GENERAL AND MICROBIOLOGICAL ASPECTS OF SOLID SUBSTRATE FERMENTATION

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Summary
We present at first some general considerations about specificity and characteristics of SSF, their advantages and disadvantages compared to LSF. We speak about micro-organisms involved in SSF processes, considering the better performances of filamentous fungi. The solid substrates and their basic macromolecular compounds are detailed in relation to this complex and heterogeneous systems. Biomass measurements are examined in detail, as so as environmental factors , both essential for studying and optimising SSF processes.

1. General considerations.

Aerobic microbial transformation of solid materials or "Solid Substrate Fermentation" (SSF) can be defined in terms of the following properties of the substrate to be transformed:
- A solid porous matrix which can be biodegradable or not but with a large surface area per unit volume, in the range of 10^3 to 10^6 m^2/l for a ready microbial growth on the solid/gas interface.

- The matrix should absorb water once or several times its dry weight with a relatively high water activity on the solid/gas interface in order to allow fast rates of biochemical processes.
- Air mixture of oxygen with other gases and aerosols should flow under a relatively low pressure and mixing the fermenting mash.
- The solid/gas interface should be a good habitat for the fast development of specific cultures of moulds, yeasts or bacteria, either by isolated or mixtures of species.
- The mechanical properties of the solid matrix should stand compression or gentle stirring as required for a given fermentation process. This requires small granular or fibrous particles which do not tend to break or stick to each other.
- The solid matrix should not be contaminated by inhibitors of microbial activities and should be able to absorb or contain available microbial foodstuffs such as carbohydrates (cellulose, starch, sugars) nitrogen sources (ammonia, urea, peptides) and mineral salts.

Typical examples of SSF are traditional fermentations such as:
- Japanese "koji" which uses steamed rice as solid substrate inoculated with solid strains of the mould *Aspergillus oryzae.*
- Indonesian "tempeh" or Indian "ragi" which use steamed and cracked legume seeds as solid substrate and a variety of non toxic moulds as microbial seed.
- French "blue cheese" which uses perforated fresh cheese as substrate and selected moulds, such as *Penicillium roquefortii* as inoculum.

- Composting of lignocellulosic fibres, naturally contaminated by a large variety of organisms including cellulolytic bacteria, moulds and *Streptomyces sp.*
- In addition to traditional fermentations new versions of SSF have been invented. For example, it is estimated that nearly a third of industrial enzyme production in Japan which is made by SSF process and koji fermentation has been modernised for large scale production of citric and itaconic acids.

Furthermore, new applications of SSF have been suggested for the production of antibiotics (Barrios & al., 1988) or enriched foodstuffs (Senez et al., 1979).

Presently SSF has been applied to large scale industrial processes mainly in Japan. Traditional *koji* , manufactured in small wooden and bamboo trays, has changed gradually to more sophisticated processes: fixed bed room fermentations, rotating drum processes and automated stainless steel chambers or trays with microprocessors, electronics sensors and servomechanical stirring, loading and discharging. The usual scale in *sake* or *miso* factories is around 1 or 2 metric tons per batch but reactors can be made and delivered by engineering firms to a capacity as large as 20 tons (Fujiwara, Ltd.).

Outside Japan, Kumar (1987) has reported medium scale production of enzymes, such as pectinases, in India. *Koji* type processes are widely used in small factories of the Far East (Hesseltine, 1972) and *koji* fermentation as been adapted to local conditions of United States and other Western countries, including Cuba (III A). In France, a new firm (Lyven SA) was recently created to commercialise a process for pectinase production from sugarbeet pulp. Blue cheese production in France is being modernised with improvements on the mechanical conditioning of cheeses, production of mould spores and control of environment conditions.

Composting which was produced for small scale production of mush rooms has been modernised and scaled up in Europe and United States. Also , various firms in Europe and USA produce mushroom spawn by cultivating aseptically Agaricus, Pleurotus or Shii-Take on sterile grains in static conditions.

New versions for SSF reactors have been developed in France (Durand et al., 1988), Cuba (Cabello, 1985; Enriquez, 1983 and Rodriguez, 1984) and fundamental studies on process engineering are being conducted in Mexico (Saucedo, 1987).

SSF is usually a batch process using heterogeneous materials with various ages, (Raimbault, 1980 and Tengerdy, 1985), giberellic acid (Agosin et al, 1987), pectinases (Kumar, 1987; Oriol, 1988), cellulases (Roussos, 1985), spores as biopesticides, flavours and frangancies and feed detoxification. All that points will be discussed during the course.

Generally, most of the recent research activity on SSF is being done in developing nations as a possible alternative for conventional submerged cultures which are the main process for pharmaceutical and food industries in industrialised nations.

SSF seems to have theoretical advantages over LSF. Nevertheless, SSF has several important limitations. Table 1 shows advantages and disadvantages of SSF compared to LSF.

Table 2 presents a list of SSF process in economical sectors of agro-industry, agriculture and Industrial fermentation. Most of the processes are commercialised in South-East Asian, African, and Latin American countries. Nevertheless, a resurgence of interest has occurred in Western and European countries over last 10 years. The future potentials and applications of SSF for specific processes are discussed in other cessions. But briefly, we can say :

