

Microbial Secondary Metabolites Production and Strain Improvement

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Received 20 November 2002; accepted 20 February 2003

Microbial 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 (SM), there are others with an enormous range of other biological activities mainly in fields like: pharmaceutical and cosmetics, food, agriculture and farming. These include compounds with anti-inflammatory, hypotensive, antitumour, anticholesterolemic activities, and also insecticides, plant growth regulators and environmental friendly herbicides and pesticides. These compounds are usually produced by liquid submerged fermentation, but some of these metabolites could be advantageously produced by solid-state fermentation. Today, strain improvement can be performed by two alternative strategies, each having distinct advantages, and in some cases all these approaches can be used in concert to increase production such as classical genetic methods with mutation and random selection or rational selection (including genetic recombination); and molecular genetic improvement methods. The latter can be applied by: amplification of SM biosynthetic genes, inactivation of competing pathways, disruption or amplification of regulatory genes, manipulation of secretory mechanisms and expression of a convenient heterologous protein. It is visualized that in the near future, genomics will also be applied to industrial strain improvement.

Keywords: secondary metabolites, new activities, classical and molecular genetic improvement

Introduction

Secondary metabolites (SM) 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 SM, 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 SM 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). Production of SM starts when growth is limited by the exhaustion of one key nutrient source: carbon, nitrogen or phosphate. 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 SM of economic importance are produced by actinomycetes, particularly of the genus *Streptomyces*, and by fungi.

Biosynthetic Families

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

- (1) Metabolites derived from shikimic acid (aromatic amino acids).

Examples are ergot alkaloids and the antibiotics candidin and chloramphenicol.

- (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 immunosuppressive agent cyclosporine.

- (3) Metabolites derived from Acetyl-CoA (and related compounds, including Krebs's cycle intermediates).

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This family can be subdivided into polyketides and terpenes. Examples of the former group include the antibiotic erythromycin, the insecticidal-antiparasitic compound avermectin and the antitumour agent doxorubicin. An example of the second group is the non cytotoxic antitumour agent taxol.

(4) Metabolites derived from sugars.

Examples of SM in this group are streptomycin and kanamycin (Smith & Berry, 1976).

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 (Demaim & Davis, 1989; Doull & Vining, 1995).

New Bioactive Compounds

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 (Table 1).

Microbial SM are now increasingly being used against diseases previously treated only by synthetic drugs, e.g. as anti-inflammatory, hypotensive, antitumour, anticholesterolemic, uterocontractants, etc. Moreover, new microbial metabolites are being used in non medical fields such as agriculture, with major herbicides, insecticides, plant growth regulators and environmental friendly herbicides and pesticides as well as antiparasitic agents.

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).

Production

Liquid Fermentation

Secondary metabolites are generally produced in industry by submerged fermentation (SmF) by batch or fed-batch culture. An improved strain of the producing microorganism is inoculated into a growth medium in flasks and then transferred to a relatively

small fermenter or "seed culture". This culture, when in rapid growth phase, is used to inoculate a fermenter tank, in the range of 30,000 to 200,000 litres, with production medium. Several parameters, like medium composition, pH, temperature, agitation and aeration rate, are controlled. The different regulatory mechanisms mentioned previously are bypassed by environmental manipulations. Hence, an inducer such as methionine is added to cephalosporine fermentations, phosphate is restricted in chlortetracycline fermentation, and glucose is avoided in penicillin or erythromycin fermentation. The fermentation processes of antibiotics regulated by carbon are now conducted 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. 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). Agitation is provided by turbine impellers at a power input of 1-4 W/litre and air has to be supplied at flow rates of 0.5-1.0 v/v per min. Exit gas is generally analyzed to monitor O₂ and CO₂ concentrations. This can provide metabolic information to regulate the feeding rates of precursors and nutrients.

Some natural antibiotics and other SM are chemically modified, in a subsequent stage to produce semi-synthetic derivatives.

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). This fermentation system has been used in several oriental countries since antiquity, to prepare diverse fermented foods from grains like soybeans or rice (Hesseltine, 1977a, b). However, different SSF systems, that could be called non-traditional have been developed in the last 15 years. A modern SSF definition is the one proposed by Lon-sane *et al* (1985)—a microbial culture that develops on the surface and at the interior of a solid matrix and in absence of free water. Today, two types of SSF can be distinguished, depending on the nature of solid phase used (Barrios-González & Mejía, 1996).

(a) Solid culture of one support-substrate phase—solid phase is constituted by a material that

Table 1—Biological activities of some microbial secondary metabolites of industrial importance

