14 Production of Antibiotics and Other Commercially Valuable Secondary Metabolites

Javier Barrios-González & Armando Mejía

14.1. INTRODUCTION

Secondary metabolites are compounds produced mainly by actinomycetes and fungi, usually late in the growth cycle (idiophase). Although antibiotics are the best known secondary metabolites, there are others with an enormous range of other biological activities. Moreover, the last two decades have been a phase of rapid discovery of new activities and development of major compounds of use in different industrial fields, mainly: pharmaceutical and cosmetics, food, agriculture and farming. Some examples are: anti-inflammatory, hypotensive, antitumor, anticholestrolemic, but also insecticides, plant growth regulators and environmental friendly herbicides and pesticides. These compounds are usually produced by liquid submerged fermentation, but many of these metabolites could be advantageously produced by solid-state fermentation.

Although solid-state fermentation (SSF) systems have been used in several oriental countries since antiquity, SSF has been transformed, in the last 25 years, for new purposes using new approaches of microbiology, biochemistry and biochemical engineering. This higher degree of control has allowed the use of SSF to produce sophisticated and valuable molecules like secondary metabolites (SMs).

Ten or even five years ago reviews on this field pointed out that SSF was an emerging technology with great potential for the production of SMs at industrial scale. The authors commented that mycelial morphology associated with the microorganisms used for secondary metabolite production is well suited for growth on a solid support. Also that SSF presents advantages like: higher product yield, often in shorter times, higher product stability, lower energy requirements; while some disadvantages, like more complicated scale-ups as well as difficulties in monitoring and controlling process parameters, were also mentioned (Barrios-González & Mejía 1996; Balakrishnan & Pandey 1996; Robinson et al., 2001).

Today, industrial SMs production by SSF is a reality. Some years ago an Indian company started industrial scale production of some secondary metabolites. It has since become a successful enterprise and the Food and Drug Administration (FDA) of the USA has approved the technology (SSF) developed by Biocon India for the production of fungal metabolites for human application (Suryanarayan 2003). In the near future the competition, between conventional SmF and SSF processes, promises to be tougher and more interesting. In the last 10 years the study of secondary metabolite (SM) production by SSF has been characterized not only by an increase in the number of publications, but also by the increase in the proportion of SMs with biological activities different from antibiotics. Another interesting feature of this stage is the surprisingly high productivity of SMs obtained in the processes designed in these studies. Ten years ago, a similar review described only one process with a production level above 7 mg/g (Barrios-González & Mejía 1996), while higher yields are quite common in recent work (see Table 2).

As was 10 years ago, SM production in solid culture was most often studied in SSF on natural substrates. This trend is represented by a considerable amount of pragmatic work directed to design high producing processes for particular SMs, most of them using agricultural products or wastes (SSF on natural substrates). Authors have looked for more efficient solid substrates for the production of particular SM. Generally, the same work is followed by the search of nutritional supplements and/or process conditions, often by sophisticated statistical methods. As a result, this new stage is characterized by a surprisingly high production yield of different SMs.

SSF has also been studied using inert solid support materials, which greatly facilitates basic studies. However, production yields as high as the ones obtained in SSF on solid substrates are also reported in these systems. Basic studies have also been performed on simplified model SSF systems, like membrane cultures (on agar media in Petri dishes). However, Rahardjo et al., (2004) have warned for a careful use of this model system since it could be artificial and not describing the actual SSF. The authors showed that this kind of culture presents a different metabolism and kinetics, in relation with SSF.

Many advantages of SSF are a consequence of the different physiology shown by fungi and other microorganisms on a solid substrate, in relation to the one presented in SmF. The molecular and physiological reasons underlying the different behavior of microorganisms in SSF are presently not well characterized, but a deeper understanding of this physiology is required to explore the possibilities for controlling or directing product formation in SSF. This information would also be of key importance to design efficient methods for strain improvement for these processes.

14.2 SECONDARY METABOLISM

14.2.1 General aspects

Secondary metabolites are compounds with varied and sophisticated chemical structures, produced by strains of certain microbial species, and by some plants. Although antibiotics are the best known SMs, there are other such metabolites with an enormous range of biological activities, hence acquiring actual or potential industrial importance. These compounds do not play a physiological role during exponential phase of growth. Moreover, they have been described as SMs in opposition to primary metabolites (like amino acids, nucleotides, lipids and carbohydrates), that are essential for growth.

A characteristic of secondary metabolism is that the metabolites are usually not produced during the phase of rapid growth (trophophase), but are synthesized during a subsequent production stage (idiophase). From studies in liquid medium, it is now known that production of SMs starts when growth is limited by the exhaustion of one key nutrient: carbon, nitrogen or phosphate source. For example, penicillin biosynthesis by *Penicillium chrysogenum* starts when glucose is exhausted from the culture medium and the fungus starts consuming lactose, a less readily utilized sugar.

Most SMs of economic importance are produced by actinomycetes, particularly of the genus *Streptomyces*, and by fungi.

14.2.2 Biosynthetic families

Microbial SMs show an enormous diversity of chemical structures. However, their biosynthetic pathways link them to the more uniform network of primary metabolism. It has been shown that SMs are formed by pathways which branch off from primary metabolism at a relatively small number of points, which define broad biosynthetic categories or families (Barrios-González et al., 2003).

1) Metabolites derived from shikimic acid (aromatic amino acids). Examples are ergot alkaloids and the antibiotics like candicidin.

2) Metabolites derived from amino acids.This family includes the β lactam antibiotics: penicillin, cephalosporins and cephamycins, as well as cyclic peptide antibiotics such as gramicidin or the immunosupressive agent cyclosporin.

3) Metabolites derived from Acetyl-CoA (and related compounds, including Krebs cycle intermediates). This family can be subdivided into polyketides and terpenes. Examples of the former group include the antibiotic

erythromycin and the hipocholesterolemic agent lovastatin. An example of the second group is the plant growth regulator gibberellic acid, as well as carotenoid pigments.

4) Metabolites derived from sugars. Examples of SM in this group are streptomycin and kanamycin.

14.2.3 Genetic regulation

Since secondary biosynthetic routes are related to the primary metabolic pathways and use the same intermediates, regulatory mechanisms such as: induction, carbon catabolite regulation and/or feedback regulate their biosynthesis. These pathway-specific regulatory mechanisms operate in conjunction with an overall control which is linked to growth rate. In submerged fermentations (SmF), these regulatory mechanisms are by-passed (or taken advantage of) by environmental manipulations. Hence, an inducer such as methionine is added to cephalosporin fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in fermentations like penicillin or erythromycin. The fermentation processes of antibiotics regulated by carbon are now conduced with slowly utilized sources of carbon, generally lactose. When glucose is used, it is usually fed at a slow, continuous rate to avoid catabolite regulation. Also nitrogen sources like soybean meal are used to avoid nitrogen (ammonium) regulation.

Although, the so called precursor effect is not a regulatory mechanism, in some cases, a precursor is used to increase one specific desirable metabolite, for example lysine is added as precursor and cofactor to stimulate cephamycin production by *Streptomyces clavuligerus* (Khetan et al., 1999; Barrios-González et al., 2003).

Studies on the molecular level have shown that genes of the biosynthetic pathways of SMs are clustered together in the genome of fungi and actinomycetes. In addition, studies have indicated that often a specific regulatory gene is embedded in these gene clusters. The co-regulation of these clusters can, in part, be explained by coordinated transcriptional control of biosynthetic genes by "narrow" or "broad" –domain transcription factors in fungi. The narrow pathway-specific regulators are usually found in the cluster and positively regulate gene expression. A typical example is AflR (aflatoxin regulator) (Woloshuk et al., 1994). Cluster regulators that are not found in the cluster itself include 2 regulators of cephalosporin production (Schmitt et al., 2004) and PENR1 for penicillins (Litzka et al., 1998).