TABLE 1. **Comparison between Liquid and Solid Substrate Fermentations.**

FACTOR	Liquid Substrate Fermentation	Solid Substrate Fermentation
Substrates	Soluble Substrates (sugars)	Polymer Insoluble Substrates: Starch Cellulose Pectines lignin
Aseptic conditions	Heat sterilisation and aseptic control	Vapor treatment, non sterile conditions
Water	High volumes of water consumed and effluents discarded	Limited Consumption of water; low Aw. No effluent
Metabolic Heating	Easy control of temperature	Low heat transfer capacity
Aeration (O2)	Limitation oby soluble oxygen High level of air required	Easy aeration and high surface exchange air/substrate
pH control	Easy pH control	Buffered solid substrates
Mecanical agitation	Good homogeneization	Static conditions prefered
Scale up	Industrial equipments Available	Need for Engineering & New design Equipment
Inoculation	Easy inoculation , continuous process	spore inoculation, batch
Contamination	Risks of contamination for single strain bacteria	Risk of contamination for low rate growth fungi
Energetic consideration	High energy consuming	Low energy consuming
Volume of Equipment	High volumes and high cost technology	Low volumes & low costs of equipments
Effluent & pollution	High volumes of polluting effluents	No effluents, less pollution
Concentration S /Products	30-80 g/l	100/300 g:l

- Potentially many high value products as enzymes, metabolites, antibiotics, could be produced in SSF. But improvements in engineering and socio-economic aspects are required because processes must use cheap substrate locally available, low technology applicable in rural region, and processes must be simplified.

- Potential exists in developed countries, but require close cooperation and exchanges between developing and industrialised countries for further application of SSF.

- The greatest socio-economical potential of SSF is the raising of living standards through the production of protein rich foods for human consumption. Protein deficiency is a major cause of malnutrition and the problem will become worse with further increases in the world population. Two ways can be explored for that:

- Production of protein-enriched fermented foods for direct human consumption. This alternative involve starchy substrates for its initial nutritional calorific value. Successful production of such food will require demonstration of economical feasibility, safety, significant nutritional improvement, and cultural acceptability.

- The second alternative consists to produce fermented products for animal feeding. Starchy fermented substrates with protein enrichment could be fed to monogastric animals or poultry. Fermented lignocellulosic substrates by increasing in the fibre digestibility could be fed to ru-

minants. In this case, the economical feasibility should be decisive in comparison to the common model using protein of soybean cake, a by-product of soybean oil.

Table 2. Main applications of SSF processes in various economical sectors

Economical Sector	Application	Examples
Agro-Food Industry	Traditional Food Fermentations	Koji, Tempeh, Ragi, Attieke, Fermented cheeses
	Mushroom Production & spawn	Agaricus, Pleurotus, Shii-take
	Bioconversion By-products	Sugar cane Bagasse Coffee pulp, Silage Composting, Detoxication
	Food Additives	Flavours, Dyestuffs, Essential Fat and organic acids
Agriculture	Biocontrol , Bioinsecticide	Beauveria, Metarhizium, Trichoderma
	Plant Growth, Hormones	Gibberellins, Rhizobium, Trichoderma
	Mycorrhization, Wild Mushroom	Plant inoculation,
Industrial Fermentation	Enzymes production	Amylases, Cellulases, Proteases, Pectinases, Xylanases.....
	Antibiotic production	Penecillin, feed & Probiotics
	Organic acid Production	Citric acid Fumaric acid Gallic acid Lactic acid
	Ethanol Production	Schwanniomyces sp. Starch Malting and Brewing
	Fungal Metabolites	Hormones, Alcaloides,

Since 15 years, the Orstom group worked on solid fermentation process for improving protein content of cassava and other tropical starchy substrates using fungi, specially from *Aspergillus* group in order to transform starch and mineral salts into fungal proteins (Raimbault, 1981).

More recently, C. Soccol working at our Orstom laboratory in Montpellier, obtained good results with fungi of the *Rhizopus* group, of special interest in human traditional fermented foods (Soccol, 1993).These works are now continued in the view of increasing knowledge about specificity of strains of *Rhizopus* able to degrade the crude granules of starch, what could be simplify drastically the process of SSF.

In another hand, the ORSTOM group is collaborating since 1981 with the Mexican UAM group on the following aspects :

- Protein enrichment of Cassava and starchy substrates
- Production of organic acids or ethanol by SSF from starchy substrate and Cassava
- Digestibility of fibres and lignocellulosic materials for animal feeding

- Degradation of caffeine in coffee pulp and ensiling for conservation and detoxification
- Enzymes and fungal metabolites production by SSF using sugarcane bagasse

Main results will be discussed further in this course by the respective speakers . We are hoping that in the future, an extended collaborative program could be fitted for a best interconnection first with all other Latin-American groups of research involved in SSF, then tentatively, create an international network including American, Asian, European and Australian groups of research.

2. Micro-organisms

Bacteria, yeasts and fungi can grow on solid substrates, and find application in SSF processes. Filamentous fungi are the best adapted for SSF and dominate in research works. The Table 3 reports some examples of SSF processes for each category of micro-organisms involved.

Bacteria are mainly involved in composting, ensiling and some food processes (Doelle et al., 1992). Yeasts can be used for ethanol and food or feed production (Saucedo et al., 1991, 1992).

But filamentous fungi are the most important group of micro-organisms used in SSF process owing to their physiological, enzymological and biochemical properties.

The hyphal mode of fungal growth and their good tolerance for low Aw and high osmotic pressure conditions make fungi efficient and competitive in natural microflora for bioconversion of solid substrates.

Koji and *Tempeh* are the two most important applications of SSF with filamentous fungi. *Aspergillus oryzae* is grown on wheat bran and soybean for *Koji* production, which is the first step of soy sauce or citric acid fermentation. Koji is a concentrated hydrolytic enzymes required in further steps of the fermentation process. Tempeh is an Indonesian fermented food produced by the growth of *Rhizopus oligosporus* on soybeans. The fermented product is consumed by people after cooking or toasting. The fungal fermentation allows better nutritive quality and degrades some antinutritional compounds contained in the crude soybean.