Activity	Examples	Producing Micro-organism
Antibacterials	Cephalosporin	<i>Acremonium chrysogenum</i>
	Cepharmycin	<i>Streptomyces clavuligerus</i>
	Chloramphenicol	<i>Streptomyces venezuelae</i>
	Erythromycin	<i>Saccharopolyspora erythraea</i>
	Kanamycin	<i>Streptomyces kanamyceticus</i>
	Tetracyclin	<i>Streptomyces aureofaciens</i>
	Penicillin	<i>Penicillium chrysogenum</i>
	Rifamycin	<i>Amycolatopsis mediterranei</i>
	Spectinomycin	<i>Streptomyces spectabilis</i>
	Streptomycin	<i>Streptomyces griseus</i>
Anticholesterolemics	Lovastatin	<i>Aspergillus terreus</i>
	Monacolin	<i>Monascus ruber</i>
	Pravastatin	<i>Penicillium citrinum</i> , <i>Streptomyces carbophilus</i>
Antifungals	Amphotericin	<i>Streptomyces nodosus</i>
	Aspergillic acid	<i>Aspergillus flavus</i>
	Aureofacin	<i>Streptomyces aureofaciens</i>
	Candididin	<i>Streptomyces griseus</i>
	Griseofulvin	<i>Penicillium griseofulvum</i>
	Nystatin	<i>Streptomyces nourse</i> , <i>S. aureus</i>
	Oligomycin	<i>Streptomyces diastachromogenes</i>
Antitumourals	Actinomycin D	<i>Streptomyces antibioticus</i> , <i>S. parvulus</i>
	Bleomycin	<i>Streptomyces verticillus</i>
	Doxorubicin	<i>Streptomyces peucetius</i>
	Mitomycin C	<i>Streptomyces lavendulae</i>
	Taxol	<i>Taxomyces andreanae</i> , plants
Enzyme inhibitors	Clavulanic acid	<i>Streptomyces clavuligerus</i>
Plants Growth Regulators	Gibberellin	<i>Gibberella fujikuroi</i>
Growth Promoters	Monensin	<i>Streptomyces cinnamomensis</i>
	Tylosin	<i>Streptomyces fradiae</i>
Herbicides	Bialaphos	<i>Streptomyces hygroscopicus</i>
Immunosuppressives	Cyclosporin A	<i>Tolypocladium inflatum</i>
	Rapamycin	<i>Streptomyces hygroscopicus</i>
	Tacrolimus (FK-506)	several <i>Streptomyces</i> species
Insecticides and Antiparasitics	Avermectin	<i>Streptomyces avermitilis</i>
	Milbemycin	<i>Streptomyces hygroscopicus</i>
Pigments	Astaxanthin	<i>Phaffia rhodozyma</i>
	Monascin	<i>Monascus purpureus</i> , <i>M. ruber</i>

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.

Fungi and actinomycetes, the main microorganisms producer of SM grow well in SSF, because the conditions are similar to their natural habitats, such as soil, and organic waste materials (Table 2).

The advantages of SSF in relation with SmF include: energy requirements of the process are relatively low, since oxygen is transferred directly to the microorganism. SM are often produced in much higher yields, often in shorter times and often sterile conditions are not required (Barrios-González *et al*, 1988; Ohno *et al*, 1993; Balakrishna & Pandey, 1996; Rosenblitt *et al*, 2000).

In SSF, parameters to control are similar to the ones controlled for SmF. Particular parameters like initial moisture content, particle size and medium

concentration have to be optimized in this culture system. It has been shown that penicillin production in a SSF impregnated bagasse system, is strongly controlled by the proportions of bagasse, nutrients and water. Combinations that supported very high penicillin yields were identified in this work (Domínguez *et al*, 2001). Interestingly, these conditions caused growth phases with different characteristics, but all allowed a slow but adequate supply of nutrients to the fungus during the idiophase, supporting a characteristic low but steady respiratory activity during production phase (Domínguez *et al*, 2000).

Reports on enzymes production suggest that high producing strains in SmF are generally poor producers in SSF (Shankaranand *et al*, 1992). Barrios-González *et al* (1993) reported that high yielding strains for SmF cannot be relied upon to perform well in SSF. This situation dictates the need to develop high-yielding strains particularly suited for SSF. These special strains can be developed faster by using hyper-producing strains developed for SmF as parental strains (Barrios-González *et al*, 1993a).

Many comparative studies between SmF and SSF claim higher yields for products made by SSF (Pandey *et al*, 1999, 2000), indicating that some of

Table 2—Secondary metabolites produced by solid state fermentation system

Metabolite	Substrate/Support	Microorganism	Use	Reference
Penicillin	Sugarcane bagasse	<i>Penicillium chrysogenum</i>	Antibiotic	Barrios-González <i>et al</i> , 1988, 1993b
Cephalosporin	rice grains	<i>Streptomyces</i> sp	Antibiotic	Wang <i>et al</i> , 1984; Jerami & Demain, 1989
Cyclosporin A	wheat bran	<i>Tolypocladium inflatum</i>	Antibiotic	Sekar & Balaraman, 1998; Ramana <i>et al</i> 1999
Cephameycin C	wheat straw	<i>Streptomyces clavuligerus</i>	Antibiotic	Kota & Sridhar, 1998
Tetracycline	sweet potato residue	<i>Streptomyces viridifaciens</i>	Antibiotic	Yang & Ling, 1989
Pyrazines	wheat and soybean	<i>Aspergillus oryzae</i>	Aroma	Serrano-Carreón <i>et al</i> , 1992
Oxycetracycline	sweet potato residue	<i>Streptomyces rimosus</i>	Antibiotic	Yang & Yuan, 1990; Yang & Wang, 1996
Iturine	Soybean curd	<i>Bacillus subtilis</i>	Antifungal	Ohno <i>et al</i> , 1993, 1996
Surfactin	Soybean curd	<i>Bacillus subtilis</i>	Surfactant, antibiotic	Ohno <i>et al</i> , 1995
Gibberellic acid	wheat bran cassava polyurethane	<i>Gibberella fujikuroi</i>	Vegetal hormone	Kumar & Lonsane, 1987a,b; Bandelier <i>et al</i> , 1997; Agosin <i>et al</i> , 1997; Tomasini <i>et al</i> , 1997
Pigments	rice grains	<i>Monascus purpureus</i>	Food and pharmaceuticals	Lontong & Suwanarit, 1990; Rosenblitt <i>et al</i> , 2000
Ergot alkaloids	Sugarcane bagasse	<i>Claviceps fusiformis</i>	Medical	Trejo <i>et al</i> , 1993

these metabolites could be commercially produced by SSF. However, commercial production application of secondary metabolites by SSF remains unexploited in Western countries, mainly due to problems associated with scale-up. Most of these problems have been studied and some solutions proposed. Taking in account these points, some bioreactors have been designed (Mitchell *et al*, 2000, 2002; Hardin *et al*, 2000; Nagel *et al*, 2001, 2002; Suryanarayan *et al*, 2001; Junter *et al*, 2002; Miranda *et al*, 2003).