Broad-domain factors are transcriptional factors that are important in integrating cellular responses to environmental parameters like C and N sources, temperature, etc. Responses to environmental signals are transmitted through (Cys2His2 zinc-finger) global transcription factors that mediate carbon (*CreA*), nitrogen and pH (*PacC*) signaling. Regulation by both narrow- and broad-domain transcription factors ensures that secondary metabolite pathways can respond to the demands of general cellular metabolism and the presence of specific pathway inducers (Keller et al., 2005).

14.2.4 New biological activities

As mentioned above, the last two decades have been a phase of rapid discovery of new activities and development of major compounds of use in different industrial fields. This new era has been driven by modern strategies to find microbial SM. Earlier whole cell assay methods, like bioassays, are being replaced by new and sophisticated, target-directed, mode-of-action screens. In this way, culture broths of new isolates are tested in key enzymatic reactions or as antagonistic or agonistic of particular receptors. This new approach relies on the knowledge of the biochemical and molecular details of different diseases or physiological processes (Barrios-González et al., 2003; Barrios-González et al., 2004). This growing wealth of bioactive compounds is usually produced by liquid submerged fermentation, but many of these metabolites could be advantageously produced by solid-state fermentation.

Table 1 presents the biological activities and applications of some of the SMs that have been produced in high yields by SSF. Other SMs that have been produced in this culture system include the coconut aroma compound, 6-pentyl-a-pyrone (Sarhy-Bagnon et al., 2000), and the cyclodepsipeptides dextruxins A and B; compounds that display insecticidal and antiviral activity (Liu & Tzeng 1999). Also, the novel tetramic acid antibiotic conoicetin, which shows a pronounced antibacterial and antifungal action, inhibiting even multidrug-resistant strains of *Staphylococcus aureus* has been produced by SSF. Interestingly, the producing fungus, *C. ellipsoida*, synthesizes this antibiotic only in SSF, although it grows well in SmF (Segreth et al., 2003).

14.3 MODERN TYPES OF SSF

Two types of SSF systems can be distinguished depending on the nature of the solid phase used.

14.3.1 SSF on solid natural substrates

This is the most commonly used (and most often described) system and involves

Table 1. Biological activities and applications of some fungal secondary metabolites that have been produced by SSF.

Secondary Metabolite	*Biological Activity*	*Remarks*	*Micro-organisms*
Lovastatin or monacolin K	Hypo-cholesterolemic	Hypercholesterolemia is the accumulation of cholesterol in blood that causes atherosclerosis (blockage of the artery), leading to coronary heart disease and hearth attack.	*Aspergillus* or *terreus*
	Potential therapeutic agent for various tumors	Lovastatin competitively inhibits the rate-limiting enzyme (HMG-Co-A) of cholesterol biosynthesis and lowers plasma cholesterol in humans (Valera et al., 2005).	*A. flavipes*
		Recently reported as antitumoral by inhibiting the synthesis of non-sterol isoprenoid compounds (Manzoni et al., 2002).	*Monascus* sp.
Cyclosporin A	Immuno-supressive majority Other: antifungal, antiparasitic and antiinflamatory	It is the drug of choice for a of human transplantation surgeries and also in the treat-ment of auto immune diseases (Rao et al., 1997)	*Tolypocladium inflatum Cylindro-carpon*
Myco-phenolic Acid	Immuno-supressive Other: antineoplasic, anti-inflamatory, antiviral, antipsoriasis and antifungal	Mycophenolic Ac. and its derivatives such as mycopyenolate mofetil, have diverse biological activities. The latter, in combination with cyclosporin A, decreases the incidence of graft rejection in humans; also it has several advantages for maintenance therapy of organ graft recipients (Sadhukhan et al., 1999)	*Penicillium brevi-compactum* And other species of *Penicillium* (3)
Nigerloxin	Antioxidant (enzyme inhibitor) Other: anti-inflammatory, antiallergic, anticancer, and antiatherogenic	Inhibitor of lipoxygenases, a family of enzymes that are involved in the pathogenesis of some diseases, such as allergy, atherosclerosis and cancer. Also a free radical scavenging property (Sekhar et al., 2005)	*Aspergillus niger*

Secondary Metabolite	*Biological Activity*	*Remarks*	*Micro-organisms*
Biopigments	Color additives for food and pharmaceuticals	Microbial pigments are highly valued since they are an alternative to other natural color additives extracted from plants or animal materials with the advantage of high productivity and without seasonal production problems. The fungus produces a mixture of pigments among which the red ones are regarded as the most important.This species can also produce the undesirable metabolite citrinin (mycotoxin).	*Monascus sp.*
Beta-carotene	Pigments and antioxidants, vitamin precursor.	These microbial pigments are difficult to produce in SSF since are intracellular. This strain accumulates them in sclerotia, which can be easily separated from solid medium (Han et al., 2003)	*Penicillium sp.*
Gibberellic	Plant growth regulator	Gibberellic acid is a potent plant growth regulator extensively used in agriculture, nurseries, greenhouses, viticulture, tea gardens, etc., for accelerating seed germination, stem elongation, induction of flowering, improvement of crop yield	*Gibberella fujikuroi* *Fusarium moniliforme*
Iturin	Antifungal	Cyclic heptapeptide connected by a long chain β-amino acid. Effective against phytopathogens	*Bacillus subtilis*
Surfactin	Surfactant	One of the most powerful biosurfactants known so far. It is also used as fibrin-clotting inhibitor (Ohno et al., 1995).	*Bacillus subtilis*

the cultivation of microorganisms on agricultural products, such as rice, wheat bran, etc.; this system is referred to as cultivation on solid substrates or on natural substrates. Cultivation on solid substrates uses materials that serve both as a support and as nutrients source. These materials are usually starchy, or lignocellulosic agricultural products such as grains and grains by-products, as well as agro corps and their residues such as cassava, potato, beans, sugar beet pulp, etc. This culture system has the advantage of using cheap unprocessed

or moderately processed agricultural raw materials. However, SSF on solid substrates has a disadvantage: the carbon source constitutes part of their physical structure. During the growth of microorganisms, the solid medium can be partially degraded, and as a result the physical characteristics of the medium change, particularly in long cultures like secondary metabolite fermentations. As a result, heat and mass transfer can be reduced (Ooijkaas et al., 2000; Barrios-González & Mejía 1996).

14.3.2 SSF on inert support

This system involves the cultivation of microorganisms on an inert support impregnated with a liquid medium. In this SSF, the inert support serves not only as a reservoir of nutrients (in a liquid phase), but also as an anchor point for the microorganism. The abovementioned disadvantage can be overcome by the use of inert supports which have a relatively constant physical structure throughout the process, enabling improved control of heat and mass transfer. Another advantage of SSF on inert supports, relative to SSF on natural substrates, is its less complicated product recovery. Extra- or even intracellular products can be extracted quite easily from the inert support, hence the products can be obtained with less impurities. Perhaps the most important advantage of this system is that it facilitates basic studies, since medium composition can be precisely designed. In this way a defined medium can be used and the effect of different compounds, on production, tested. Besides, the liquid medium can be extracted from the inert support at any time of the culture and its components analyzed.

From the applied side, this system allows the design of adequate production media, and mass balances for more-advanced process modeling and process control are more easily performed, since concentrations of all the nutrients in the production medium are known. On the other hand, SSF on inert support has been used with two variants: a) SSF on natural inert support, such as sugar cane bagasse; b) SSF on artificial inert support, such as polyurethane foam, polystyrene, amberlite, vermiculite, perlite, pozolano particles (volcanic material) and clay granules (Ooijkaas et al., 2000; Baños et al., 2007a).

The potential of SSF on impregnated inert supports as a commercial system has been analyzed. One conclusion is that the higher costs of defined media make solid substrate the preferred choice, especially for low-cost products. However, for high-added-value products, such as secondary metabolites, SSF on inert supports can be used because the medium costs are normally a fraction of the overall production costs. Ooijkaas et al., (2000) give, as an example, the cost calculation for spore production on defined media on inert support. The authors show that fermentation costs account for less than 20% of the

production costs. In addition, when downstream processing, formulation and registration are also considered, the medium costs will become even less important. Furthermore, it is known that downstream processing can markedly affect the overall production costs owing to recovery losses. Because downstream processing is simplified and improved with inert supports, it is anticipated that this will affect the overall production costs in a positive way, and consequently outweighing the higher medium costs (Ooijkaas et al., 2000).