The hyphal mode of growth gives to filamentous fungi a major advantage over unicellular micro-organisms in the colonisation of solid substrates and for the utilisation of available nutrients. The basic mode of fungal growth is a combination of apical extension of hyphal

tips, plus the generation of new hyphal tips through branching. An important feature is that although extension occurs only at the tip at a linear and constant rate, the frequency of branching make the growth of the total biomass at exponential kinetic pattern, mainly in the first steps of the vegetative stage. That point is of importance for the modelling of the growth, and we will be discussed further.

The hyphal mode of growth gives also the filamentous fungi the power to enter into the solid substrates. The cell wall structure attached to the tip and the branching of the mycelium ensure firm and solid structure. The hydrolytic enzymes are excreted at the hyphal tip, without large dilution like in the case of LSF, that makes very efficient the action of hydrolytic enzy-

mes and allows penetration into most solid substrates. Penetration increases the accessibility of all available nutrients within particles.

Table 3. Main groups of micro-organisms involved in SSF processes.

Microflora	SSF Process
Bacteria	
Bacillus sp.	Composting, Natto, amylase
Pseudomonas sp.	Composting
Serratia sp	Composting
Streptoccus sp.	Composting
Lactobacillus sp.	Ensiling, Food
Clostidrium sp.	Ensiling, Food
Yeast	
Endomycopsis burtonii	Tape, cassava, rice
Saccharomyces cerevisiae	Food, Ethanol
Schwanniomyces castellii	Ethanol, Amylase
Fungi	
Alternaria sp.	Composting
Aspergillus sp.	Composting, Industrial, Food
Fusarium sp.	Composting, gibberellins
Monilia sp.	Composting
Mucor sp.	Composting, Food, enzyme
Rhizopus sp.	Composting, Food, enzymes, organic acids
Phanerochaete chrysosporium	Composting, lignin degradation
Trichoderma sp.	Composting, Biological control, Bioinsecticide
Beauveria sp., Metharizium sp.	Biological control, Bioinsecticide
Amylomyces rouxii	Tape cassava, rice
Aspergillus oryzae	Koji, Food, citric acid
Rhizopus oligosporus	Tempeh, soybean, amylase, lipase
Aspergillus niger	Feed, Proteins, Amylase, citric acid
Pleurotus oestreatus, sajor-caju	Mushroom
Lentinus edodes	Shii-take mushroom
Penicilium notatum, roquefortii	Penicillin, Cheese

Fungi can not transport the macromolecular substrate, but the hyphal growth allows a close contact between hyphae and substrate surface. The fungal mycelium synthesise and excrete high quantities of hydrolytic exoenzymes. The resulting contact catalysis is very efficient and the simple products are in close contact to the mycelium where they can enter across the cell membrane for biosynthesis and fungal metabolic activities. This contact catalysis by enzymes can explain the logistic model of fungal growth commonly observed (Raimbault, 1981). Also that point will be discussed further.

3. Substrates

All solid substrates have a common feature: their basic macromolecular structure. In general. substrates for SSF are composite and heterogeneous products from agriculture or by-products of agro-industry. This basic macromolecular structure (e.g. cellulose, starch, pectin, lignocellulose, fibres etc..) confers the properties of a solid to the substrate. The structural macromolecule may simply provide an inert matrix within which the carbon and energy source (sugars, lipids, organic acids) are adsorbed (sugarcane bagasse, inert fibres, resins). But generally the macromolecular matrix represents the substrate and provide also the carbon and energy source.

Preparation and pre-treatment represents the necessary steps to convert the raw substrate into a form suitable for use, that include:

-size reduction by grinding, rasping or chopping

- physical chemical or enzymatic hydrolysis of polymers to increase substrate availability by the fungus.

- supplementation with nutrients (phosphorus, nitrogen, salts) and adequation to pH and moisture content, through a mineral solution

- Cooking or vapour treatment for macromolecular structure pre-degradation and elimination of major contaminants. Pre-treatments will be discussed under individual applications.

The most significant problem of SSF is the high heterogeneity which makes difficult to focus one category of hydrolytic processes, and leads to poor trials of modelling. This heterogeneity is of different nature:

- non-uniform substrate structure (mixture of starch, lignocellulose, pectin)

- Variability between batches of substrates limiting the reproducibility

- Difficulty of mixing solid mass in fermentation, in order to avoid compactation, which causes non uniform cultivation, gradients of temperature, pH and moisture with virtual impossibility to obtain a representative sample.

Each macromolecular type of substrate presents different kind of heterogeneity:

Lignocellulose occurs within plant cell walls which consists of cellulose microfibrils embedded in lignin, hemicellulose and pectin. Each category of plant material contain variable proportion of each chemical compounds. Two major problems can limit lignocellulose breakdown:

- cellulose exists in four recognised crystal structures known as celluloses I,II,III and IV. Various chemical or thermal treatment can change the amorphous form of cristalinity.

- different enzymes are necessary in order degrade cellulose, e.g. endo and exo-cellulases plus cellobiase

Pectins are polymers of galacturonic acid with different ratio of methylation and branching. Exo-and endo pectinases and demethylases hydrolyse pectin in galacturonic acid and metha-

nol. Hemicellulases are divided in major three groups: xylans, mannans and galactans. Most of hemicellulases are heteropolymers containing two to four different types of sugar residue.

Lignin represents between 26 to 29% of lignocellulose, and is strongly bounded to cellulose and hemicellulose, hiding them and protecting them from the hydrolase attack. Lignin peroxydase is the major enzyme involved in lignin degradation. *Phanerochaete chrysosporium* is the most recognised fungi for lignin degradation.

So the lignocellulose hydrolysis is a very complex process. Effective cellulose hydrolysis requires the synergetic action of several cellulases, hemicellulases and lignin peroxydases.