In South-East Asia, where SSF is more common, research attention was directed towards the industrialization of this culture method. In India, a fermentation industry, started industrial production of microbial enzymes and secondary metabolites by SSF. Suryanarayan (2002) designed a solid state bioreactor in which the system is contained, and the fermentation product can be extracted from the solid matrix without opening the reactor. Finally this reactor is operated automatically. Since January 2001, this reactor is being used to produce lovastatin, the first secondary metabolite produced industrially by SSF.

Strain Improvement

The science and technology of 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. Typical examples include removal of unwanted cometabolites, improved utilization of inexpensive carbon and nitrogen sources or alteration of cellular morphology to a form better suited for separation of the mycelium from the product and/or for improved oxygen transfer in the fermenter.

Today, strain improvement can be performed by two alternative strategies: 1) Classical genetic methods (including genetic recombination); and 2) Molecular genetic methods.

Each has distinct advantages, and in some cases all these approaches can be used in concert to increase production.

Classical Genetic Methods

Strain development by this strategy has typically relied on mutation, followed by random screening. After this, careful fermentation tests are performed and new improved mutants are selected. Mutation can be carried out with physical mutagens like UV-light or chemical mutagens like N-methyl-N'-nitro-N-nitrosoguanidine or ethyl methanesulphonate (Baltz, 1999). This empirical approach has a long history of success, 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). Other examples include fungal or actinomycetal cultures capable of producing metabolites in quantities as high as 80 g/l (Rowlands, 1984; Vinci & Byng, 1999).

The advantage of mutation/selection is simplicity, since it requires little knowledge of the genetics, biochemistry and physiology of the product biosynthetic pathway. Moreover, it does not need sophisticated equipment and requires minimal specialized technical manipulation. Another important advantage is effectiveness, since it leads to rapid titer increases.

A drawback of this strategy is that it is labour intensive. In the last 10-15 years, these random screening methods have been replaced by less empirical, directed selection techniques or rational selection.

Rational selection. Rational screening allows for significant improvement in the efficiency of the selection stage. In this process, a selection is made for a particular characteristic of the desired genotype, different from the one of final interest, but easier to detect. In its more effective form, a rational screen will eliminate all undesirable genotypes, allowing very high numbers of isolates to be tested easily.

The design of these methods requires some basic understanding of the product metabolism and pathway regulation. This knowledge can be used to propose environmental conditions, or the addition of a chemical that could be a chromogenic or selective reagent, a dye or an indicator organism. For example, a toxic precursor of penicillin (phenylacetic acid) was added to the agar medium, where the sensitive parent strains were prevented from growing, while only resistant mutants propagated. In this case, 16.7% of the resistant mutants produced more antibiotic than the parental strain (Barrios-González *et al*, 1993b).

In another example, carotenoids have been shown to protect the yeast, *Phaffia rodofzyma* from singlet oxygen damage (oxidative stress). Combination of

Rose Bengal and thymol (oxidation/reduction reaction detection) in visible light has been used to select carotenoid over-producing strains (Schroeder & Johnson, 1995a, b).

Vinci and Byng (1999) have given some examples of selection by rational screening. These include resistance to chloroacetate, fluoroacetate or chloroacetamide for overproduction of polyketides; resistance to 2-deoxyglucose to overcome glucose repression; resistance to methylammonium chloride to overcome repression by ammonium ion, and arsenate resistance to overcome phosphate repression.

Micro-organisms possess regulatory mechanisms which control production of their metabolites, thus preventing overproduction. For primary metabolite production, microbiologists have found that eliminating or decreasing the particular mechanism (deregulating) in the microbe causes overproduction of the desired product. However, factors that turn on secondary product formation are complex (induction, feedback regulation, nutritional regulation by source of carbon, nitrogen and/or phosphorus as well as a global physiological control), most of which are bypassed by nutritional manipulations of the culture. Some success has been achieved by applying concepts derived from mutation of regulatory controls of primary metabolism. For example, a way to produce feed-back resistant mutants of primary metabolism is to select for analogue-resistant mutants. The analogue technique has been successfully applied to secondary metabolism. The fungi, *Penicillium chrysogenum* and *Acremonium chrysogenum* are producers of the β -lactamic antibiotics penicillin and cephalosporin, respectively, which are derived from amino acid precursors. Mutants resistant to analogs of lysine and methionine yielded a much higher frequency of superior strains (Elander & Lowe, 1992). In a similar manner, Pospisil *et al* (1998) evaluated analog resistant mutants of monensine over-producing strains of *Streptomyces cinnamonensis*. When a secondary metabolite like an antibiotic is itself a growth inhibitor, the antibiotic can be used to select resistant cultures, some of which are superior producers (Elander & Vournakis, 1986).

Genetic recombination methods are represented by sexual or parasexual crosses in fungi and conjugation in actinomycetes. However, it is very often performed by protoplast fusion in both organisms (Elander & Lowe 1992). This strategy becomes an important complement to mutagenesis, once several independent

lineages of mutants have been established. It represents a means to construct strains with many different combinations of mutations that influence production. A situation where recombination (by protoplast fusion) of related species of actinomycetes or related species of fungi seems particularly attractive is when one strain has been subjected to years of genetic development and produces high levels of a SM, and the other is a new isolate that produces low levels of a new SM. The productivity of the newly identified SM may be increased by generating recombinants from the two strains.

Molecular Genetic Methods

To carry out these strategies, some biochemical and molecular genetic tools, including identification of the biosynthetic pathway, adequate vectors and effective transformation protocols for the particular species have to be developed or made available. After this, the biosynthetic gene or genes have to be cloned and analyzed. Molecular biology of actinomycetes and fungi has been successfully developed to a degree that its application to industrial strain improvement is now a reality.

Genetic engineering methods have also provided the tools to know in detail the nature of the modifications that have occurred during the decades of genetic improvement of industrial strains (mainly by random mutagenesis).