From the analysis of studies of SM production by SSF on solid substrates and on inert support, conclusions are beginning to emerge, often complementary, which allow a clearer vision of how to produce SMs on SSF, as well as its potential to the future.

14.4 DEVELOPMENT OF SSF PROCESSES FOR THE PRODUCTION OF SMS

14.4.1 Selection of solid substrates

The possibility of altering the medium composition and investigating the influence of certain medium components is limited when using natural substrates. Modifying the type and concentration of the medium is indirectly carried out by changing the grain or agricultural residue, by mixing agricultural products with each other and/or by supplementation with nutrients solution. The solid substrate role is also related to physical structure and thus water retention capacity and porosity. Studies to develop a production system for a particular SM on SSF on solid substrate generally start by establishing the best (basic) solid substrate. This is done by comparing SM production yields on different agricultural products or residues, like: wheat bran, barley or wheat rawa. Analyzing the research work of the last years, that achieved high yields of fungal SMs (Table 2), it is possible to draw several conclusions about the choice of solid substrate.

Wheat bran is the most frequently used solid substrate and also a very effective one. Five of the 10 more productive processes, for different fungal SMs, used WB. Moreover, it is has also been successful in processes with actinomycetes and (endospore forming) bacteria (Table 3), since 3 of the 7 more productive processes use wheat bran. This can be further illustrated by the case of rifamycin SV production process, where an impressive yield of 32 mg/g was achieved in 7 days (Krishna et al., 2003).

Wheat rawa is a substrate that has been used lately as solid substrate for the production of some SM of fungi and actinomycetes with excellent results. The only example of the use of this substrate in the production of fungal metabolites is in the biosynthesis of cephalosporin C where a very high production

Table 2. Production yields, of fungal SMs, by SSF on different natural substrates and by SSF on inert supports.

SSF System	*Metabolite*	*Micro-organism*	*ProductionSSF (mg/g)*	*Supplements*	*Strain*	*Reference*
Artificial inert support	Lovastatin	*A. terreus* mutant EOM-36	27.89	Impregnated liquid medium	Mutant from TUB-F514	Baños et al., (2007b)
Wheat Rawa	Cephalosporin C	*A. chrysogenum*	22.28	Soluble starch 1% Yeast extract, minerals	Improved strain	Adinarayana et al., (2003b)
Artificial inert support	Lovastatin	*A. terreus* TUB-F514	19.95	Impregnated liquid medium	Wild isolate	Baños et al., (2007a)
WB	Lovastatin	*A. flavipes*	16.78		Improved industrial strain	Valera et al., (2005)
Rice	Lovastatin (monacolin K)	*Monascus sp.*	12	Only nutrients in the seed culture	Improved strain	Xu, G. et al., (2004)
Natural inert support (sugar cane bagasse	Penicillin G	*P. chrysogenum*	10.5	Impregnated liquid medium	Improved	Barrios-Gonzalez et al., (1993)
Rice Bran	Griseofulvin	*P. griseofulvum*	9.732	Glucose 3%, yeast extract 0.05%, minerals and precursor	Improved strain	Saykheda & Singhal (2004)
WB	Gibberellic Ac.	*G. fujikuroi*	6.8	Soluble starch 20%, minerals	Improved strain	Agosin et al., (1995)
WB	Nigerloxin	*A.niger*	5.06	Trisodium citrate 5%	Wild isolate	Rao et al., (2005)
WB	Cyclosporin A	*Tolypocladium inflatum*	4.84	Millet flour 20%, jowar flour 10%	Improved strain	Murthy et al., (1999)
WB	Mycophenolic Ac.	*P. brevicompactum*	3.286	Mannitol 3%, ragi flour 25%, millet flour 10%, $(NH_4)_2HPO_4$ 4%, casein hydrolisate 2%		Sadhukhan et al., (1999)

Table 3. SM production, by actinomycetes and *Bacillus sp*, in SSF on different natural substrates.

SSF System	*Metabolite*	*Micro-organism*	*Production SSF (mg/g)*	*Supplements*	*Strain*	*Reference*
Wheat Bran	Rifamycin SV	*A. mediterranei*	32	Peanut de-oiled cake 0.1%, barbital	Improved strain	Krishna, et al., (2003)
Wheat Rawa	Neomycin	*Streptomyces marinensis*	17.150	Dextrin 1.41 %, raspberry seed powder 6.49%, minerals 17.26 ml%	Improved strain	Adinarayana et al., (2003b)
Wheat Rawa/ Cotton seed de-oiled cake 1/1	Cephamycin	*S. clavuligerus*	15	CSL 9%, sunflower cake 0.5%		Kota & Sridhar (1999)
Ground nut (peanut) shells	Tetracyclin	*Streptomyces sp.*	13.18	Soluble starch 10%, peanut meal 10%, $(NH_4)_2SO_4$ 1%, NaCl 0.5% Cl^-donor	Wild isolate	Asagbra et al., (2005)
Corn-cob	Oxitetracycline	*S. rimosus*	7.5	Rice bran 20% or $(NH_4)_2SO_4$, 1% minerals	Improved strain	Yang & Swei (1996)
Wheat Bran/ Soy bean meal (1:3)	Bacitracin	*Bacillus lichen-iformis*	4.82 iu/g			Farzana, et al., (2005)
Wheat Bran	Iturin	*Bacillus subtilis*	3.6	Glucose, minerals	Wild isolate	Ohno et al., (1992)

(22.28 mg/g) was achieved in a relatively short period (5days) (Adinarayana et al., 2003). With actinomycetes a good example of the use of wheat rawa is the production of 17.15 mg of neomycin per g of dry medium by Adinarayana et al., (2003b), second highest in the actinomycets group. Kota & Sridhar (1999) used wheat rawa in combination (1:1) with cotton seed de-oiled cake, reaching a yield of 15 mg of cephamycin per g, also in 5 days.

Few other solid substrates have given high yields. One case is the use of rice bran by Saykhedkar & Singhal (2004) to produce the antifungal griseofulvin

(9.732 mg/g) in 9 days. The use of groundnut (peanut) shells as a solid substrate to produce 13.18 mg/g of tetracycline in 5 days is interesting (Asagbra et al., 2005). This substrate, because of its cellulosic nature and physical structure, approaches to an inert support, as does the solid substrate (corn-cob) used for the production of oxytetracyclin (7.5 mg/g in 8 days); both fermentations performed with actinomycetes. The use of rice as solid substrates has given good results only when the fungus *Monascus sp.* is used. Rice is a typical substrate for this fungus, since it is used since antiquity in a traditional fermentation to produce the Chinese food additive red rice. A relatively high production level of lovastatin (monacolin K) (12 mg/g) was obtained with the use of rice as a solid substrate by a strain of this fungus, although in a relatively long time (35 days) (Xu et al., 2004). Hence, rice has also been the solid substrate used in processes to produce biopigments (Carvalho et al.,, 2006) and in a process to produce lovastatin together with γ-aminobutyric acid (a hypotensive drug) aiming to produce a multifunctional dietary supplement (Su et al., 2003).

Logical substrates, for certain fungi, have worked well for the production of their SMs. In this way, ergot alkaloids production by *Claviceps* species was higher in SSF on rye than on impregnated bagasse (Trejo et al., 1992; Trejo et al., 1993; Barrios-González & Mejía 1996).

On the other hand, it is interesting to note that some particular SMs are not synthesized in some natural substrates. The study on nigerloxin production by SSF found that this metabolite was not produced when corncob, rice husks, bagasse or tapioca wastes were used as solid substrates.

In other studies, where different solid substrates were compared, lovastatin production was higher in wheat bran, followed by barley and much less produced in gram bran or bagasse. Worst results were obtained using fruit waste like orange and pineapple epicarp (6 times lower production), which do not seem like a good option for SM production (Valera et al., 2005). In cephalosporin C, production was better with wheat rawa, and a bit lower with wheat bran. Comparatively lower yields were obtained with rice bran.