But lignocellulose is a very abundant and cheap, natural, renewable material, so a lot of works were dedicated to micro-organisms breakdown, specially fungal species.

Starch is another very important and abundant natural solid substrate. Many micro-organisms are capable to hydrolyse starch, but generally the efficient hydrolysis requires previous gelatinization. Some recent works concern the raw (crude or native) starch like it occurs naturally.

The chemical structure of starch is relatively simple compared to lignocellulose substrates. Essentially starch is composed of two related polymers in different proportion following plant material: amylose (16-30%) and amylopectin (65-85%). Amylose is a polymer of glucose linked in a -1,4 bonds mainly in linear chains. Amylopectin is a large highly branched polymer of glucose including also a -1,6 bonds at the branch points.

Within the plant, cell starch is stored in the form of granules located in amyloplasts, intracellular organelles surrounded by a lipoprotein membrane. Starch granules are highly variable in size and shape depending on the plant material. Granules contain both amorphous and crystalline internal regions in respective proportions of about 30/70 . During the process of gelatinization, starch granules swell when heated in the presence of water, which involves the breaking of hydrogen bonds, especially in the crystalline regions.

Many micro-organisms can hydrolyse starch , specially fungi which are suitable for SSF application involving starchy substrates. Glucoamylase, a-amylase, b-amylase, pullulanase and isoamylase are involved in the processes of starch degradation. Mainly a-amylase and glucoamylase are of importance for SSF.

a-amylase is an endo-amylase attacking a-1,4 bonds in random fashion which rapidly reduce molecular size of starch and consequently its viscosity and liquefaction. Glucoamylase occurs almost exclusively in fungi including *Aspergillus* and *Rhizopus* groups. This exo amylase produces glucose units from amylose and amylopectin chains.

Micro-organisms generally prefer gelatinised starch. But large quantity of energy is required for gelatinization, and it would be attractive to use organisms growing well on raw (ungelatinised) starch. Different works are dedicate to isolate fungi producing enzymes able to degrade raw starch, as has been done by Soccol et al (1991), Bergmann et al. (1988) and Abe et al. (1988).

In our lab we developed many studies concerning SSF of cassava, a very common tropical starchy crop, in the view of upgrading protein content, both for animal feeding using *Aspergillus sp.* or better for direct human consumption, using *Rhizopus*. Table 4 indicates the protein enrichment with different fungi.

Table 4. Protein enrichment of Cassava by various selected strains of fungi. (Raimbault et al., 1985)

Inoculum		Time (h)	Composition (% dry basis)	
Strain	Source		Protein	Total sugar
Aspergillus niger no. 10	Cassava	25	18.5	35.6
Aspergillus awamori no. 12	*Koji*	30	16.5	35.1
Aspergillus usamii no. M140	*Koji*	30	15.6	29.5
Monilia sitophila no. 27	Fanol	42	15.1	32.3
Rhizopus sp. no. 7	Cassava	48	14.9	39.3
Aspergillus oryzae no. M84	*Koji*	30	14.8	30.0
Aspergillus sp. no. B1	Banana	30	14.7	39.1
Aspergillus sp. no. T1	*Tempeh*	30	14.5	34.0
Aspergillus niger no. 51	Cassava	30	14.3	34.5
Aspergillus sp. no. 14	Cassava	30	14.2	37.9
Aspergillus terricola no. R3	*Ragi*	30	14.1	40.9
Aspergillus sp. no. M101	*Tempeh*	30	14.0	31.4
Aspergillus sp. no. 72	Banana	30	13.8	28.2
Aspergillus awamori no. 13	*Koji*	48	13.0	38.8
Aspergillus sp. no. M147	*Koji*	30	12.7	32.4
Aspergillus niger no. 17	Cassava	30	12.0	45.2
Aspergillus sp. no. 39	Banana	30	11.1	40.0
Aspergillus sp. no. M82	*Tempeh*	30	10.9	38.0
Raw cassava	—	—	2.50	90.00

Initial water content 50%, temperature of incubation 35°C.

Recently good results were obtain by Soccol for the protein enrichment of cassava and cassava bagasse using selected strains of *Rhizopus,* producing biotransformed starchy flours containing 10-12% of good protein, comparable to cereal. Such biotransformed Cassava flour can be used as cereal substitute for breadmaking until 20% without sensible change for the consumer .

4. Biomass Measurement

Biomass is a fundamental parameter in the characterisation of microbial growth. Its measurement is essential for kinetic studies on SSF. Direct determination of biomass in SSF is very difficult due to problems of separation of the microbial biomass from the substrate. This is especially true for SSF processes involving fungi, because the fungal hyphae penetrate into and bind the mycelium tightly to the substrate. On the other hand, for the calculation of growth rates and yields it is the absolute amount of biomass which is important. Methods that have been used for biomass estimation in SSF belong to one of the following categories.

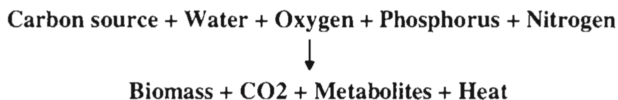
Direct evaluation of biomass

Complete recovery of fungal biomass is possible only under artificial circumstances in membrane filter culture, because the membrane filter prevents the penetration of the fungal

hyphae into the substrate (Mitchell et al, 1992). The whole of the fungal mycelium can be recovered simply by peeling it off the membrane and weighing it directly or after drying. This method obviously cannot be used in actual SSF. However, it could find application in the calibration of indirect methods of biomass determination. Indirect biomass estimation methods should be calibrated under conditions as similar as possible to the actual situation in SSF. The global mycelium composition could be appreciate through analysis of the mycelium cultivated in LSF in conditions as close as possible than SSF cultivation.