Characterization of high producing strains. The genes responsible for antibiotic biosynthesis are grouped together in clusters in most fungi and actinomycetes. It has been found that in industrial penicillin production strains, like *P. chrysogenum* AS-P-78 or P2, the cluster of penicillin biosynthetic genes is amplified in a tandem array. In these strains, a DNA region of ~106.5 kb (containing these genes) has been amplified between 5 and 7 times, while only one copy is found in the original isolate (NRRL 1951). The sequence TTTACA has been found flanking the amplified region, as well as linking the different copies. In the much higher-producing industrial strain, *P. chrysogenum* E1, there are 12 to 14 copies of the biosynthetic cluster, being the size of the amplified region of only ~57.9 kb, in this case (Fierro *et al*, 1995). Penicillin production correlates well with the number of copies of the biosynthetic genes present in them. It indicates that this cluster amplification has been an important factor in achieving the great production increases during the long process of development (by

mutation and selection) of these strains. But not the only one, since the intermediate level producer, Wisconsin 54-1255, displays a 15-20 fold higher production than the wild type, but they both have just one copy of the biosynthetic genes.

These and other findings have influenced the strategies that are being used to apply genetic engineering to strain improvement of antibiotics and other SM producing strains.

Targeted duplication or amplification of SM production genes. Although this method has not yet been harnessed as a general method to improve product yields, there are some encouraging reports, both in actinomycetes and in fungi. This strategy can be divided into two different approaches: targeted gene duplication (or amplification) and whole pathway amplification.

A prerequisite for the former is to identify the rate limiting step in the biosynthetic pathway and to clone the gene. Ideally, the first step would be to identify a neutral site in the chromosome where genes can be inserted without altering the fermentation properties of the strain. Then the neutral site is cloned and incorporated into the vector with the antibiotic gene. In this way, after transformation, the gene is inserted into the chromosomal neutral site by homologous recombination (Baltz, 1998).

An example of the neutral site cloning was the targeted duplication of the *tylF* gene that encodes the rate limiting O-methylation of macrocin in the tylosin biosynthesis in an industrial production strain of *Streptomyces fradiae*. Transformants that contained two copies of the *tylF* gene produced 60% more tylosin than the parental strain (Solenberg *et al*, 1996; Baltz *et al*, 1997).

It is important to note that in many organisms, particularly industrial antibiotic producing fungi, homologous recombination is not a frequent event (or not easy to achieve). In these cases the plasmid integrates in different sites in the different transformants obtained. However, a very simple screening for high producers among them will indicate the cases where the gene integrated in an adequate site of the chromosome.

With the development of genetic tools for fungi, including more efficient transformation techniques, first in *Aspergillus nidulans* (Yelton *et al*, 1984) and later in *Acremonium chrysogenum* (Peñalva *et al*, 1985; Queener *et al*, 1985; Skatrud, 1987) and *P. chrysogenum* (Beri & Turner, 1987; Cantoral *et al*,

1987; Sánchez *et al*, 1987), the gene amplification effect was studied in these organisms.

Skatrud and coworkers (1989) successfully amplified the gene *cefEF* of the cephalosporin pathway in *A. chrysogenum*. This caused a decrease in the intermediate penicillin N and a 30% increase in cephalosporin C production. Even better results (3 fold cephalosporin C production increase) were obtained when gene *cefG* (last step in the pathway) was amplified in *A. chrysogenum* C10 (Gutiérrez *et al*, 1991).

Kennedy & Turner (1996), working with *A. nidulans*, performed a variation of this strategy: promoter replacement. That is, exchanging the gene's promoter for a stronger and/or less regulated one, hence obtaining the same effect as with gene amplification. They performed a promoter fusion to the first gene of the pathway *pcbAB* resulting in a 30 fold increase in penicillin yields. It is important to note, however, that, penicillin production in this model organism is very small compared with the strains of *P. chrysogenum*.

Integration of additional copies of the second or the third gene of this three steps pathway, has not had an important effect on penicillin yields (Barredo, 1990; Fernández, 1997). However, introduction of additional copies of these two genes together in the original fragment caused a 40% increase in the penicillin low producing strain *P. chrysogenum* Wis. 54-1255 (Veenstra *et al*, 1991). Recently, the introduction of the complete penicillin cluster in the same strain was studied. Transformants were isolated with production increases of 124 to 176% (Theilgaard *et al*, 2001).

There are two reports of gene cluster amplifications in actinomycetes leading to yield enhancements (Gravius *et al*, 1994; Peschke *et al*, 1995).

Inactivation of competing pathways. Molecular genetics also provides the means to block a pathway that competes for a common intermediate, key precursors such as cofactors, reducing power and energy supply. Such strains could be able to channel the precursors to the SM biosynthesis. This can be done by transposon mutagenesis in actinomycetes, gene disruption or by inserting an antisense synthetic gene.

α -amino adipic acid, is one of the 3 amino acid precursors of penicillin biosynthesis, and it is also a branching point, leading to the synthesis of lysine. Disruption of gene *lys2* of *P. chrysogenum*, which connects α -amino adipic towards lysine, has generated auxotrophs of the amino acid that show 100% increase in penicillin yields (Casqueiro *et al*, 1999). In microorganisms where homologous recombination is

not easy to achieve, such as *P. chrysogenum*, inactivation of a gene could probably be done easier by transforming with an antisense gene or an antisense oligonucleotide.

Regulatory genes. A task much more complicated than identifying the biosynthetic pathway and cloning the corresponding genes is investigating its regulation at a molecular level. However, the same molecular genetics tools are allowing important advances in this complicated field.

It is encouraging that the amplification of a regulatory gene (*ccaR*), required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*, results in a 3 fold overproduction of both industrial β -lactam compounds. (Pérez-Llarena *et al*, 1997). Moreover, disruption of negatively acting regulatory gene *mmv* of methylenomycin biosynthesis increased production 17 fold, whereas introduction of a single copy of the positively acting gene *actIII* raised the synthesis of actinorhodine 35-fold in *Streptomyces coelicolor* (Hobbs *et al*, 1992; Gramajo *et al*, 1993; Bibb, 1996). Research with actinomycetes is more advanced in this area, where transposon mutagenesis appears to be a useful procedure to identify (disrupt) and clone regulatory genes (Solenberg & Baltz, 1994; Baltz, 2001).