14.4.2 SSF on inert support systems

The use of supports impregnated with liquid medium offers additional flexibility when designing the medium for optimal production of metabolites. SM production studies on inert support have used media recommended for SmF and optimized its overall concentration. The optimum is usually between 2 and 2.5 times the concentration used in SmF (Barrios-González et al., 1988, Domínguez et al., 2000, Sarhy-Bagnon et al., 2000, Baños et al., 2006a). Once the concentration level is established, optimal concentration of individual nutrients can be determined

by, for example, central composite designs and response surface analysis methodology. In this way optimum values can be found with less number of experiments, as is done in SSF on solid substrate (see next section).

Although SSF on inert support is very convenient for basic studies, previous review work already indicated that solid fermentation on impregnated bagasse (natural inert support) could be very productive, with the case of penicillin (13 mg/g) (Barrios-González & Mejía 1996; Barrios-González et al., 1993). Very recent results show that artificial inert supports can have an excellent performance for the production of SMs.

Baños et al., (2006a) designed a lovastatin production process by SSF on artificial inert support that reached 19.95 mg/g in 7 days. These studies were performed with *A.terreus* TUB F-514, a wild strain isolated from a soil sample from Irak by Dr. Szakacs. The production of this strain, in SmF with exactly the same medium, was only 0.62 mg/ml. This strain was also tested in SSF on sugarcane bagasse impregnated with the same liquid medium, and final yield was 8.615 mg/g. It is yet not clear if the relatively better performance of the artificial inert support system is a general feature or if it depends on the particular fungus and metabolite. In a second stage of this work, *A.terreus* TUB F-514 was mutated and a mutant strain selected by rational screening methods (see Strain Improvement section). The new mutant was cultured in the artificial support system producing 27.89 mg of lovastatin per g (Baños et al., 2006b). On the other hand, in our earlier studies on penicillin production by SSF, bagasse was impregnated with a concentrated liquid medium which included some insoluble components. Results indicated that penicillin production was strongly controlled by the proportions of bagasse, nutrients and water. Interestingly, high antibiotic production was obtained with different combinations, but low bagasse content (10 to 12%) of the solid medium was a common feature (see the end of section 4.4.3).

14.4.3 Optimization of nutritional factors

14.4.3.1 Selection of supplements

In SSF on solid substrates, modification of the type and concentration of nutrients is done indirectly by selection of the solid substrate. However, a further and finer balance of the solid medium composition, for optimal metabolite production, is done by the addition of supplements. Besides complementing the C and N sources and minerals in the substrate, these supplements can contain inductors or precursors. C sources can be mono-, disaccharides or polysaccharides like starch. N sources are usually inorganic or organic with grains or cereal flours (mixed C and N sources). Typically, this stage is performed by testing a number of potential C, N and complex organic sources, as well

as minerals. Their impact on production can be evaluated, at a fixed concentration, by Plackett-Burman design, and compared with a control without supplements. In this way the "effective nutrients" are identified. In a second stage, the effective nutrients concentration is optimized by a response surface methodology (Murthy et al., 1999; Adinarayana et al., 2003a).

Research work has shown that the optimization of supplements has played a key role in increasing production yields with different metabolites and solid substrates. During optimization of cephalosporin C production by SSF on wheat rawa, production of the antibiotic increased 216% after the optimization of additional C and N sources; while these operations accounted for a 94% increase in cyclosporin A production (Table 4). However, there are also examples of very high yields on wheat bran without supplements. Valera et al., (2005) indicated that for lovastatin production, supplementation with external C sources like sucrose and lactose and N sources such as ammonium sulphate and ammonium nitrate, inhibited the metabolite production.

As can be seen in Tables 2, 3 and 4, although a great number of supplements have been tested, a relatively small group is giving good results. Often soluble starch has been successfully used as additional C source, as has yeast extract as N source. Also some cereal or grain flours like millet, ragi (finger millet) or jowar (sorghum) or seeds de-oiled cakes like peanut (groundnut) or sunflower de-oiled cakes have given good results as complex organic sources. Nevertheless, another conclusion is that each microorganism and metabolite has different requirements. These requirements go from no supplements for *A. flavipeps* in lovastatin fermentation, passing through the requirement of only trisodium citrate for nigerloxin production by *A.niger* to very complicated requirements for *P. brevi-compactum* in the production of mycophenolic acid. (these SSFs on wheat bran).

Another aspect to have in mind when developing solid media for SM production is that studies in liquid culture have shown that C, N or P sources can have a negative effect on SM production, due to regulatory mechanisms such as carbon catabolite repression. Earlier work of our group, studying aflatoxin production on cassava SSF, showed that when the concentration of phosphate and ammonium were reduced in the solid medium, aflatoxin production increased proportionally (Barrios-González et al., 1990). Since aflatoxin biosynthesis is regulated by nitrogen and phosphate, these results suggested that regulatory mechanisms were also active in SSF. Making use of the advantage of SSF on inert support for basic studies, catabolite repression of penicillin biosynthesis was studied in SSF on sugarcane bagasse, and compared with parallel experiments in SmF. Regulatory thresholds were estimated in both systems by carefully correlating

Table 4. Impact of supplements optimization on secondary metabolites production by SSF

Solid Sub-strate	*Metabolite*	*Supplements*	*Increase mg/g*	*Increase %*	*Reference*
Wheat Rawa	Cephalospor in C	Salts solution soluble starch yeast extract	2.805 - 3.962 7.045 - 14.054 14.058 - 22.281	41.2 99.54 58.5	Adinarayana et al., (2003b)
Wheat Bran	Cyclosporin A	Millet & jowar flours, mineral	2.6 to 5.043	94	Murthy et al., (1999)
Wheat Bran	Rifamycin SV	Groundnut de-oiled cake, barbiturate	12 to 19	58	Krishna et al., (2003)
Wheat Rawa	Neomycin	Raspberry seed powder	7.457 to 10.755	44.2	Adinarayana et al., (2003a)

glucose uptake kinetics with the initiation of penicillin biosynthesis. Results (SmF = 28 – 20 g/l; SSF = 36 – 14 g/l) showed that penicillin biosynthesis is also regulated by carbon catabolite regulation in SSF and at similar thresholds as the ones observed in SmF (García et al., 1993). These studies indicate that mechanisms that regulate the biosynthesis of secondary metabolites also play a role in solid culture. Hence, glucose should be used carefully in fermentations regulated by C as inorganic N and phosphate sources in fermentations regulated by N or phosphate. Regulatory mechanisms in SSF could explain the success in the use of polysaccharides like starch and/or complex nitrogen organic supplements, that bypass these mechanisms by slowly dosing glucose or ammonium.

14.4.3.2 Use of inductors and precursors

In liquid culture medium, the addition of a precursor (biosynthetic pathway intermediate) that is rate limiting, during antibiotic and other SM biosynthesis, brings about an increase in production and/or directs the synthesis preferentially towards one of the products of the related metabolites that would be formed otherwise. No specific studies on this subject have been performed in SSF, however, the effect of triptophane on alkaloid production by SSF reported by Trejo et al., (1992), as well as the increase in tetracycline production by the addition of methionine indicates a precursor effect (Barrios-Gonzálelz &Mejía 1996). In our own research on penicillin production, phenylacetic acid has been used as a precursor for penicillin G. HPLC analysis of extracts from SSF samples have shown that only penicillin G is produced and no evidence of the synthesis of other penicillins has been found (unpublished results). Recently,

Saykhedkar & Singhal (2004) reported that the addition of 0.1% of choline chloride, as a precursor, produced a 76% increase in the yield of griseofulvin in SSF. This compound is a methyl donor and increases the rate of biosynthesis in liquid cultures. This information indicates that the precursor effect also manifests in SSF, so has to be considered with the supplements. On the other hand, it probable that barbital, added to rifamycin SSF (Krishna et al., 2003), was not necessary due to good aeration in this culture system. It was previously thought that barbital was a precursor in the synthesis of rifamycin V, since it increased its production (decreasing rifamycin W concentration in the culture broth) in industrial SmFs. In a previous work we showed that barbital effect was indirect by partially inhibiting respiratory chain, hence liberating more O_2 towards biosynthesis, for the oxidation step leading to rifamycin B in a viscous industrial fermentation with low O_2 transfer (Mejía et al., 2003).

