Malaysian Journal of Microbiology Vol 1 (1) 2005, pp. 1-6

REVIEW ARTICLE

Secondary Metabolites Production by Solid-State Fermentation

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ABSTRACT

Microbial secondary metabolites are useful high value products with an enormous range of biological activities. Moreover, the past two decades have been a phase of rapid discovery of new activities and development of major compounds for use in different industrial fields, mainly pharmaceuticals, cosmetics, food, agriculture and farming. Many of these metabolites could be produced advantageously in industry by solid–state fermentation (SSF). Two types of SSF can be distinguished, depending on the nature of the solid phase used: 1) Solid cultures of one support-substrate phase in which solid phase is constituted by a material that assumes, simultaneously, the functions of support and of nutrients source; and 2) Solid cultures of two substrate-support phases: solid phase is constituted by an inert support impregnated with a liquid medium. Besides good production performance, two phases systems have provided a convenient model for basic studies. Studies in our laboratory, as well as in others, have shown that physiology of idiophase (production phase) in SSF share several similarities with the physiology in liquid medium, so similar strategies must be adapted for efficient production processes. However, our studies indicate the need to develop special strains for SSF since overproducing strains, generated for liquid fermentation, cannot be relied upon to perform well in SSF. On the other hand, there are important parameters, specific for SSF, that have to be optimized (pretreatment, initial moisture content, medium concentration and aeration). Respiration studies of secondary metabolites SSF, performed in our laboratory, have shown more subtle aspects of efficient production in SSF. This indicates that there are certain particularities of physiology in SSF that represent the point that needs a better understanding, and that promise to generate knowledge that will be the basis for efficient processes development and control strategies, as well as for the methodology to generate hyperproducing strains, particularly suited for SSF.

Keywords: secondary metabolites, solid-state fermentation, fungi, actinomycetes

INTRODUCTION

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 secondary metabolites, there are other such metabolites with an enormous range of biological activities, hence acquiring actual or potential industrial importance (Table 1). These compounds do not play a physiological role during exponential phase of growth. Moreover, they have been described as secondary metabolites in opposition to primary metabolites (like amino acids, nucleotides, lipids and carbohydrates), that are essential for growth.

Secondary metabolites and 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). Studies in liquid culture show that production of Secondary metabolites 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 Secondary metabolites of economic importance are produced by actinomycetes, particularly of the genus *Streptomyces*, and by fungi.

BIOSYNTHESIS

Microbial secondary metabolites 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 secondary metabolites are formed by pathways which branch off from primary metabolism at a relatively small number of points, which define broad biosynthetic categories or families.

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Table 1: Biological activities of some microbial secondary metabolites of industrial importance (modified from Barrios-Gonzales *et al.*, 2004b)

Activity and some examples	Producing microorganisms
Antibacterials	
Cephalosporin	*Acremonium chrysogenum*
Cephamycin	*Streptomyces clavuligerus*
Chloramphenicol	*Streptomyces venezuelae*
Erythromycin	*Saccharopolyspora erythraea*
Kanamycin	*Streptomyces kanamyceticus,*
Tetracycline	*Streptomyces aureofaciens*
Penicillin	*Penicillin chrysogenum*
Ryfamicin	*Amycolatopsis mediterranei*
Spectinomycin	*Streptomyces spectablis*
Streptomycin	*Streptomyces griseus*
Anticholesterolemics	*Aspergillus terreus*
Lovastatin	*Monascus ruber*
Monacolin	*Penicillium citrinum,*
Pravastatin	*Streptomyces carbophilus*
Antifungals	
Amphotericin	*Streptomyces nodosus*
Aspergillic acid	*Aspergillus flavus*
Aureofacin	*Streptomyces aureofaciens*
Candicidin	*Streptomyces griseus*
Griseofulvin	*Penicillium griseofulvum*
Nystatin	*Streptomyces nourse,* *Streptomyces aureus*
Oligomycin	*Streptomyces diastachromogenes*
Antitumorals	
Actinomycin D	*Streptomyces antibioticus* *Streptomyces parvulus*
Bleomycin	*Streptomyces verticillus*
Doxorubicin	*Streptomyces speuceticus*
Mitomycin C	*Streptomyces lavendulae*
Taxol	*Taxomyces andreanae*
Plant enzyme inhibitor	*Streptomyces clavuligerus*
Clavulanic acid	
Plant growth regulators	
Gibberellin	*Gibberella fujikuroi*
Growth promoters	
Monensin	*Streptomyces cinnamonensis*
Tylosin	*Streptomyces fradiae*
Herbicidal	
Bialaphos	*Streptomyces hygroscopicus*
Immunosuppresives	
Cyclosporine A	*Tolypoclaudium inflatum*
Rapamycin	*Streptomyces hygroscopicus*
Tacrolimus (FK-506)	Several *Streptomyces* species
Insecticides and antiparasitics	
Avermectin	*Streptomyces avermitilis*
Milbermycin	*Streptomyces hygroscopicus*
Pigments	
Astaxanthin	*Phaffia rhodozyma*
Monascin	*Monascus purpureus* *Monascus ruber*