Microscopic observations can also represent good way to appreciate fungal growth in SSF. Naturally, optic examination is not possible at high magnitude but only at stereo microscope. Scanning Electron Microscope (SEM) is an useful manner to observe the mode of growth in SSF. New approach and researches are developed for image analysis by computing software in order to evaluate the total length or volume of mycelium on SEM photography. Another new approach very promising is the Confocal Microscopy based on specific reaction of fungal bio-mass with specific fluorochrome probes. Resulting 3D images of biomass can open new way to appreciate and may be measure biomass *in situ* in a near future.

Anyway, direct measurement of exact biomass in SSF is a very difficult question, then other approaches were preferred by workers. For that we can consider the global stoechiometric equation of the microbial growth:



Each component is under strict variation of others when all coefficients are maintained cons-tants. For that measuring one of them can indicate the evolution of the others.

Metabolic measurement of the biomass

- Respiratory metabolism

Oxygen consumption and carbon dioxide release result from the respiration, the metabolic process by which aerobic micro-organisms derive most of their energy for growth. These metabolic activities are therefore growth associated and can be used for the estimation of biomass biosynthesis.

As carbon compounds within the substrate are metabolised, they are converted into biomass and carbon dioxide. Production of carbon dioxide causes the weight of fermenting substrate to decrease during growth, and the amount of weight lost can be correlated to the amount of growth that has occurred.

Growth estimation based on carbon dioxide release or oxygen consumption assumes that the metabolism of these compounds is completely growth associated, which means that the amount of biomass produced per unit of gas metabolised must be constant. Sugama and Okazaki (1979) reported that the ratio of mg CO_2 evolved to mg dry mycelia formed by *Aspergillus oryzae* on rice ranged from 0.91 to 1.26 mg CO_2 per mg dry mycelium. A gradual

increase in the ratio was observed late in growth due to endogenous respiration. Drastic changes can be observed for the respiratory quotient which commonly changes with the

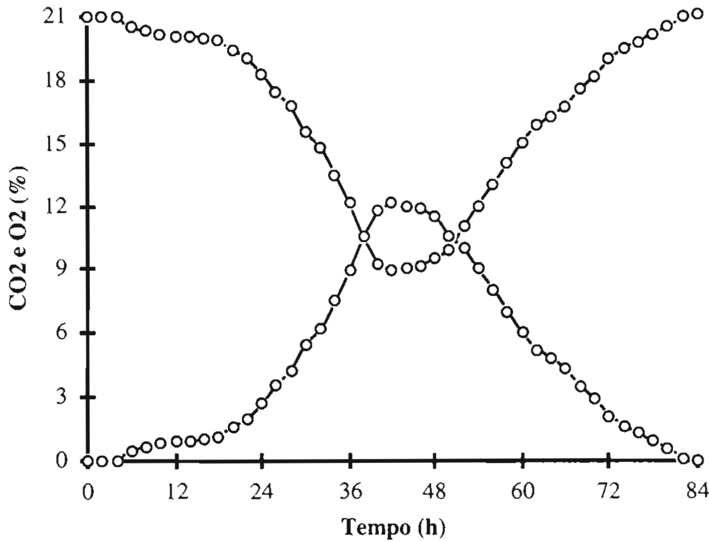


Fig. 1.- Kinetic evolution of CO2 and O2 in air flow during fermentation of *Rhizopus* on cassava.

growth phase, i.e: germination, rapid and vegetative growth, secondary metabolism, conidiation and degeneration of the mycelium.

The measurement of either carbon dioxide evolution or oxygen consumption is most powerful when coupled with the use of a correlation model. The term correlation model is used here to denote a model which correlates biomass with a measurable parameter. Correlation models are not growth models as such since they make no predictions as to how the measurable parameter changes with time. The usefulness of correlation models is that by following the profile for the change in the parameter during growth, a biomass profile can be constructed.

Application of these correlation models involving prediction of growth from oxygen uptake rates or carbon dioxide evolution rates requires the use of numerical techniques to solve the differential equations. A computer and appropriate software is therefore essential. If both the monitoring and computational equipment is available then these correlation models provide a powerful means of biomass estimation since continuous *on-line* measurements can be made. Other advantages of monitoring effluent gas concentrations with paramagnetic and infrared analysers include the ability to monitor the respiratory quotient to ensure optimal substrate oxidation, the ability to incorporate automated feedback control over the aeration rate, and the non-destructive nature of the measurement procedure.

The metabolic activity in SSF is so important that we have dedicated a special lecture to study all theoretical and practical aspects of respirometric measurement of fungal biomass cultivated

in SSF. Other speaker also will present a lot of data concerning lab and scale up experiences of respirometric measurement for several applications. More, during the practical training on the afternoons, you shall practice the laboratory methodology that we have specially design to study fungal growth on SSF based on the gas chromatography analysis of the effluent gas.

- Production of extracellular enzymes or primary metabolites

Another metabolic activity which may be growth associated is extracellular enzyme production. Okazaki and co-workers (1980) claim that the a-amylase activity was directly proportional to mycelial weight for *Aspergillus oryzae* grown in SSF on steamed rice. For growth of *Agaricus bisporus* on mushroom compost, mycelial mass was directly proportional to the extracellular laccase activity for 70 days (Wood, 1979). In our works we observed generally a good adequation between growth and hydrolytic enzymes as amylases, cellulases or pectinases (see annexed list).

In another hand, we observed frequently a good correlation between mycelial growth and organic acid production, which can be measured by the pH measurement or *a posteriori* correlated by HPLC analysis on extracts. In the case of *Rhizopus*, Soccol demonstrated a close correlation between fungal protein (Biomass) and organic acids (citric, fumaric, lactic or acetic).