14.4.4 Optimization of environmental factors

14.4.4.1 Substrate pretreatment

Natural substrates and even inert supports generally require some kind of pretreatment to make their chemical constituents more accessible and their physical structure more susceptible to mycelial penetration and adhesion. Moreover, it also improves water retention capacity (penetration and distribution). Early work in or laboratory was directed towards using sugar cane bagasse as an inert support for the production of penicillin by *P.chrysogenum*, but growth and production was extremely low. Microscopic observations, using colored water, indicated that the liquid was not penetrating the bagasse fibers. It was noticed that, after thermal pretreatment in the autoclave, water could penetrate the fibers. When this pretreated bagasse was used for SSF, abundant growth and antibiotic production was finally obtained (unpublished results).

Solid substrates are generally moistened with distilled water (that can include salts solution) and then autoclaved from 20 minutes to 1 hour. A variation that can be used with wheat bran is acid pretreatment. Since the early studies on gibberellic acid, performed by Kumar & Lonsane (1987), the authors described an acid pretreatment: mixing the wheat bran with 60% mineral salts-acid solution of 0.2 N acidity (HCl) before autoclaving. Recent work of Krishna et al., (2003) showed that rifamycin SV production increased nearly 300% when the substrate was treated with 0.2 M HCl. Although similar growth was obtained when wheat bran was pretreated by moistening with distilled water before autoclave, antibiotic production was much lower. The authors comment that acid pretreatment probably influenced the breakdown of starch and protein, and improved the water distribution. Also Sekar et al., (1997) reported that cyclosporin A production in SSF was increased from 0.16 to 1.14 mg/g by acid pretreatment of the substrate (wheat bran).

14.4.4.2 Particle size

After pretreatment, solid substrates are usually ground and sieved to a certain average particle size. For wheat bran particle sizes around 0.4 – 0.7 mm are usually employed in recent research. However, in early studies, Kumar & Lonsane (1987) reported an increase in gibberellic acid production with the use of 0.3-0.4 cm, in relation with smaller particles. With wheat rawa, Ellaiah et al., (2004) found coarse size (> 0.84 mm) to give the best results in neomycin production.

14.4.4.3 Moisture content and water activity

Water is the main component of microorganisms, and in SSF has a key role in enzymes, nutrients and products diffusion through the solid matrix. However, the importance of this parameter appears to be more critical for SM formation, probably since initial conditions must generate an adequate environment for production phase (idiophase) after the rapid growth phase (Barrios-González & Mejía 1996). In SSF, the best initial moisture content (IMC) value depends on the nature of the substrate, mainly on its water retention capacity. Hence, different substrates will show different ranges of IMC for optimal metabolites production. Recent studies on SM production by SSF do not show very impressive production increases by optimizing IMC anymore. This is because the optimal ranges for the most usual substrates are already known, so the initial values are not far from the optimum. Thus, the study using wheat rawa obtained best production at 80% IMC, although different metabolites were studied using *S.marinensis*, *S.clavuligerus* and *A.chrysogenum*.

Works with wheat bran show optimum values at around 70% (cyclosporin C and mycophenolic acid), while best nigerloxin and lovastatin production were obtained at 65% IMC. It is interesting to note that rifamycin SV SSF fermentation was carried out at 80% IMC with very high yields. Less common solid substrates showed optimum values at different IMC: groundnut shells 67% > corncob 65.5 > rice 53 to 65% >rice bran 50%.

Water activity (a_w) is related to the concentration of solutes in a liquid medium and represents the unbounded, and therefore available water. Through indirect calculations it has been proposed that in cassava SSF, A_w decreases during the culture (Oriol et al., 1988a; Oriol et al., 1988b). This has been recently confirmed in a wheat bran SSF system (Corona et al., 2005), showing that water availability for the microorganism decreases strongly during the solid culture of *Gibberella fujikuroi*. Conversely, higher gibberellic acid production yields were obtained at higher A_w values (1 - 0.96), although this last experiment was performed on a model system (agar culture on Petri dish). Interestingly, Sekar et al., (1998)

obtained a higher yield of cyclosporin A (1.92 mg/g), when the solid culture was incubated in a chamber with controlled relative humidity at the highest value (95%); while SSF incubated at lower values gave lower yields. Cultures incubated at 80% relative humidity only produced 0.98 mg/g and inspection of the solid culture indicated dryer zones where growth was poor.

Oriol et al., (1988b), working with *A. niger* in SSF using sugarcane bagasse with absorbed liquid medium, showed that Aw controls growth rate in SSF, being this parameter higher at higher water activity values. The authors found that when IMC was varied between 40 and 75%, but keeping Aw of the medium constant, growth rate was not modified ($m=0.4\ h^{-1}$). However, Barrios-González et al., (1988), using the same SSF system and keeping Aw constant, showed that IMC had a very important impact on penicillin production level in SSF. In the same work, experiments were performed in which nutrients concentration was increased (decreasing Aw), keeping IMC constant at 70%. It was found that the use of very concentrated media favors the antibiotic production in SSF. This was an important effect, since penicillin yields increased 5-fold in 2X concentrated medium (twice the recommended concentration for SmF). Conversely, the use of concentrated media negatively affected production in SmF. In a later study, using the same system, it was observed that the same IMC could be reached by using different combinations of the solid phase (represented by the nutrients + support). And that this gave different penicillin production yields. It was observed that when using different combinations of support nutrients and water, higher penicillin yields were obtained in those conditions when support was kept low (10-12.5%). It was not clear why such different combinations could create a favorable environment for production during idiophase (Domínguez et al., 2001). Respirometric analysis (derivative form) showed that growth phase (trophophase) is observed like a steadily rising peak (CO_2 production or O_2 uptake). The transition to idiophase is seen when the rising peak falls, indicating that growth was limited by exhaustion of the key nutrient (in that case glucose). Idiophase is seen as a usually lower and wider second peak, it is during this period that secondary metabolites are synthesized.

With the above mentioned information, it was possible to identify a high production respiratory profile. Results showed that a low and steady respiration profiles during idiophase (relatively horizontal and wide second peak) correlated with high penicillin production. While higher and variable respiration patterns in this phase were associated with low production conditions (Domínguez et al., 2000).

These studies show that in SSF, like in liquid culture, idiophase starts when growth is limited by the exhaustion of a key nutrient and that productive

idiophases are characterized by low and constant growth rates. The way to achieve this, in the case of penicillin SSF, was the use of conditions (low support proportion) that permit a slow but constant nutrients supply during idiophase, supporting slower but constant growth rates for longer periods. Very probably, similar respiration patterns are being obtained in the high yielding SSF processes for SMs described in previous sections. It is also possible that other SSF variables can also be manipulated to achieve this high production respiration pattern. On the other hand, a practical consideration on this subject is that evaporation is an effective mechanism for the removal of metabolic heat produced during the cultivation in large-scale SSF reactors (Gutiérrez-Rojas et al., 1996). However, evaporation can affect water activity (Aw) and might have and adverse effect on microbial metabolism (Weber et al., 1999; Larroche & Gros 1992; Oriol et al., 1988). Replenishment of the lost water is therefore required. In non-mixed systems this is impossible, so solid substrates should be able to release a large amount of water without affecting the Aw (Weber et al., 1999). The alternative is mixing of the medium, which enables the possibility of homogenous water addition besides promoting mass and heat transfer.