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

2) Metabolites derived from amino acids. Like the β-lactam antibiotics: penicillin, cephalosporins and cephamycins, as well as the immunosupressive agent cyclosporine; and 3) Metabolites derived from Acetyl-CoA (and related compounds, including Krebs cycle intermediates). This family can be subdivided into polyketides like erythromycin and avermectin; and terpenes like gibberellic acid; and 4) Metabolites derived from sugars. Examples of secondary metabolites in this group are streptomycin and kanamycin (Barrios-González *et al.*, 2003).

REGULATION

Since secondary biosynthetic routes are related to the primary metabolic pathways and use the same intermediates, regulatory mechanisms i.e. induction, carbon catabolite regulation and/or feedback regulation, apparently operate in conjunction with an overall control which is linked to growth rate (Demain, 1983; Sánchez and Demain, 2002; Barrios-González *et al.,* 2004a).

SOLID-STATE FERMENTATION

Solid-state fermentation (SSF) holds an important potential for the production of secondary metabolites (Barrios-González *et al*, 1988, Tomasini *et al*, 1997; Robinson *et al*, 2001).

Although SSF on grains like rice or soy bean has been performed in the East since antiquity, different SSF systems (non-traditional) with potential for secondary metabolites production have been developed in the last 20 years (Table 2).

Today, two types of SSF can be distinguished, depending on the nature of solid phase used (Barrios-González and Mejía, 1996):

a) Solid culture of one support-substrate phase: solid phase is constituted by a material that assumes, simultaneously, the functions of support and of nutrients source. Agricultural or even animal goods or wastes are used as support-substrate.
b) Solid culture of two substrate-support phase: solid phase is constituted by an inert support impregnated with a liquid medium. Inert support serves as a reservoir for the nutrients and water. Materials as sugarcane bagasse pith or polyurethane can be used as inert support.

In our experience two phase systems have provided a convenient model for basic studies (since the media used in submerged fermentation (SmF) can be used in SSF, making comparisons possible. Also, the effect of the presence of different compounds can be determined, and the liquid fermented broth can be extracted (by pressing) and analyzed at any time. Moreover, some of these systems, like sugarcane bagasse, allow very high metabolites production levels.

Table 2: Secondary metabolites produced by solid state fermentation system.

Microorganism (Substrate for SSF system)	Product (concentration µg/g)	Time (days)	Reference
Penicillium chrysogenum Impregnated support	Penicillin 13,000	3	Barrios-González *et al.*, 1993a
Streptomyces viridifaciens Sweet potato residues	Tetracycline 4,720	5	Yang *et al.*, 1989
Acremonium chrysogenum Barley	Cephalosporin 950	10	Jermini *et al.*, 1989
Sugarcane bagasse	Cephalosporin 5,000	4	Cuadra *et al.* 2004
Bacillus subtilis Wheat bran	Iturine 3,660	2	Ohno *et al.*, 1992
Claviceps fusiformis Impregnated Support	Ergot 960	-	Trejo *et al.*, 1992
Rye	Alkaloids 2,080	-	
Monascus sp. Rice	Monacolin K 11,000	20	Ganrong, *et al.*, 2003
Aspergillus terreus Impregnated support	Lovastatin 20,000	7	Baños *et al.*, 2004
Penicillium brevicompactum Wheat bran	Mycophenolic Ac. 3,286	6	Sadhukan *et al.*, 1999

The advantages of some SSF systems, in relation with SmF, include energy requirements of the process are relatively low, since oxygen is transferred almost directly to the microorganism. Secondary metabolites are often produced in much higher yields, in shorter times and sterile conditions are not required (Barrios-González *et al*, 1988; Ohno *et al*, 1993, Balakrishna and Pandey, 1996, Rosenblitt *et al*, 2000). Recently, Biocon India Ltd. started production of three valuable microbial secondary metabolites on industrial scale by SSF. The Food and Drug Admistration (FDA) of the USA has approved the solid-state fermentation technology developed by Biocon for production of human drugs of fungal origin. In the future the race between SMF and SSF will become tougher (Suryanarayan, 2003).