Biomass Components

The biomass can also be estimated from measurements of a specific component, until the composition of the biomass is constant and stable and the fraction of the component be representative.

Protein content:

The most readily measured biomass component is protein. We used the protein content (as determined by the Lowry method) to measure the growth rate of *Aspergillus niger* on cassava meal (Raimbault and Alazard, 1980). For growth of *Chaetomium cellulolyticum* on wheat straw the TCA insoluble nitrogen was determined using the Kjeldahl method (Laukevics et al. 1984), biomass protein was then calculated as 6,25 times this value. In all cases the protein content of the biomass was assumed to be constant. Biomass protein contents measured by the biuret method were consistent with those measured by the Kjeldahl method. But unfortunately the biuret method was not suitable for application to SSF itself because of non-specific interference by the starch from the substrate. The Folin method is more sensitive and allowed a greater dilution of the sample which avoided interference from the starch in the substrate. For that we choose the Folin technique to measure protein enrichment in starchy substrates .

Nucleic acids

DNA production has been used to estimate the biomass of *Aspergillus oryzae* on rice (Bajracharya & Mudgett. 1980). The method was calibrated using the DNA contents of fungal mycelia obtained in submerged culture. DNA contents were higher during early growth and then decreased. levelling off as stationary phase was approached. The method was corrected for the DNA content of the rice, which did not change since *Aspergillus oryzae* did not produce extracellular DNases. DNA or RNA methods are reliable only if there is little nucleic acid in the substrate. and if no interfering chemicals are present .

Glucosamine

A useful method for the estimation of fungal biomass in SSF is the glucosamine method. This method takes advantage of the presence of chitin in the cell walls of many fungi. Chitin is a poly-Nacetylglucosamine. Interference with this method may occur with growth on complex agricultural substrates containing glucosamine in glucoproteins (Aidoo et al, 1981).

The accuracy of the glucosamine method for determination of fungal biomass depends on establishing a reliable conversion factor relating glucosamine to mycelial dry weight (Sharma et al, 1977). However, the proportion of chitin in the mycelium will vary with age and the environmental conditions. Mycelial glucosamine contents ranged from 67 to 126 mg per g dry mycelium. Another disadvantage of the glucosamine method is the tedious extraction procedures and processing times of over 24 hours which make it inconvenient to perform.

Ergosterol

Ergosterol is the predominant sterol in fungi. Glucosamine estimation was therefore compared with the estimation of ergosterol for determination of the growth of *Agaricus bisporus* (Matcham et al, 1985). In solid cultures directly proportional relationships for glucosamine and ergosterol against linear extension of the mycelium were obtained. Determination of ergosterol was claimed to be more convenient than glucosamine. It could be recovered and separated by HPLC and quantified simply by spectrophotometer, providing a sensitive index of biomass at low levels of growth. HPLC was necessary to separate the ergosterol from sterols endogenous to the solid substrate. However, Nout et al. (1987) showed that the ergosterol content of *Rhizopus oligosporus* varied from 2 to 24 micrograms per mg dry biomass, depending on the culture conditions. Ergosterol content was influenced by aeration. phase of growth and substrate composition. They concluded that it was an unreliable method for following growth of *Rhizopus otigosporus* in SSF.

Physical measurement of biomass

Peñaloza (1990) used another physical parameter to evaluate mycelial growth, based on the difference in the electric conductivity between biomass versus the substrate. Good correlation with biomass was obtained and a model was proposed.

Recently Auria et al.(1990) monitored the pressure drop in a packed bed during SSF of *Aspergillus niger* on a model solid substrate consisting of ion exchange resin beads. Pressure drop was closely correlated with protein production. Pressure drop is a parameter which is simple to measure and can be measured on-line. Further studies are required to determine whether the use of pressure drop in monitoring growth in forcefully aerated SSF bioreactors is generally applicable. An interesting point of this physical technique resides in the fact that it is sensible to the conidiation: early conidiophore stage make the pressure drop drastically and a breaking point can be easily observed.

In conclusion, the measurement of biomass in SSF is important to follow the kinetics of growth in relation to the metabolic activity. Measurement of metabolic activity by carbon

dioxide evolution or oxygen consumption can be generally applied, whereas extracellular enzyme production will only be useful when enzyme production is reasonably growth-associated.

Vital staining with fluorescein diacetate has potential in providing basic information as to the mode of growth of fungi on complex solid surfaces as this method can show the distribution of metabolic activity within the mycelium. But it can not be measured on line.

On the other hand, in the production of protein enriched feeds, the protein content itself is of greater importance than the actual biomass concentration, and the variation in biomass protein content during growth becomes less relevant.

Overall, oxygen uptake and carbon dioxide evolution methods are probably the most promising techniques for biomass estimation in aerobic SSF as they provide on-line information. The monitoring and computing equipment is relatively expensive and will not be suitable for low technology or rural applications. None method is ideally suited to all situations so the method most appropriate to the particular SSF application must be chosen on the basis of simplicity, cost and accuracy. The best choice could be to cross two or three, or more, techniques for measurement of various parameters, and the total balance could be highly correlated to the actual biomass.

5. Environmental Factors

Environmental factors such as temperature, pH, water activity, oxygen levels and concentrations of nutrients and products significantly affect microbial growth and product formation. In submerged stirred cultures environmental control is relatively simple because of the homogeneity of the suspension of microbial cells and of the solution of nutrients and products in the liquid phase.

The low moisture content of SSF enables a smaller reactor volume per substrate mass to be used for microbial cultivation than with submerged cultures and also simplifies recovery of the product (Moo-Young et al., 1983). Serious problems, however, are encountered in respect of mixing, heat exchange, oxygen transfer, moisture control and the localisation of pH gradients and nutrient and product levels as a consequence of the heterogeneity of the culture.