14.4.4.4 Aeration

One strategy used to assure adequate oxygen availability to the solid culture, is diffusive aeration by using static shallow beds of solid medium. Working with wheat bran SSF, Sekar & Balaram (1999) found that when the thickness of the bed was increased from 1 to 2 cm, there was a significant reduction in yield of cyclosporin A; indicating a limitation in available oxygen and moisture (trays were incubated in controlled relative humidity chambers) as the depth increased. Rao et al., (2005) studied the area of exposed surface on production of nigerloxin, by varying the ratio of the quantity of wheat bran to the flask volume. The optimum yield of the product was obtained at a ratio of 1:20.

The second aeration strategy is to employ forced aeration in more complex fermenters. Valera et al., (2005) studied this parameter in lovastatin production in a 2 l reactor with a load of 200 g of solid culture. Lovastatin yield improved with airflow rate, reaching a maximum at 1 vvm. Carvalho et al., (2006) also found an optimum of 1 ml of air per gram of wet substrate per minute, in the production of *Monascus* pigments in rice SSF, using a system of aerated glass columns. Although in the latter study, higher aeration rates had a deleterious effect on production.

14.4.4.5 Mixing

Mixing of the medium prevents heterogeneity of the solid medium composition and mycelial age. It breaks long mycelial nets, generating shorter mycelia in similar physiological state (Barrios-González & Mejía 1996). Mixing enables the possibility of homogenous water addition besides promoting mass and heat transfer. However, it has been reported that some fungi do not tolerate mixing in SSF (Mudgett 1986), possibly since this operation not only can break mycelia but can disturb mycelial attachment to the solid medium. Our group studied the effect of mixing on a penicillin production on impregnated bagasse (unpublished results and Barrios-González & Mejía 1996). Results showed a positive effect of manual mixing once a day on product formation (4, vs 5.7 mg/g) and, interestingly, an important increase in metabolic activity: 0.32 g of total CO2/g dry culture vs 0.44 g/g at 96 h (Fig.1). Since in these experiments mixing operation included unpacking each column fermenter, mixing and re packing, it was noticed that the solid culture showed moisture loss. When the experiment was performed restituting moisture loss, even higher penicillin production yields were reached (Fig. 2). Image analysis indicated that the microbial population was formed by shorter mycelia (approximately 200 vs 500 μ), that presented higher branching frequency (branching every 67 ± 25 vs 240 ± 70 μ). In other words, a higher number of growing hyphal tips, giving rise to a higher metabolic activity and probably to a greater secretion surface, as well as greater physiological homogeneity of mycelia in the solid culture (Barrios-González & Mejía 1996). This way of creating a higher branching frequency is an alternative to the genetic approach (See Stain Improvement section). In a subsequent work, using a 2 Kg rotating drum-type solid fermenter, it was confirmed that mixing had a positive effect on penicillin production. However, highest production was obtained with a mixing time of 2 minutes per day (at 5 rpm), while production decreased slightly with 4 minutes per day, and no antibiotic was produced with 20 minutes of mixing per day (Miranda et al., 2003). Recently, Valera et al., (2005) also used intermittent stirring in a 2 l reactor for the production of lovastatin.

The case of griseofulvin is interesting since high antibiotic production (9.732 mg/g) was obtained by SSF on rice bran that was incubated under very severe agitation conditions, at 250 rpm in flasks (Saykhedar & Singhal 2004). This is quite uncommon and probably the only antecedent is the very early work carried out by Hesseltine and the group in NRRL with aflatoxin production on rice SSF. The authors found a very high increase in production when a flask with rice fermentation was agitated in shaker (200 rpm) as if it contained liquid medium. A relatively very high concentration of aflatoxins was obtained in this way (1.5 mg/g) (Hesseltine 1972).

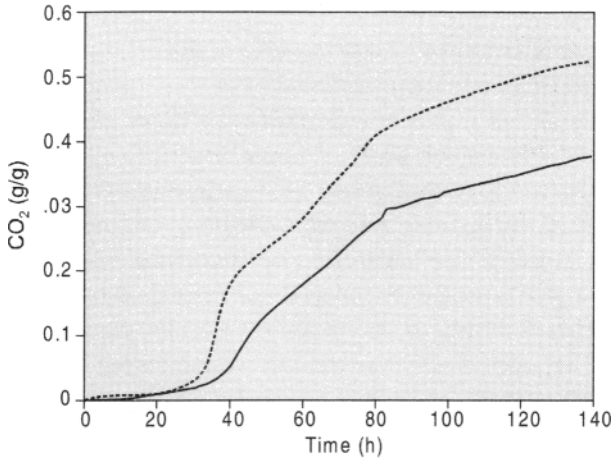

Fig. 1 Respiration (integrated form) of *Penicillium chrysogenum* P2 during SSF; static (—), and mixing once a day (- - -).

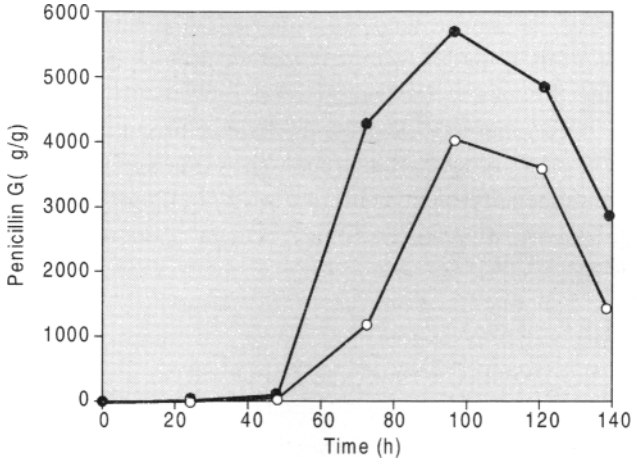

Fig. 2 Penicillin production by SSF: mixed once every 24 h (●) and static (○).

14.4.4.6 pH

It is considered that global pH of the liquid phase of a SSF can be considerably different than the local pH values on the solid surfaces where growth is taking place, due to the superficial charge effects and the ionic equilibrium modified by the effect of solute transport (Mudgett, 1986). A general procedure to for pH measurement in SSF is to determine global pH, after suspension of a sample in a 10 times grater volume of water. However, there are no comparative studies (SSF vs SmF) on the effect of pH on secondary metabolism. Very recent results in our laboratory indicated that this global value of pH in the solid culture relates very well with the pH sensed by the fungus in the SSF.

Studying cephalosporin C biosynthesis in bagasse SSF, it was found that pH is a key parameter since it only allows biosynthesis in a relatively narrow range of pH values. If this control was also displayed in SmF this phenomenon could be used as a very precise biosensor to describe the microenvironment sensed by the fungus in SSF. Comparative studies were performed, using the same strain and exactly the same medium composition. Results showed that behavior of pH was similar in both culture systems and, more important, that the antibiotic synthesis was also controlled by pH in SmF and by exactly the same range of pH (Cuadra et al., 2007).

14.4.4.7 Inoculum type and size

In SSF inoculum must be distributed homogeneously and must be high enough to assure predominance of the strain. Two different strategies are commonly being used, that is, inoculating with spores or inoculating with mycelium that comes from a previous stage of liquid culture (seed culture). Among the studies in which spore inoculum was used, the work on alkaloid production on rye grains is interesting since the effect of inoculum size was determined (Trejo, 1992). Inoculum size was varied between 2 X 10^6 and 2 X 10^8 spores/g of dry medium. Results showed that, by increasing inoculum size, lag phase (or germination) was slightly reduced, while growth rate was increased. Optimum concentration for alkaloid production was 2 X 10^7 spores/g. Interestingly, lovastatin production by SSF on wheat bran was performed with a similar inoculum size i.e. 2.5 X 10^7 spores/g (Valera et al., 2005).

Also, SSF with actinomycetes have been inoculated with spores. Asagbra et al., (2005) tested the effect of inoculum size on the production of tetracycline on peanut shells SSF. The authors found 10^8 spores/g to be optimal, while the use of 10^{10} and specially lower that 10^6 produced lower yields. Kota & Sridhar (1999) also found 10^8 spores/g to be an optimum inoculum level for the production of cephamycin C. On the other hand, SSFs for SMs production are often inoculated with mycelium generated in a previous stage of liquid seed culture. Using a strain of the fungus *Achremonium chrysogenum*, Adinarayana et al., (2003b) found that inoculating cephalosporin C SSF with 10% w/v of seed culture produced best results. In these experiments, increasing inoculum size from 5 to 10 % gave a production increase of 25%. In the case of cyclosporin A, Sekar et al., (1998) found that size and type of inoculum had significant impact on the immunosuppressant yield in SSF. The culture inoculated with mycelium (30%) was significantly higher than the culture inoculated with spores.