ENVIRONMENTAL FACTORS

A careful analysis of the literature confirms the importance of initial moisture as a key process variable in SSF. Hence, this parameter has to be optimized for secondary metabolites production, as well as for primary metabolites, enzymes or biomass production. In practice, other key process variables are usually optimized or controlled in SSF: pretreatment, particle size, temperature and initial pH, aeration, inoculum size, and some times mixing (Barrios-González and Mejía, 1996).

Moisture content and water activity (Aw).

The early reports on mycotoxin production in SSF as well as reports on the newer processes confirm the importance of moisture content as a fundamental parameter.

The particular initial moisture content range depends on the water retention capacity of the solid medium, which is lowest in grain (traditional type) SSF systems, with values around 35%. In starchy flour systems it is around 50% and in wheat bran between 50 and 60%. The impregnated support systems like sugarcane bagasse allow the usage of the highest initial moisture contents (up to 78%) with optimum values around 70%. Recent work in our laboratory with artificial supports indicated that initial moisture content as high as 85% can be used with polyurethane foam.

Oriol *et al* (1988) studying growth of *A.niger* in SSF using sugarcane bagasse with absorbed liquid medium found that Aw of the liquid phase controls growth in SSF having direct effect on growth rate and an inverse effect on germination time, while initial moisture content does not have any effect on these parameters. However, Barrios-González *et al* (1993b) showed that initial moisture content has an important influence on the penicillin production levels reached in this SSF system. The authors modified initial moisture (between 60 and 78%) keeping Aw constant in a similar way as done by Oriol *et al* (1988). In cultures with initial moisture content of 70 and 73%, high production rates were observed also during the last part of the fermentations, which allowed the cultures to reach penicillin titers of 1,120 µg/g of dry medium, while cultures with lower or higher initial moisture content reached 280 µg/gdm. In the same work, experiments were performed in which nutrients concentration was increased (decreasing Aw), keeping initial moisture content of 70%. It was found that, unlike trophophase (growth phase) the use of very concentrated media favors the antibiotic production in SSF. This was an important effect since production increased 5-fold in 2X medium (twice the concentration recommended for SmF). Conversely, the use of concentrated media had a negative effect on production in SmF.

RESPIRATION STUDIES

Our group performed respiration studies of penicillin SSF Domínguez *et al.* (2000). Respiratory activity plotted in a derivative curve (ml CO_2/g min *vs* t), showed that in this and other secondary metabolites production starts when growth (respiratory activity) is limited. In this way, this curve can be divided into an initial sharp peak (trofophase), (when this peak falls penicillin production starts) and after a second peak, which is generally shorter and much wider presenting a long negative slope that falls steadily until it almost reaches zero. Penicillin production continues during most of the second peak. In this work, manipulating the proportions of support in the solid medium, several high production conditions were detected, and one high production respiratory profile was identified. This profile showed lower and more stable respiration

rates, particularly during the second peak (idiophase). In other words, the different conditions that permitted high penicillin production generated the similar metabolic pattern. It appears that, under these conditions, nutrients were less exposed and mass transfer declined, causing slower nutrients uptake. These conditions probably allow an adequate and constant nutrients supply during a longer period of time, supporting slower but constant growth rates that are more adequate for product biosynthesis. Later results, with other secondary metabolites, suggest that other parameter like aeration or pH can be used to control growth.

NUTRITIONAL FACTORS

Earlier research in our laboratory has shown the mechanisms regulating secondary metabolite production in liquid culture, are also active in SSF. Aflatoxin biosynthesis by *Aspergillus flavus* and *Aspergillus parasiticus* is regulated by phosphate and nitrogen source. Barrios-González *et al.* (1990), studying aflatoxin biosynthesis in cassava SSF, showed that aflatoxin production decreased in solid medium with increasing concentrations of nitrogen and/or phosphate sources, without affecting growth. Other experiments, in two phases SSF showed that penicillin biosynthesis is triggered by the exhaustion of glucose in the solid medium, at a similar threshold as in liquid fermentation (Miranda *et. al.*, 2003). These and other works indicate that regulatory mechanisms are also active in SSF. A practical application of these findings is that, when a solid medium composition is designed, we must consider to by-pass these regulatory mechanisms by avoiding repressing C, N or P sources (of the particular secondary metabolite) and including inductors.

