.8	76.3	77.3
	80	78.0	77.8	78.0
	103	79.4	78.3	79.5
	143	78.9	79.3	80.1

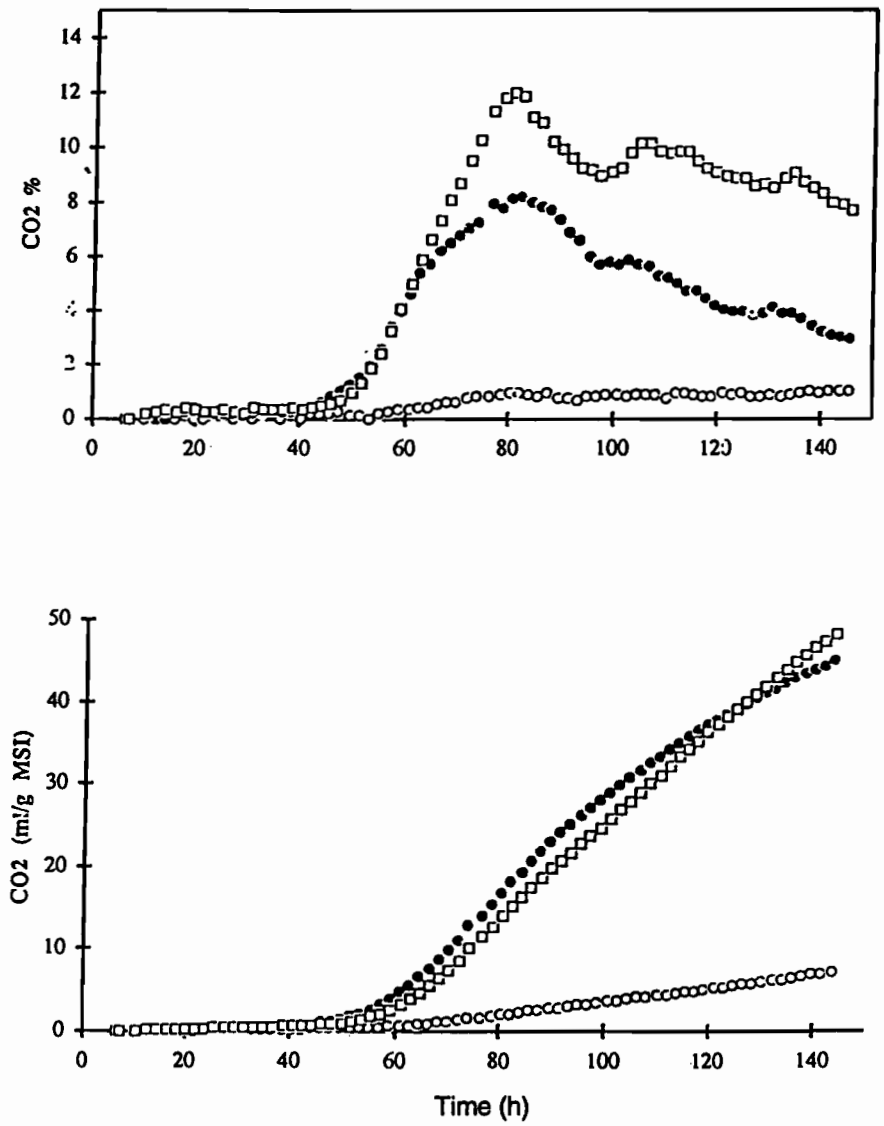


Fig. 4 : Influence of the ratio of urea and ammonium nitrogen on the evolution of carbon dioxide by *P. opuntiae* C73 on bagasse support media (**A**: ○ ; **B**: ● and **C**: □) in column fermenter. MSI = Initial Dry Matter.

CONCLUSIONS

Sugar cane pith bagasse as inert support, after impregnation with nutritive solution, seves as a better source of inoculum of *P. opuntiae* C73, as compared to the conventional potato dextrose agar medium. The changes in the pH of the inoculum medium can be overcome by using appropriate ratios of urea and ammonium nitrogen in the medium. Activated bagasse may prove as more efficient spawn and avoid reliance on cereal grains. Work on these aspects is in progress. This culture technique can be used for study physiology and metabolism of different mushrooms and ectomycorrhizal fungi mycelial growth.