However, it is important to note that spores were produced in SmF. Also that, as authors indicated, the fermentation inoculated with spores lagged approximately

2 days behind the one inoculated with mycelium, so yields might have not been so different if this culture had been sampled for 2 more days. Also, the authors did not test higher spore inoculum size. In any case, experiments performed in this work showed that increasing inoculum size (with seed culture) from 20 to 30% caused a 50% increase in cyclosporin A production. In a subsequent report, this group optimized the mycelium inoculum to 60% of seed liquid culture.

Very high inoculum sizes with mycelium have also been used in fermentations with actinomycetes. Krishna et al., (2003) obtained very high rifamycin yields in SSF on wheat bran. The process was inoculated with 40% seed culture. Moreover, the yield obtained with this inoculum size was 42% higher than the one obtained in cultures inoculated with 10%. Ellaiah et al., (2004) also observed a strong influence of high (mycelial) inoculum size on the production of neomycin.

14.5 MICROBIAL PHYSIOLOGY IN SSF

The special physiology displayed by fungi and other microorganisms in SSF is presently poorly characterized. In fact, many advantages of SSF are a consequence of the different physiology shown by microorganisms in solid medium, in relation to the one presented in SmF. Several differences (and advantages) have been described for enzymes production: SSF provides higher productivities, less prone to problems with substrate inhibition and yields enzymes with a higher temperature or pH stability (Hölker et al., 2004). In relation with SMs production by SSF, a clear difference is higher production, often in shorter periods. Moreover, sometimes the SM is produced only by SSF, even though the organism also displays good growth in SmF (Segreth et al., 2003).

The molecular and physiological reasons underlying the different behavior of microorganisms in solid medium are presently not well characterized. A few works have studied the physiological differences that arise during the growth of microbial cells in the two types of culture systems (Biesebeke et al., 2002). In *Aspergillus oryzae*, water activity seems to play an important role in this context. This is apparent from the accumulation of polyols: glycerol, erythritol and arabitol in SSF conditions. Furthermore, glycerol and erythritol dehydrogenases were induced by osmotic stress conditions (Ruijter et al., 2004). Molecular differences in gene expression, depending on the fermentation technique, have been described by Ishida et al., (2000). The authors found *A.oryzae* has two different genes that code for glucoamylase: *glaA* and *glaB*; and that the latter is expressed only under SSF conditions, while glaA is synthesized in SmF. *gla B* was induced by low Aw, high temperature and physical barriers to hyphal extension; while promoter motifs were identified. Although, in the experiments

performed by Biesebeke et al., (2005) *glaB* was induced by maltose rather than by low Aw.

Very recently our group performed molecular studies, comparing lovastatin production by *A.terreus*, in SSF and SmF. Results showed that the expression of a gene related to the adaptation of the fungus to low Aw conditions (glaw) was approximately 5 times higher in SSF. Also, that the expression of genes of lovastatin biosynthetic pathway was 3 times higher in SSF, and expressed for a longer period. Higher expression of the gene related to low Aw seemed to be a characteristic of the physiology of SSF and maybe related to higher expression of lovastatin genes. However, in this experiment a more concentrated medium (2.5 times) was used in SSF, in relation to the one used in SmF. Hence, another experiment was performed where exactly the same concentration of medium was used in both systems (2.5X). Results indicated that glaw induction was due to medium concentration and not to the culture system. Yet, in these new conditions, lovastatin genes expression in SmF was 10 times lower than in SSF, indicating that high lovastatin (SM) production is characteristic of the special physiology of SSF. However, this expression is not due to low Aw, but to other (unknown) factors of SSF (Barrios-González et al., 2007). These results show that higher production in SSF is partially explained by higher transcription of the biosynthetic genes in SSF, but this might not be the only factor, since transcription was 3 times higher in SSF, while specific lovastatin production in SSF was 13 times higher than in SmF (see next section).

14.6 STRAINS FOR SSF

One of the major positive aspects of SSF is that metabolites are, in many cases, produced at much higher yields than by SmF (Barrios-González et al., 1998; Barrios-González et al., 1993; Barrios-González & Mejia 1996; Balakrishnan & Pandey 1996; Roibinson et al., 2001). Recent reports on SM production by SSF, where production in SmF has been determined too, also support this claim. The parameter used in Table 5 to make this comparison is "relative production", which is calculated simply by dividing production in SSF by production in SmF. This value gives a rough idea of how many times is production in SSF higher than production in SmF, and hence how efficiently is this particular strain expressing its production potential in solid culture (Barrios-González et al., 1993). This table shows that fungi, actinomycetes and even bacteria (*Bacillus*) can be very efficient for SM production in SSF. Although different products and species are shown, relative productions are very high and range from 6.7 to 97. This value is lower in *P.chrysogenum* strains developed for SmF (last two examples). It is important to note that precise comparisons using the same medium composition can only be done in SSF on inert support. Earlier studies of our group indicated that biochemical and physiological responses of

Table 5. Production yield of some secondary metabolites by SSF and SmF.

Metabolite	*Micro-organism*	*SSF system*	*Production SSF(mg/g)*	*Production SmF(mg/ml)*	*Relative Production (PS/PL)*	*Reference*
Griseo-fulvin	P. griseofulvum	Millet	9.732	0.1	97	Saykhedkar et al., (2004)
6-Pentyl-alpha – pyrone	*Tricho-dermahar-zianum*	Sugar cane bagasse (N.I.S)	2.8	0.056	50	Sahry-Bagnon et al., (2000)
Lovastatin	*A. terreus*	A.I.S.	27.89	0.804	34.7	Baños et al., (2007b)
Lovastatin	*A.terreus*	A.I.S.	19.95	0.620	32	Baños et al., (2007a)
Iturin	*Bacillus subtilis*	Wheat bran	3.66	0.15	24.4	Ohno et al., (1992)
Cyclo-sporin A	*T. cylindro-sporum*	Wheat bran	1.4	0.08	17.5	Sekar et al., (1997)
Rifamycin SV	*A. medi-terranei*	Wheat bran	32	2	16	Krishna et al., (2003
Penicillin	*P. chryso-genum* P2-4	Sugarcane bagasse	10.555	0.977	10.8	Barrios-González et al., (1993)
Monacoli K	*Monsascus sp.*	Rice	11	1.65	6.7	Xu et al., (2004)
Penicillin	*P. chryso-genum* P2	Sugarcane bagasse	2.082	0.83	2.5	Barrios-González et al., (1993)
Penicillin	*P.chryso-genum* Industrial strain	Sugarcane bagasse	13	9.804	1.3	Barrios-González et al., (1993)

many microorganisms differ greatly from those in SmF, leading to discrepancies in their production level displayed in both culture systems (Barrios-González et al., 1993; Barrios-González & Mejía 1996). In that work we studied penicillin production, of several strains of *P.chrysogenum*, in SmF and in SSF. These were strains, of different production levels, that had been developed for SmF and included an industrial strain. Some data of those experiments was included at the end of Table 5 (see *P.chrysogenum* P2 & Industrial). The first conclusion of this study was that higher penicillin yields in SSF could be achieved with strains that were higher producers in liquid medium. In this way, 1.345 mg/g were obtained with Wisconsin 54-1255, a strain that produced only 0.56 mg/

ml in SmF, while 2.084 mg/g were reached in SSF with strain P2, which was a higher producer than the latter in SmF (0.83 mg/ml). Moreover, the industrial strain reached the highest production level of 13 mg/g in SSF. However, this was in a certain sense, disappointing since it was not so far from the production displayed by this strain in SmF (9.804 mg/ml). When we calculated relative productions, from this data, we were able to draw a second conclusion: higher producing strains, developed for SmF, tend to express their production potential less efficiently in SSF (lower PS/PLs) than do lower producing strains, that are closer to the wild type and that show higher PS/PL values. This means that during the course of the genetic improvement programs the industrial hyperproducing strains, developed for SmF, have been loosing some (unknown) functions that are important for good adaptation and performance in solid medium (Barrios-González et al., 1993).