The latter characteristic of SSF renders the measurement and control of the above mentioned parameters difficult, laborious and often inaccurate, thereby limiting the industrial potential of this technology (Kim et al., 1985). Due to these problems, the micro-organisms that have been selected for SSF are more tolerant to a wide range of cultivation conditions (Mudgett, 1986).

Moisture content and Water activity (Aw)

SSF process can be defined as microbial growth on solid particles without presence of free water. The water present in SSF systems exists in a complexed form within the solid matrix or as a thin layer either absorbed to the surface of the particles or less tightly bound within the capillary regions of the solid. Free water will only occur once the saturation capacity of the solid matrix is exceeded. The moisture level at which free moisture becomes apparent varies

considerably between substrates, however, and is dependant upon their water binding characteristics. For example, free water is observed when the moisture content of solid substrates such as maple bark exceeds 40% and when it exceeds 50-55% in rice and cassava (Oriol et al, 1988). With most lignocellulosic substrates free water becomes apparent before the 80% moisture level is reached (Moo-Young et al, 1983).

The moisture levels in SSCF processes which vary between 30 and 85% has a marked effect on the growth kinetics, as shown on Figure 1 (Oriol et al, 1988). The optimum moisture level for the cultivation of *Aspergillus niger* on rice was 40%, whereas on coffee pulp the level was 80%, which illustrates the unreliability of moisture level as a parameter for predicting the growth of a micro-organism. It is now generally accepted that the water requirements of micro-organisms should be defined in terms of the water activity (Aw) of the environment rather than the water content of the solid substrate. This parameter is defined by the ratio of the vapour pressure of the water in the substrate (p) to the vapour pressure of pure water (p_0) at the same temperature, i.e Aw = p/po. This concept is related to other parameters such as relative humidity (%RH = 100 x Aw) and water potential (psi = RT/V. ln Aw; where R is the ideal gas constant, T is the absolute temperature and V is the mol volume of water) (Griffin, 1981).

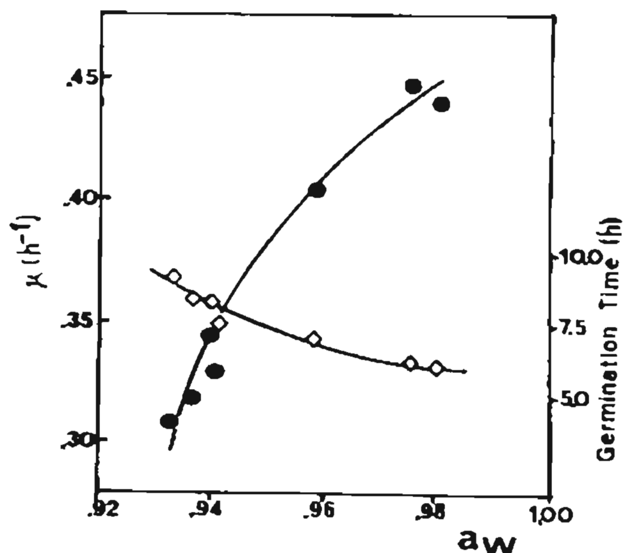


Fig. 2 **Evolution of the specific growth rate (—●—) and of the germination time (—◇—) as a function of the initial water activity of the medium**

The reduction of Aw has a marked effect on microbial growth. Typically, a reduction in Aw extends the lag phase, decrease the specific growth rate, and results in low amount of biomass

produced (Oriol et al. 1988) as it is shown in fig.2. In general, bacteria require higher values of Aw for growth than fungi, thereby enabling fungi to compete more successfully at the Aw values encountered in SSC processes. With the exception of halophilic bacteria, few bacteria grow at Aw values below 0.9 and most bacteria investigated show considerably higher minimum Aw values for growth. Some fungi. on the other hand, only stop growing at Aw values as low as 0.62 and a number of fungi used in SSC processes have minimum growth Aw values between 0.8 and 0.9 .

The optimum moisture content for growth and substrate utilisation is between 40 and 70% but depended upon the organism and the substrate used for cultivation. For example. cultivation of *Aspergillus niger* on starchy substrates such as cassava (Raimbault & Alazard, 1980) and wheat bran (Nishio et al. 1979) was optimal at moisture levels considerably lower than on coffee pulp (Penaloza et al. 1985) or sugarcane bagasse (Roussos et al., 1989), possibly because of the greater water holding capacity of the latter substrate (Oriol et al. 1988). The optimum Aw for growth of a limited number of fungi used in SSF processes was at least 0.96 whereas the minimum growth Aw was generally greater than 0 9. This suggests that fungi used in SSF processes are not especially xerophilic. The optimum Aw values for sporulation by *Trichoderma viride* and *Penicillium roqueforti* were lower than those for growth (Gervais et al.. 1988). Maintenance of the Aw at the growth optimum would permit fungal biomass to be produced without sporulation.

Temperature and Heat Transfer

Stoechiometric global equation of respiration is highly exothermic and heat generation by high levels of fungal activity within the solids lead to thermal gradients because of the limited heat transfer capacity of solid substrates. In aerobic processes, heat generation may be approximated from the rate or C02 evolution or O2 consumption. Each mole of C02 produced during the oxidation of carbohydrates released 673 Kcal . That is for why it is of high interest to measure CO2 evolution during a SSF process, because it is directly relied to the risk of elevation of temperature. Detailed calculation of the relation between respiration, metabolic heat and temperature were discussed in early works on SSF with *Aspergillus niger* growing on cassava or potato starch (Raimbault, 1981). The overall rate or heat transfer may be limited by the rates of intra- and inter-particle heat transfer, by the rate at which heat is transferred from the particles surface to the gas phase. The thermal characteristics of organic material and the low moisture content in SSF are special difficult conditions for heat transfer. Saucedo-Castaneda and co-workers (1990), developed a mathematical model for evaluating the fundamental heat transfer mechanism in static SSF and more specifically to assess the importance of convection and conduction in heat dissipation. Saucedo will explain in his lecture how this model could be used as a basis for automatic control of static bioreactors.