Basic knowledge on regulatory mechanisms will also present the opportunity to delete negatively *cis* acting regulatory elements in the promoter region, as well as insertion of activating sequences.

Secretion mechanisms. This is another point now under study with an important potential for molecular strain improvement. In fact several protein-hyperproducing yeast strains have been constructed by increasing specific genes of the secretion path (like genes *kar2* and *pdi1*) or by disruption of genes like *pmr1*.

Enhanced *bipA* (*kar2* analogue in filamentous fungi) mRNA levels have been observed in various *Aspergillus* strains expressing recombinant extracellular proteins. (Punt *et al*, 1998; Sägt *et al*, 1998). However, the correlation between BiP induction and secretion efficiency remains unclear. *pdiA* genes, encoding protein disulphide isomerase, also are potential targets for secretion pathway manipulation. Noticeable differences in the *Trichoderma reesei* *pdiA* expression levels were observed under conditions supporting high levels of protein secretion compared to those supporting low levels of protein secretion (Saloheimo *et al*, 1999).

Unfortunately, the amplification of these genes in *Aspergillus niger* has not succeeded in increasing heterologous proteins production in the fungus (Conesa *et al*, 2001).

Expression of heterologous enzyme activities. An alternative strategy for strain improvement is to incorporate a new enzymatic activity in the strain (heterologous gene) that will lead to the formation of a new related product of industrial interest. This could only be obtained through a difficult and expensive process of chemical synthesis. Transformation of *A. chrysogenum* with a D-aminoacid oxidase of *Fusarium solani* and a cephalosporin acylase from *Pseudomonas diminuta*, caused the direct synthesis of 7-ACA, the substrate for the production of semi-synthetic cephalosporins (Isogai *et al*, 1991).

When an oxygen transporter bacterial protein, similar to hemoglobin, was introduced in *A. chrysogenum*, transformants were isolated with increased cephalosporin C production yields (De Modena *et al*, 1993).

Another example is the disruption of gene *cefEF* in an industrial strain of *A. chrysogenum*, and the integration of the gene *cefE* from *Streptomyces clavuligerus*. The transformants obtained could produce great amounts of desacetoxicephalosporin C, product that can easily be transformed into the other precursor of semi-synthetic cephalosporins, 7-ADCA (Velasco *et al*, 2000).

Combinatorial biosynthesis. Another interesting strategy is the development of novel antibiotics, produced by using non conventional compounds as substrates of the biosynthetic enzymes of the microorganism. These enzymes can be modified or mutated in such a way as to increase their affinity for those unnatural substrates.

Generation of new antibiotics can also be performed by the so called combinatorial biosynthesis. In this case, different activity modules of enzymes like polyketide synthases can be rearranged by genetic engineering to obtain a microbial strain that synthesizes an antibiotic with novel characteristics. An Eli Lilly research group engineered *Streptomyces toyocaensis*, the producer of the non-glycosylated heptapeptide (similar to teicoplanin core) to produce hybrid glycopeptides. They expressed the glycosyltransferase genes from vancomycin- and chloroeremomycin-producing strains of *A. orientalis* in this organism, generating a novel monoglycosylated derivative (Solenberg *et al*, 1997).

Perspectives

It is visualized that discoveries of new antibiotics and other SM, useful in the medical field as well as in other productive activities, will continue at a fast rate, driven by the new target-directed strategies to find microbial SM. Generation of new SM will also be performed by the so called combinatorial biosynthesis. Economic production of these compounds will depend on the fermentation production process and on the application of adequate strain improvement methods.

Even though molecular genetic improvement is just starting to become a practical reality, the next important scientific and technological advance is already appearing on the horizon, challenging researchers imagination and creativity.

The end of the human genome project has liberated a great technical potential for DNA sequencing. Part of this capacity is now being directed to sequencing the genomes of model microorganisms. The complete genomes of *E. coli*, the yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe*, and of other 50 microorganisms, have already been sequenced; while the sequencing of the fungi *Aspergillus nidulans* and *Neurospora crassa* are in progress. After this group, the turn is of microorganisms of industrial importance. Moreover, the entire genomic sequences of *Streptomyces coelicolor* and *Streptomyces avermitilis* have very recently been published (Omura *et al*, 2001; Bentley *et al*, 2002).

Hence, the challenge is to apply this huge amount of information to genetic improvement strategies and methods (genomics). The knowledge (availability) of the complete nucleotide sequence of a species, supported by the genomic sequence information and functional annotations of many other microbial genomes, will enable us to identify all the genes present in SM producing microorganism. This information will facilitate metabolic reconstruction; that is the prediction of the pathways (genes) associated with the particular SM biosynthesis, like the synthetic pathway itself, precursor biosynthesis, cofactors biosynthesis, reducing power, regulatory circuits, etc. This information could be useful in designing rational screens.

In a very modern approach to molecular genetics strain improvement, this information will facilitate rapid testing of the metabolic reconstruction predictions by gene disruption analysis. Genes whose disruption causes a decrease in product yield should be amplified. The inactivation of genes encoding for a

competing function or a negative regulatory element should cause an increase in product titers. In this way, genes that should be amplified and genes that should be inactivated can be identified. On the other hand, multiple transcript analysis by DNA micro arrays, of different strains and environmental and physiological conditions, will provide additional and complementary information about the relevance of many genes.

In a near future, a number of genetic and molecular genetics methods will be available to improve fermentation product yields and other strain characteristics. Some are effective and simple (like mutation and selection), others are more expensive and sophisticated and have been applied successfully in a few industrial cases, but with high theoretical potential. The choice of approaches which should be taken will be driven by the economics of the biotechnological process, and the genetic tools available for the strain of interest.