ACKNOWLEDGMENTS

This work was financiated by the french government. Projet MRT/ORSTOM N° 90.L.0724. Authors thank Dr. B.K. Lonsane, CFTRI-Mysore, for his critical and very constructive discussions.

REFERENCES

Aslam Azizi, K., Shamala, T.R. and Sreekantiah, KR. 1990. Cultivation *of Pleurotus sajor-caju* on certain agro-industrial wastes and utilization of the residues for cellulase and D-xylanase production. *Mush. J. Tropics*, 10: 21-26.

Bisaria, R., Madan, M. and Bisaria, V.S. 1987. Mineral content of the mushroom *Pleurotus sajor-caju* cultivated on different agro-residues. *Mush. J. Tropics*, 7: 53-60.

Chang S.T. and Miles 1991. World production of edible mushrooms. *Muschroo J.* 504: 15-15.

Dubois, M., Gilles, K.A., Hamilton, J.K., Rebers, P.A. and Smith, F. 1956. Colorimetric method for determination of sugars and related substances. *Analytical Chem.* 28: 350-356.

Fritsche, G. 1981. Some remarks on the breeding, maintenance of strains and spawn of *Agaricus bisporus* and *A. bitorquis*. Mushroom Science XI. *Proceedings of the Eleventh International Scientific Congress on the cultivation of Edible Fungi*, Australia, pp. 367-385.

Guillen, F., Martinez, A.T. and Martinez, M.J. 1990. Production of hydrogen peroxide by aryl-alcohol oxidase from the ligninolytic fungus *Pleurotus eryngii. Appl. Microbiol. Biotechnol.*, 32 : 465-469.

Goltapeh, E.M. and Kapoor, J.N. 1981. New substrates for spawn production of button mushroom *Agaricus bisporus* (Lange) Singer. Mushroom Science XI. *Proceedings of the Eleventh International Scientific Congress on the cultivation of Edible Fungi*, Australia, pp. 281-285.

Gupta, V.K. and Langar, P.N. 1988. *Pleurotus florida* for upgrading the nutritive value of wheat straw. *Biological Wastes*, 23 : 57-64.

Guzman, G. and Martinez-Carrera, D. 1986. *Pleurotus* growing on coffee-pulp in a semi-industrial plant. *Mush. J. Tropics*, 3: 7-10.

Kannan, K. and Oblisami, G. 1990. Enzymology of ligno-cellulose degradation by *Pleurotus sajor-caju* during growth on paper-mill sludge. *Biological Wastes*, 33 : 1-8.

Khanna, P. and Garcha, H.S. 1985. Physiological studies on *Pleurotus* spp. I. Nitrogen utilization. *Mush. Newsletter Tropics*, 5:16-21.

Martinez, A.T., Gonzalez, A.E., Martinez, M.J., Guillen, F. and Barrasa, J.M. 1992. Biotecnología en la industria papelera: utilización de los hongos y sus enzimas en la fabricación de papel y en el tratamiento de efluentes. *Rev. Iberoam. Micol.* 9: 76-80.

Martinez-Carrera, D., Guzman, G. and Soto, C. 1985. The effect of fermentation of coffee pulp in the cultivation of *Pleurotus ostreatus* in México. *Mush. J. Tropics*, 1: 21-28.

Martinez-Carrera, D., Morales, P., Soto, C., Murrieta, E. and Guzman, G. 1986. Cultivo de *Pleurotus ostreatus* sobre hojas usadas en la extracción de aceites esenciales. *Rev. Mex. de Micología*, 2: 119-124.

Martinez-Carrera, D., Morales, P. and Sobal, M. 1989. Produccion de hongos comestibles sobre pulpa de café a nivel comercial. *In* Roussos, S., Licona, F.R. and Gutierrez, R.M. (Eds.), *I Seminario Internacional de Biotecnologia en la Industria Cafetalera.* Memorias, Xalapa, Ver. Mexico, p. 177-184.