Some aspects of more recent research also support this conclusion. Some of the studies reported in the last years used wild isolates to produce SMs by SSF with surprisingly high production yields. One example is the 5.06 mg of nigerloxin/g reached by Rao et al., (2005) using a wild isolate of *A.niger*. Asagbra et al., (2005) compared tetracycline production by SSF of several collection strains with the antibiotic yield of a locally isolated strain (*Streptomyces sp.*). Since this wild isolate displayed higher tetracycline titers in this culture system the authors optimized conditions and obtained 13.18 mg of tetracycline in a 6 days fermentation. This production level by a wild isolate would be practically impossible in a SmF process. Furthermore, our group designed a lovastatin production process in SSF on artificial inert support that reached 19.95 mg/g in 7 days (Baños et al., 2006a). This very high production level was achieved with *A.terreus* TUB F-514, a wild strain (isolated from a sample of soil from Irak by Dr. G. Szakacs). In this study the production of this strain in SmF was also determined. Lovastatin yield obtained in liquid medium was only 0.62 mg/ml, giving a relative production of 32; indicating that the strain is very well suited for solid culture. Of course this parameter is very broad since one value is mg of SM per g of dry solid medium that is divided by another one that is in mg of SM per ml of culture medium. In fact, from a physiological point of view, the only precise comparison can only be done by calculating specific production. Unfortunately, this parameter is seldom reported since information of biomass concentration in solid is needed. Due to the advantages of SSF on inert support, this work could report that each mg of mycelium from SSF produced 816 mg of lovastatin, while each mg of mycelium from SmF only produced 59 mg of lovastatin; a specific production more than13 times higher in SSF. This indicates that these microbes present a different physiology in SSF, and one characteristic of this special physiology is the higher SM production in this culture system (see section 5).

14.7 STRAIN IMPROVEMENT FOR SSF

The science and technology of genetically manipulating and improving microbial strains, in order to enhance their metabolic capacities for biotechnological applications, are referred to as strain improvement. The microbial production strain can be regarded as the heart of a fermentation industry, so improvement of the production strain(s) offers the greatest opportunities for cost reduction without significant capital outlay (Parekh et al., 2000). Moreover, success in making and keeping a fermentation industry competitive depends greatly on continuous improvement of the production strain(s). Improvement usually resides in increased yields of the desired metabolite. However, other strain characteristics can also be improved. Genetic improvement of the production strain has a long history of success in SmF industry, best exemplified by the improvement of penicillin production, in which modern reported titles are 50 g/l, an improvement of at least 4,000 fold over the original parent (Peberdy 1985). Strain improvement has been performed mainly by mutation and selection. However, other procedures like sexual and parasexual crosses and protoplast fusion have also been employed in the last 20 years. More recently, genetic engineering strategies are also starting to be applied to strain improvement (Barrios-González et al., 2003).

14.7.1 Classical genetic methods

In relation to SSF, although wild isolates perform surprisingly well in SSF, genetic improvement of these strains is also very important or even inevitable for the development of competitive SSF processes. In this sense, it is significant that, from the 10 more productive SSF processes for fungal SMs presented in Table 3, eight used an improved strain. In fact, in 3 of the cases the strain improvement was described in the publication. It is also meaningful that the 3 most productive SSF process for SMs of actinomycetes (Table 4) also used improved strains. In SSF, it is now clear that special strains, particularly suited for SSF, have to be generated for SM production (Barrios-González et al., 1993) as well as for enzyme production (Shankaranand et al., 1992). What is not so clear is what methods and strategies should be used.

14.7.1.1 Starting from hyperproducing strains for SmF

According to the conclusions of the above mentioned study with *P.chrysogenum* (Barrios-González et al., 1993), one form of generating high yielding strains for SSF processes could be starting from an available hyperproducing strain for SmF and isolating mutants that can perform well in SSF (high PS/PL). Ideally, these mutants will combine high production capacity with efficient performance in SSF. In this work, we started from *P.chrysogenum* P2, a strain for SmF, with a discrete SSF production level of 2.084 mg/g (PS/PL of 2.5). By screening spontaneous mutants it was possible to obtain mutant P2-4 which

produced 10.555 mg of penicillin/g in SSF, showing a relative production of 10.8 (times higher than in SmF) (Table 5). Mutants with even higher relative production (16.7) were also isolated in this study, but since they came from a low producer (Wisconsin), its final production in SSF was lower than that of P2-4. Apparently, the genes coding for the characteristics useful for adaptation to solid environment were not completely lost.

14.7.2 Genetic improvement of selected wild isolates

A strategy that has produced excellent results is screening of the best wild isolates and then subject them to a mutation and selection program. In 1999, Murthy et al., evaluated four strains of *Tolypocladium inflatum*, deposited in the standard culture collection centers (ATCC), for production of cyclosporin A in SSF. They selected strain ATCC 34921, which produced 0.459 mg/g, and from it developed a mutant strain to produce high titers of this metabolite in SSF. The authors carried out 3 sequential stages of mutation and selection, using UV light, epichlorohydin and protoplast generation and regeneration. The selected high yielding mutant strain DRCC 106 produced 1.031 mg of cyclosporin A/g, which represented a production increase of 125%. Under optimum fermentation conditions this strain produced 4.834 mg/g. Our group used this strategy in lovastatin production by SSF. *A.terreus* TUB F-514, was isolated and screened by Szakács et al., (1998). In our laboratory, a SSF process on artificial inert support was developed in which this strain produced 19.95 mg/g. In a second stage, the strain was mutated with UV light and a new mutant strain was isolated by a rational screening method (based on the theoretical environmental constraints found by microorganisms in SSF), that produced 27.89 mg/g also in 7 days. In fact, the mutant strain displayed a slightly higher relative production value of (34.7) than the parental (32) (Table 5).

14.7.3 Molecular genetic improvement

It is important to advance in the understanding of the particular environment faced by fungi and actinomycetes when growing in a solid culture, and the characteristics or functions that allow microorganisms to adapt and perform better in solid culture. In this way researchers will be able to design efficient rational screening methods to obtain superior mutants for SSF. Furthermore, if specific genes involved in its adaptation to the solid environment and/or that have an impact in its productivity are identified, molecular improvement could also be achieved. This would be done by cloning the genes and introducing several copies into the microorganism.

Recently, Biesebeke et al., (2005) studied the relation between the number of hyphal tips and enzyme secretion during the growth of *A.oryze* in SSF. The

authors reasoned that growth of the fungus on a solid substrate involves modification of the substrate by secreted enzymes and subsequent penetration of the hyphae in the substrate. Since enzymes secretion occurs around the apical and subapical region of the advancing hyphal tip, an increase in branching frequency (hyphal tips) should give a higher enzyme producing strain. To prove this, the authors cloned 2 genes related to branching frequency and made 2 constructions to disrupt the genes (*pclA* and *pg/pi-tp*). The disrupted strains displayed an increased hyphal branching and produced 50% more amylase, 100% more glucoamylase and 90% more protease than the parental *A.oryzae* strain in SSF on wheat kernels. Unfortunately enzyme production of these strain and the mutants was not evaluated in SmF, so it is not so clear if this is a molecular improvement method for SSF or if it is a general one.

In another example of molecular improvement our group constructed a cosmid vector for fungi, and cloned the genes of the whole penicillin biosynthetic pathway. This construction was used to transform strains of *P.crysogenum* and penicillin production in SmF and SSF was evaluated. Preliminary results indicate that at least one transformant of strain P2 presented a 421% production increase to reach 12 mg/g in 92 h, while production increase in SmF was much lower.

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