References

- Agosin E *et al*, 1997. Production of gibberellins by solid substrate cultivation of *Gibberella fujikuroi*. in *Advances in Solid state fermentation*, edited by S Roussos, B K Lonsane, M Raimbault & G Viniegra-González. Kluwer Academic Publishers, The Netherlands. Pp 355-366.
- Balakrishnan K & Pandey A, 1996. Production of biologically active secondary metabolites in solid state fermentation. *J Sci Ind Res*, **55**, 365-372.
- Baltz R H, 1997. Molecular genetic approaches to yield improvement in actinomycetes. *Drug Pharm Sci*, **82**, 49-62.
- Baltz R H, 1998. New genetics methods to improve secondary metabolite production in *Streptomyces*. *J Ind Microbiol Biotechnol*, **20**, 360-363.
- Baltz R H, 1999. Mutagenesis. in *Encyclopedia of Bioprocess technology: Fermentation, biocatalysis and separation*, edited by M C Flickinger & S W Drew. Wiley, New York. Pp 307-311.
- Baltz R H, 2001. Genetics methods and strategies for secondary metabolite yield improvement in actinomycetes. *A van Leeuwenhoek*, **79**, 251-259.
- Bandelier S *et al*, 1997. Production of gibberellic acid by fed batch solid state fermentation in an aseptic pilot scale reactor. *Proc Biochem*, **32**, 141-145.
- Barredo, 1990. Análisis de una región del genoma de *Penicillium chrysogenum* que contiene los genes *pcbC* y *penDE*. Ph D Thesis. University of León, Spain.
- Barrios-González J & Mejia A, 1996. Production of secondary metabolites by solid-state fermentation. *Biotechnol Annu Rev*, **2**, 85-121.
- Barrios-González J *et al*, 1988. Penicillin production by solid state fermentation. *Biotechnol Lett*, **10**, 793-798.
- Barrios-González J *et al*, 1993a. Development of high penicillin producing strains for solid-state fermentation. *Biotechnol Adv*, **10**, 793-798.

- Barrios-González, J *et al*, 1993b. Penicillin production by mutants resistant to phenylacetic acid. *J Ferm Bioeng*, **76**, 455-458.
- Barrios-González J *et al*, 2003. Production of Antibiotics. in Concise Encyclopedia of Bioresource Technology, edited by Ashok Pandey. The Haworth Press Inc., Binghamton, New York (in press).
- Bentley S D *et al*, 2002. Complete genome sequence of the model actinomycete *Streptomyces coelicolor* A3(2). *Nature (Lond)*, **417**, 141-147.
- Beri R K & Turner G, 1987. Transformation of *Penicillium chrysogenum* using the *Aspergillus nidulans* *amdS* gene as a dominant selective marker. *Curr Genet*, **11**, 6390-6411.
- Bibb M, 1996. Colworth Prize Lecture. The regulation of antibiotic production in *Streptomyces coelicolor* A3(2). *Microbiology*, **142**, 1335-1344.
- Cantoral J M *et al*, 1987. High frequency transformation of *Penicillium chrysogenum*. *BioTechnology*, **5**, 494-497.
- Casqueiro J *et al*, 1999. Gene targeting in *Penicillium chrysogenum*: Disruption of the *lys2* gene leads to penicillin overproduction. *J Bacteriol*, **181**, 1181-1188.
- Conesa A *et al*, 2001. The secretion pathway in filamentous fungi: A biotechnological view. *Fungal Genet Biol*, **33**, 155-171.
- DeModena J A *et al*, 1993. The production of cephalosporin C by *Acremonium chrysogenum* is improved by the intracellular expression of a bacterial hemoglobin. *BioTechnology*, **11**, 926-929.
- Domínguez M *et al*, 2000. Respiration studies of penicillin solid-state fermentation. *J Biosci Bioeng*, **89**, 409-413.
- Domínguez M *et al*, 2001. Optimization of bagasse, nutrients and initial moisture ratios on the yields of penicillin in solid-state fermentation. *World J Microbiol Biotechnol*, **17**, 751-756.
- Elander R P & Vournakis J N, 1986. Enetic aspects of overproduction of antibiotics and other secondary metabolites. in Overproduction of Microbial metabolites, edited by Z Vaneck & Z Hostalek. Butterworth, Boston. Pp 63-79.
- Elander R P & Lowe D A, 1992. Fungal Biotechnology: An overview. in Handbook of Applied Mycology, Vol. 4, edited by D K Arora, R P Elander & K G Mukerji. Marcel Dekker, New York. Pp 1-34.
- Fernández F J, 1997. Caracterización de la expresión de los genes implicados en la biosíntesis de penicilina: Reacción enzimática catalizada por el producto del último gen (*penDE*) de la ruta biosintética de penicilina. Ph D Thesis. University of León, Spain.
- Fierro F *et al*, 1995. The penicillin gene cluster is amplified in tandem repeats linked by conserved hexanucleotide sequences. *Proc Natl Acad Sci USA*, **92**, 6200-6204.
- Gramajo H C, *et al*, 1993. Stationary-phase production of the antibiotic actinorhodin in *Streptomyces coelicolor* A3(2) is transcriptionally regulated *Mol Microbiol*, **7**, 837-845.
- Gravius B *et al*, 1994. The 387 kb linear plasmid of *Streptomyces rimosus* and the interactions with the chromosome. *Microbiology*, **140**, 2271-2277.
- Gutiérrez S *et al*, 1991. Expression of the *penDE* gene of *Penicillium chrysogenum* encoding isopenicillin N acyltransferase in *Cephalosporium acremonium*: production of benzylpenicillin by the transformants. *Mol Gen Genet*, **225**, 56-64.
- Hardin M T *et al*, 2000. Approach to designing rotating drum bioreactors for solid-state fermentation on the basis of dimensionless design factors. *Biotechnol Bioeng*, **67**, 274-282.
- Hesseltine C W, 1977a. Solid-state fermentation Part 1. *Proc Biochem*, **12**, 24-27.
- Hesseltine C W, 1977b. Solid-state fermentation Part 2. *Proc Biochem*, **12**, 29-32.
- Hobbs G *et al*, 1992. An integrated approach to studying regulation of production of the antibiotic methylenomycin by *Streptomyces coelicolor* A3(2). *J Bacteriol*, **174**, 1487-1494.
- Isogai T *et al*, 1991. Construction of a 7-aminocephalosporanic acid (7ACA) biosynthetic operon and direct production of 7ACA in *Acremonium chrysogenum*. *BioTechnology*, **9**, 188-191.
- Jermi M F G & Demain A L, 1989. Solid state fermentation for cephalosporin production by *Streptomyces clavuligerus* and *Cephalosporium acremonium*. *Experientia*, **45**, 1061-1065.
- Junter B *et al*, 2002. Use of frozen bagged seed inoculum for secondary metabolites and biocover conversion processes at the pilot scale. *Biotechnol Bioeng*, **79**, 628-640.
- Kennedy J & Turner G, 1996. δ -(L- α -amino adipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Mol Gen Genet*, **253**, 189-197.
- Khetan A *et al*, 1999. Precursor and cofactor as a check valve for cephamycin biosynthesis in *Streptomyces clavuligerus*. *Biotechnol Prog*, **15**, 1020-1027.
- Kota K P & Sridhar P, 1998. Solid state cultivation of *Streptomyces clavuligerus* for producing cephamycin C. *J Sci Ind Res*, **57**, 587-590.
- Kumar P K R & Lonsane B K, 1987a. Gibberellic acid by SSF: consistent and improvement yields. *Biotechnol Bioeng*, **30**, 267-271.
- Kumar P K R & Lonsane B K, 1987b. Extraction of gibberellic acid from dry mouldy bran produced under solid-state fermentation. *Proc Biochem Biotechnol*, **33**, 139-143.
- Lonsane B K *et al*, 1985. Engineering aspects of solid-state fermentation. *Enzyme Microbiol Technol*, **7**, 258-265.
- Lotong N & Suwanarit P, 1990. Fermentation of ang-kak in plastic bags and regulation of pigmentation by initial moisture content. *J Appl Bacteriol*, **68**, 565-570.
- Miranda L O *et al*, 2003. Efecto del mezclado, la adición de agua y lactosa en la producción de penicilina por *Penicillium chrysogenum* en fermentación en estado sólido usando un biorreactor dinámico. *Información Tecnológica*, **14**(1)(in press).
- Mitchell D A *et al*, 2000. New developments in solid-state fermentation. II Rational approaches to the design, operating and scale-up of bioreactors. *Proc Biochem*, **35**, 1211-1225.
- Mitchell D A *et al*, 2002. The potential for establishment of axial temperature profiles during solid-state fermentation in rotating drum bioreactors. *Biotechnol Bioeng*, **80**, 114-122.
- Nagel F J *et al*, 2001 Temperature control in a continuously mixed bioreactor. *Biotechnol Bioeng*, **72**, 219-230.
- Nagel F J *et al*, 2002 Simultaneous control of temperature and moisture content in a mist bioreactor for solid-state fermentation. *Biotechnol Bioeng* (in press)