Martinez-Ortiz, P. and Roussos, S. 1991. Production de blanc de *Pleurotus cornucopiae* par fermentation en milieu solide sur rafles de mais au Mexique: Mise au point de la méthode de culture en FMS. *Projet MRT/ORSTOM N°90.L.0724. Rapport N°2*, ORSTOM, Montpellier.

Olivier, J.M., Laborde, J., Guinbertau, J. and Poitou, N. 1991. La culture de champignons, Armand Colin, Paris.

Oriol, E., Schettino, B., Viniegra-Gonzalez, G. and Raimbault, M. 1988. Solid state culture of *Aspergillus niger* on support. *J. Ferment. Technol.* 66: 57-62.

Raimbault, M. and Alazard, D. 1980. Culture method to study fungal growth in solid fermentation. *European J. Appl. Microbiol.*, 9: 199-209.

Roussos, S., Martinez, P. and Olivier, J-M 1990. Production de blanc de *Pleurotus cornucopiae* par fermentation en milieu solide sur rafles de maïs au Mexique. *Projet MRT/ORSTOM N° 90.L.0724. Rapport N°1*, ORSTOM, Montpellier.

Roussos, S., Raimbaul,t M., Saucedo-Castañeda, G., Viniegra-Gonzalez, G. and Lonsane, B.K. 1991. Kinetics and ratios of carboxy-methyl cellulase and filter paper activities of the cellulolytic enzymes produced by *Trichoderma harzianum* on different substrates in solid state fermentation. *Micol. Neotrop. Apl.* 4: 19-40.

Sannia, G., Limongi, P., Cocca, E., Buonocore, F., Nitti, G. and Giardina, P. 1991. Purification and characterization of a veratryl alcohol oxidase enzyme from the lignin degrading basidiomycete *Pleurotus ostreatus*. *Biochim. et Biophys. Acta*, 1073: 114-119.

Saucedo-Castañeda, G., Lonsane, B.K., Navarro, J.M., Roussos, S. and Raimbault, M. 1992. Potential of using a simple fermenter for biomass built up, starch hydrolysis and ethanol production: Solid state fermentation system involving *Schwanniomyces castellii* , *Appl. Biochem. Biotechnol.* 36: 47-61.

Saucedo-Castañeda, G., Trejo-Hernandez, M.R., Lonsane, B.K., Navarro, J.M., Roussos, S., Dufour, D. and Raimbault, M. 1993. On-line monitoring and control system for CO_2 and O_2 concentrations in aerobic and anaerobic solid state fermentations. *Process Biochem.* 29: 13-24.

Soccol, C., Marin, B., Raimbault, M. and Lebeault, J.M. 1994. Breeding and growth of *Rhizopus* in raw cassava by solid state fermentation. *Appl. Microbiol. Biotechnol.* 41: 330-336.

Trejo-Hernandez, M.R., Lonsane, B.K., Raimbault, M. and Roussos, S. 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029c in solid state fermentation system: Influence of the composition of liquid medium used for impregnating sugar cane pith bagasse. *Process Biochem.* 28: 23-27.

Valsameda, M., Martinez, M.J. and Martinez, A.T. 1991. Kinetics of wheat straw solid-state fermentation with *Trametes versicolor* and *Pleurotus ostreatus* - lignin and polysaccharide alteration and production of related enzymatic activities. *Appl. Microbiol. Biotechnol.*, 35: 817-823.

Yang, Q.Y. and Jong, S.C. 1987. A quick and efficient method of making mushroom spawn. *Proceedings of the Twelfth International Congress on the Science and Cultivation of Edible Fungi.* Braunschweig, Germany, pp. 317-324.

Zadrazil, F. 1975. Influence of CO_2 concentration on the mycelium growth of three *Pleurotus* species. *Europ. J. Appl. Microbiol.* 1: 327-335.

Roussos Sevastianos, Bresson Estelle, Saucedo-Castaneda G., Martinez P., Guinberteau J., Olivier J.M. (1997).

Production of mycelial cell inoculum of Pleurotus opuntiae on natural support in solid state fermentation.

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, 483-500. FMS-95 : Solid State Fermentation : International Symposium, 2., Montpellier (FRA), 1997/02/27-28. ISBN 0-7923-4732-3