STRAINS FOR SSF

The development of highly producing microbial strains is a prerequisite for efficient biotechnological processes. One major positive aspect of SSF is that metabolites are, in many cases, produced at much higher yields than in liquid culture, also called submerged fermentation (SmF). Shankarananda *et al* (1992) observed discrepancies in the levels of α-amylase produced by different strains of *Bacillus* in SSF in relation to SmF. They concluded that cultures (strains), which are good producers in SmF cannot be relied upon to perform well in SSF. Working with *Penicillium chrysogenum*, our group showed that higher penicillin producing strains (developed for SmF) tend to be the higher producers in SSF. However, it was determined that lower producing strains (closer to the wild type) tend to be more efficient to produce in SSF, i.e. these strains produced several times more in SSF than in SmF up to 17 times more (or a relative production of 17) in this study (Barrios-González *et al.*, 1993a). Conversely, the relative production of the high producing strains ranged from 0.4 to 4 times more in SSF. In studies, with other SM like lovastatin we have found that a strain, that was recently isolated from nature, displayed a relative production of 63 in a 2-phase SSF system (Banos *et al.*, 2004).

This implicates that there is one or several characteristics that allow high production in SSF, which are more frequently found in lower production strains. These characteristics are unknown, but are probably lost during the genetic improvement programs used to generate these strains, since the aim has been high productivity in SmF.

Although in this sense, higher yielding strains (for SmF) tend to be less efficient in SSF, we were able to isolate mutants from these strains (P-2 and ASP-78) particularly suited for SSF that displayed a very good performance in solid culture. With some mutants we were able to produce 10.5 mg penicillin/g dry medium, representing production increases, in relation with the parental strains, of between 500 to 620 % (Barrios-González *et al.*, 1993a).

MOLECULAR STRAIN IMPROVEMENT

Present research in our laboratory is evaluating different strategies for the molecular improvement for strains particularly suited for SSF. In relation to the strategy of increasing the biosynthetic genes dosage, we are evaluating the effect of increasing the penicillin biosynthetic genes copy number in *P.chrysogenum*, in its performance in liquid and in SSF. To do this we have constructed a plasmid (pUAMJC1) with the second and third genes of the pathway (*pcb*C and *pen*DE), and transform *P.chrysogenum* Wisconsin 54-1255 and the higher producer P-2. Penicillin production of these transformants was evaluated in liquid fermentation as well as in SSF. Results indicate that, using this strategy, only a moderate increase in penicillin production was achieved. The best transformant of the Wisconsin strain (TW2) displayed a production increase of 30% in this culture system, while the best on from P-2 showed a production increase of 14%. Surprisingly, no production increase was observed in SSF by these transformants. It appears thus, that these two genes are not rate limiting for penicillin production.

In a new stage we have constructed a fungal cosmid and cloned the whole biosynthetic pathway (*pcb*AB, *pcb*C and *pen*DE). Since the first gene is abnormally long (11.5 kb), it is very difficult to work with it *in vitro*, so the cosmid clone is going to be used directly to transform *P. chrysogenum* strains.

In the cephalosporin line we have observed that an important problem is that *Acremonium chrysogenum* does not present satisfactory growth in SmF. Our efforts aiming to find the conditions to obtain good growth of this fungus in SSF, at the levels we observe with *Penicillium* or *Aspergillus* strains, have not yet been successful. A molecular genetic approach here has been to construct a transgenic strain of *P.chrysogenum*, conserving the penicillin genes that are common to both pathways (*pcb*AB and *pcb*C), but containing additional genes, from *A.chrysogenum*, needed to synthesize cephalosporin C (*cef*D, *cef*EF *and cef*G). Characterization of these transformants is in progress.

CONCLUSIONS

Secondary metabolites are high value products of use in different industrial fields. Some can be produced advantageously by SSF. Moreover, these products can be obtained in different SSF systems.

Two phases SSF systems are ideal for basic studies, and very high yields are also achieved. From studies in these systems, we now know that, when a solid medium composition is designed, we must consider to by-pass the regulatory mechanisms (of the particular secondary metabolite) by avoiding repressing C, N or P sources, while including inductors.

Some fermentation parameters to optimize in SSF are similar to the ones controlled in SmF (pH, aeration, mixing, etc.). Others are particular to SSF, like moisture content, medium concentration, etc.

In relation with the nature of idiophase in solid medium, respiratory studies with penicillin production in two phases SSF indicated that factors (like low bagasse proportion in the solid medium), which propitiates a slow and steady dosification of nutrients generate better conditions for production of secondary metabolites in solid medium.

On the other hand, it has been found that hyperproducing strains, developed for liquid fermentation cannot be relied upon to perform well in SSF, so methods have to be developed to generate overproducing strains particularly suited for SSF.

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