- Ohno A *et al*, 1993. Production of the antifungal peptide antibiotic, iturin, by *Bacillus subtilis* N B22. *Biotechnol Lett*, **14**, 1165-1168.
- Ohno A *et al*, 1995. Production of a lipopeptide antibiotic, surfactin, by recombinant *Bacillus subtilis* in solid state fermentation. *Biotechnol Bioeng*, **47**, 209-213.
- Ohno A *et al*, 1996. Use of soybean curd residue, okra, for solid state substrate in the production of a lipopeptide antibiotic, iturin A, by *Bacillus subtilis* NB22. *Proc Biochem*, **16**, 483-489.
- Omura S *et al*, 2001. Genome sequence of an industrial microorganism *Streptomyces avermitilis*: deducing the ability of producing secondary metabolites. *Proc Natl Acad Sci USA*, **98**, 12215-12220.
- Pandey A P *et al*, 1999. Solid state fermentation for the production of industrial enzymes. *Curr Sci*, **77**, 149-162.
- Pandey A P *et al*, 2000. New developments in solid state fermentation: I-Bioprocesses and products. *Proc Biochem*, **35**, 1153-1169.
- Parekh S *et al*, 2000. Improvement of microbial strains and fermentation processes. *Appl Microbiol Biotechnol*, **54**, 287-301.
- Peberdy J F, 1985. Biology of Penicillins. in *Biology of Industrial Microorganisms*, edited by A Demain & N Solomon. Benjamin Cummings, Menlo Park. Pp 407-431.
- Peñalva M A *et al*, 1985. Studies on transformation of *Cephalosporium acremonium*. in *Molecular Genetics of Filamentous Fungi*, edited by W E Timberlake. & Alan R. Liss, New York. P 59.
- Perez-Llarena F J *et al*, 1997. A regulatory gene (*ccaR*) required for cephamycin and clavulanic acid production in *Streptomyces clavuligerus*: amplification results in overproduction of both beta-lactam compounds. *J Bacteriol*, **179**, 2053-2059.
- Peschke U *et al*, 1995. Molecular characterization of the lincomycin-production gene cluster of *Streptomyces lincolnensis* 78-11. *Mol Microbiol*, **16**, 1137-1158.
- Pospisil S *et al*, 1998. Depression and altered feedback regulation of valine biosynthesis pathway in analogue-resistant mutants of *Streptomyces cinnamonensis* resulting in 2-ketoisovalerate excretion. *J Appl Microbiol*, **85**, 9-16.
- Punt P J *et al*, 1998. Analysis of the role of the gene *bipA*, encoding the major endoplasmic reticulum chaperone protein in the secretion of homologous and heterologous proteins in black Aspergilli. *Appl Microbiol Biotechnol*, **50**, 447-454.
- Queener S W *et al*, 1985. A system for genetic transformation of *Cephalosporium acremonium*. in *Microbiology*, edited by L. Lieve. American Society for Microbiology, Washington DC. Pp 468-472.
- Ramana M M V *et al*, 1999. Cyclosporin A production by *Tolypocladium inflatum* using solid state fermentation. *Proc Biochem*, **34**, 269-280.
- Robinson T *et al*, 2001. Solid-state fermentation: a promising microbial technology for secondary metabolite production. *Appl Microbiol Biotechnol*, **55**, 284-289.
- Rosenblitt A *et al*, 2000. Solid substrate fermentation of *Monascus purpureus*; Growth, carbon balance and consistency analysis. *Biotechnol Prog*, **16**, 152-162.
- Rowlands R T, 1984. Industrial strain improvement: mutagenesis and random screening procedures. *Enzyme Microbiol Technol*, **6**, 3-10.
- Sagt C M *et al*, 1998. Impaired secretion of a hydrophobic cutinase by *Saccharomyces cerevisiae* correlates with an increased association with immunoglobulin heavy-chain binding protein (BiP). *Appl Environ Microbiol*, **64**, 316-324.
- Saloheimo M *et al*, 1999. The protein disulphide isomerase gene of the fungus *Trichoderma reesei* is induced by endoplasmic reticulum stress and regulated by the carbon source. *Mol Genet*, **262**, 35-45.
- Sánchez F *et al*, 1987. Transformation in *Penicillium chrysogenum*. *Gene*, **51**, 97-102.
- Schroeder W A & Johnson E A, 1995a. Singlet oxygen and peroxyl radicals regulate carotenoid biosynthesis in *Phaffia rhodozyma*. *J Biol Chem*, **270**, 18374-18379.
- Schroeder W A & Johnson EA, 1995b. Carotenoids protect *Phaffia rhodozyma* against singlet oxygen damage. *J Ind Microbiol*, **14**, 502-507.
- Sekar C & Balaraman K, 1998. Optimization studies of the production of cyclosporine A by solid state fermentation. *Biol Eng*, **18**, 293-296.
- Serrano-Carreón L *et al*, 1992. A simple protocol for extraction and detection of pyrazines produced by fungi in solid state fermentation. *Biotechnol Tech*, **6**, 19-22.
- Shankaranand V S *et al*, 1992. Idiosyncrasies of solid-state fermentation system in the biosynthesis of metabolites by some bacterial and fungal cultures. *Proc Biochem*, **27**, 33-36.
- Skatrud P L *et al*, 1987. Efficient integrative transformation of *C. acremonium*. *Curr Genet*, **12**, 337-348.
- Skatrud P L *et al*, 1989. Use of recombinant DNA to improve production of cephalosporin C by *Cephalosporium acremonium*. *Bio/Technology*, **7**, 477-485.
- Solenberg P J & Baltz R H, 1994. Hyper-transposing derivatives of the streptomycete insertion sequence IS493. *Gene*, **147**, 47-54.
- Solenberg P J *et al*, 1996. Transposition mutagenesis in *Streptomyces fradiae*: Identification of a neutral site for the stable insertion of DNA by transposon exchange. *Gene*, **168**, 67-72.
- Solenberg P J *et al*, 1997 Production of hybrid glycopeptide antibiotics in vitro and in *Streptomyces toyocaensis*. *Chem. Biol*, **4**, 195-202.
- Suryanarayan S, 2001. Solid State Fermentation. *US Pat 6197573B1*. 6 March 2001.
- Suryanarayan S, 2002. Current industrial practice in solid matrix fermentations for secondary metabolite production. The Biocon India Experience. *Biochem Eng J of Solid State Ferm.* (in press).
- Theilgaard H *et al*, 2001. Quantitative analysis of *Penicillium chrysogenum* Wis54-1255 transformants overexpressing the penicillin biosynthetic genes. *Biotechnol Bioeng*, **72**, 379-388.
- Tomasini A *et al*, 1997. Gibberellic acid production using different solid state fermentation systems. *World J Microbiol Biotechnol*, **13**, 203-206.
- Trejo H M R *et al*, 1993. Spectra of ergot alkaloids produced by *Claviceps purpurea* 1029C in solid state fermentation system: Influence of the composition of liquid medium used for