Heat removal is probably the most crucial factor in large scale SSF processes , and conventional convection or conductive cooling devices are inadequate for dissipating metabolic heat due to the poor thermal conductivity of most solid substrates and result in non acceptable temperature gradients. Only evaporative cooling devices provide sufficient heat elimination. Although the primary function of aeration during aerobic solid state cultivations was to supply oxygen for cell growth and to flush out the produced carbon dioxide, it also serves a critical function in heat and moisture transfer between the solids and the gas phase. The most

efficient processes for temperature control consists in evaporating water, what needs in return to complete the loss to avoid desiccation.

Maintaining a constant temperature and moisture content in large scale solid substrate cultures is generally difficult , but as you will realise some alternative equipment begin to fit that function, and all that will be discussed by Perez and Saucedo. The reactor type can have a large influence on the quality of temperature control achieved. It depends highly of the type of SSF: static on clay or vertical exchangers, drums or mechanically agitated with parameters controls, all that aspect will be discussed in cessions about Engineering aspects of SSF.

pH control and risks of contamination.

The pH of a culture may change in response to microbial metabolic activities. The most obvious reason is the secretion of organic acids such as citric, acetic or lactic acids, which will cause the pH to decrease, in the same way than ammonium salts consumption. On the other hand, the assimilation of organic acids which may be present in certain media will lead to an increase in pH, and urea hydrolysis result in an alcalinisation. The changes in pH kinetics depends also highly on the micro-organism. With *Aspergillus sp., Penicillium sp.* , and *Rhizopus sp.* the pH can drop very quickly until less than 3.0; for another type of fungi, like *Trichoderma, Sporotrichum, Pleurotus sp.* the pH is more stable between 4 and 5. Besides, the nature of the substrate influence highly pH kinetics, due to the buffering effect of lignocellulosic materials.

In our case we used a mixture of ammonium salt and urea to regulate the pH decrease during *A. niger* growth on starchy substrates (Raimbault, 1980). A degree of pH control may be obtained by using different ratios of ammonium salts and urea in the substrate. Hydrolysis of urea liberates ammonia, which counteracts the rapid acidification resulting from uptake of the ammonium ion (Raimbault & Alazard. 1980). In this manner, we obtained optimal growth of *Aspergillus niger* on granulated cassava meal when using a 3:2 ratio (on a nitrogen basis) of ammonium to urea. We observed that during the first stage of the cultivation the pH increased as the urea was hydrolysed. During the subsequent rapid growth ammonium assimilation exceeded the rate of urea hydrolysis and the pH decreased, but increased again in the stationary phase. During the cultivation the pH remained between the limits of about pH 5 to pH 6.2, whereas a lower urea concentration resulted in a rapid decrease in pH.

In a same way, pH adjustment during the cultivation of *Trichoderma viride* on sugar-beet pulp by spraying with urea solutions was effective due to the urease activity of the micro-organism causing an increase in pH at pilot plant level experimentation (Durand et al. 1988).

Finally, in a process of cultivation of filamentous fungi or yeasts, bacterial contamination may be minimised or prevented by employing a suitably low pH.

Aeration

Aeration fulfils four main functions in solid state processes, namely (i) to maintain aerobic conditions, (ii) for carbon dioxide desorption, (iii) to regulate the substrate temperature and (iv) to regulate the moisture level . The gas environment may significantly affect the relative levels of biomass and enzyme production. In aerobic submerged cultures oxygen supply is

often the growth limiting factor due to the low solubility of oxygen in water. In contrast, a solid state process allows free access of atmospheric oxygen to the substrate, aeration may be easier than in submerged cultivations because of the rapid rate of oxygen diffusion into the water film surrounding the insoluble substrate particles and also the very high surface of contact between gas phase, substrate and aerial mycelial. The control of the gas phase and air flow is a simple and practical mean to regulate gas transfer and generally no oxygen limitation are observed in SSF processes. when the solid substrate is particular. It is important to maintain a good balance between the three phases gas, liquid and gas in SSF processes (Auria, 1989; Saucedo et al. 1984). Modelling mass transfer in SSF is a key to keep good conditions for the development of the mycelium. By this very simple aeration process, it is also possible to induce metabolic reaction, either by water stress, heat stress or temperature changes, all processes that can be drastically change biochemical, physiological or metabolic behaviour.

6. Conclusion

SSF is a well adapted process for cultivation of fungi on natural vegetal materials which are breakdown by excreted hydrolytic enzymes. In contrast with LSF, in SSF processes, water related to the water activity is a limiting factor, both parameters no involved in LSF where water is in large excess. On the other hand, oxygen is a limiting factor in LSF but not in SSF where aeration is facilitated by the porous and particular structure and high surface contact area which facilitate transfers between gas and liquid phases.

SSF are aerobic processes where respiration is a predominant processes for energy supply to the mycelium; but it can cause severe limitation of the growth when heat transfer is not efficient enough causing rapid elevation of the temperature.

Is the reason why it is so important to study and control respirometry in SSF . We developed a laboratory technique to measure CO2 and O2 *on line* in SSF. A special lecture will be dedicated to the theory, modelling and basic concept of respirometry. Also it will be organise training cessions at the lab, to practice respirometric measurement and kinetics analysis.

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