- impregnating sugar cane pith bagasse. *Proc Biochem*, **28**, 23-27.
- Veenstra A E *et al*, 1991. Strain improvement of *Penicillium chrysogenum* by recombinant DNA techniques. *J Biotechnol*, **17**, 81-90.
- Velasco J *et al*, 2000. Environmentally safe production of 7-aminodeacetoxycephalosporanic acid (7-ADCA) using recombinant strains of *Acremonium chrysogenum*. *Nat Biotechnol*, **18**, 857-861.
- Vinci V & Byng G, 1999. Strain improvement by non-recombinant methods. in *Manual of Industrial Microbiology and Biotechnology*, edited by A L Demain & J E Davies. American Society for Microbiology, Washington DC, Pp 103-113.
- Wang H H *et al*, 1984. Cephalosporin production by SSF of rice grains. *J Microbiol Immunol*, **17**, 55-69.
- Yang S S & Ling M Y, 1989. Tetracycline production with sweet potato residue by solid state fermentation. *Biotechnol Bioeng*, **33**, 1021-1028.
- Yang S S & Wang J Y, 1996. Morphogenesis, ATP content and oxytetracycline production by *Streptomyces rimosus* in solid substrate cultivation. *J Appl Bacteriol*, **80**, 545-550.
- Yang S S & Yuan S S, 1990. Oxytetracycline production by *Streptomyces rimosus* in solid state fermentation of sweet potato residue. *W J Microbiol Biotechnol*, **6**, 236-244.
- Yelton MM *et al*, 1984. Transformation of *Aspergillus nidulans* by using a *trpC* plasmid. *Proc Natl Acad Sci USA*, **81**, 1470-1474.