Chapter 7
Solid-State Fermentation of Agricultural Residues for the Production of Antibiotics

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7.1 Introduction

The agro-industrial residues are generated globally and a major portion is left unutilised, leading to loss of biomass and an environmental pollution problems. Wide agricultural practices and activities of agro-based industries produce tonnes of by-products, viz., sugarcane bagasse, sweet sorghum, citrus and agave, seeds and peels, rice, barley, wheat and oat straw, corn straw and corncobs. Based on the nutritional properties, the agro-industry residues are classified into two major groups: fibrous residues (higher and lower digestibility) and brans. High digestibility fibrous residues include citrus pulp, corn gluten bran, soy husk and brewing residues, and those with low digestibility include sugarcane bagasse, cereal, wheat, corn, cotton, soy and peanut husk. Brans include rice, peanut, soy and cotton (Graminha et al. 2008). Considering the properties and chemical constitution, these agricultural residues can be used as a natural bioresource for the production of bioactive compounds such as secondary metabolites from various selected microorganisms.

In microbial cultures, the end of primary growth phase initiates the synthesis of secondary metabolites. Various groups of secondary metabolites can play many different roles, such as antibiotics, toxins, ionophores and bioregulators, and involve in intra- and interspecific signalling. These metabolites represent some of the most important industrial products and possess tremendous economic importance. Most certainly, the antibiotics are the best-known subdivision of this group of metabolites. Several species of filamentous fungi and actinomycetes followed by other bacteria, such as *Bacillus*, *Pseudomonas*, myxobacteria and cyanobacteria, have been used in utilising the agricultural residues through fermentation techniques due to their ability to grow on particle surfaces as sources of carbon and energy and

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S.K. Brar et al. (eds.), *Biotransformation of Waste Biomass into High Value Biochemicals*,
DOI 10.1007/978-1-4614-8005-1_7,

Table 7.1 Examples of bacterial and fungal biosynthesis of secondary metabolite, antibiotics

Class	Antibiotics	Producing microorganisms	Reference
Aminoglycoside	Streptomycin	*Streptomyces griseus*	Miyake et al. (1990)
Aminonucleoside	Puromycin	*Streptomyces alboniger*	Tercero et al. (1996)
β-Lactams	Cephalosporin	*Acremonium chrysogenum*	Teijeira et al. (2011)
	Cephamycin C	*Streptomyces clavuligerus*	Devi and Padma (2000)
	Cephabacin	*Lysobacter lactamgenus*	Sohn et al. (2001)
	Penicillin	*Penicillium chrysogenum*	Barrios-Gonzalez et al. (1993)
	Nocardicin A	*Nocardia uniformis*	Jeanne and Craig (2009)
Polyketide	Actinorhodin	*Streptomyces coelicolor*	Elibol (2004)
	Enterocin	*Enterococcus faecium*	Kumar and Srivastava (2011)
	Rifamycin B	*Amycolatopsis mediterranei*	Venkateswarlu et al. (2000)
	Tetracenomycin	*Streptomyces glaucescens*	Gramajo et al. (1991)
Polypeptide	Actinomycin	*Streptomyces chrysomallus*	Haese and Keller (1988)
Macrolide	Carbomycin	*Streptomyces thermotolerans*	Epp et al. (1987)
	Erythromycin	*Saccharopolyspora erythraea*	El-Enshasy et al. (2008)
	Oleandomycin	*Streptomyces antibioticus*	Quirs and Salas (1995)
	Oxytetracycline	*Streptomyces rimosus*	Yang and Swei (1996)
	Pimaricin	*Streptomyces natalensis*	Recio et al. (2006)
	Spiramycin	*Streptomyces ambofaciens*	Karray et al. (2010)
	Tetracycline	*Streptomyces aureofaciens*	Asanza et al. (1997)
	Tylosin	*Streptomyces fradiae*	Bate et al. (2000)
Lipopeptide	Iturin	*Bacillus subtilis*	Shih et al. (2008)
	Surfactin	*Bacillus subtilis*	Wei et al. (2007)
Anthracyclines	Daunorubicin	*Streptomyces peucetius*	Otten et al. (1995)
Streptogramin	Pristinamycin	*Streptomyces pristinaespiralis*	Mehmood et al. (2012)
Aminocyclitol	Spectinomycin	*Streptomyces spectabilis*	Hyun et al. (2000)
Cyclopentanoid	Methylenomycin	*Streptomyces coelicolor*	Obanye et al. (1996)

produce important secondary metabolites including antibiotics (Marinelli and Marcone 2011). Initial researches are in the view that secondary metabolites have no functional importance in the growth of the producing cultures. However, the current opinions have changed that every secondary metabolite is synthesised to confer a survival advantage for the producing organism in a particular habitat (Brakhage et al. 2009). One of the predominant secondary metabolites, antibiotic, have greater importance in human health and possess a high pharmaceutical and commercial importance (Table 7.1). The alarming rise of multidrug-resistant pathogens and its infections has prompted a desperate search for novel antibiotics. It is noteworthy that microorganisms have the ability to meet the challenges of change, e.g. a new

streptogramin antibiotic etamycin produced by an actinomycetes species against methicillin-resistant *Staphylococcus aureus* (Haste et al. 2010).

A wide variety of agricultural residues are readily available as underutilised resources, which can be considered as inexpensive renewable carbon source for the commercial production of secondary metabolites (Poonam Singh and Pandey 2009). Bioconversion of agricultural residues for antibiotic production would hold a prominent position in future fermentation technologies, mainly because of its cost-effectiveness, eco-friendliness and feasibility in both developed and developing countries. Finally, the catabolism and utilisation of agricultural residues represent an important contribution to the implementation of biotechnology concepts and to the reduction of environmental problems associated with the disposal of solid wastes.

7.2 Antibiotics

One of the greatest achievements of medical science was the discovery of antibiotics which have profound importance on human health. Antibiotics are one of the best-known groups of the secondary metabolites synthesised by microorganisms, which are active against other microorganisms. Antibiotics affect a multitude of targets and essential cellular functions, which include DNA replication (actinomycin and griseofulvin), transcription (rifamycin), translation by 70S (S-Svedberg sedimentation mass value) ribosomes (chloramphenicol, tetracycline, erythromycin and streptomycin), transcription by 80S ribosomes (cyclohexamide), transcription by 70S and 80S ribosomes (puromycin and fusidic acid), cell wall synthesis (cycloserine, bacitracin, penicillin, cephalosporin and vancomycin) and cell membrane disruption (polymyxin and amphotericin, ionophores such as gramicidin, lonomycin and monensin) (Indu 2006).

Due to its enormous importance in human health care, demand for antibiotics is increasing worldwide. Moreover, continuous efforts are being made to decrease its production cost by process optimisation using raw materials like agricultural residues through different fermentation processes like SmF and SSF. Inexpensive substrates, such as agricultural residues and agro-industrial waste products have been found to be very valuable for economy and appropriate for biotechnological process. Their usage as substrate has widely opened the potential to reduce production costs up to 60 % by reducing the cost of raw material during fermentations (Lotfy 2007). The importance of agro-industrial residues in SSF system for the production of antibiotics and other secondary metabolites has gained much recognition in recent years (Mahalaxmi et al. 2010). Antibiotic production using SSF requires very minimum energy and less investment cost, and recently it has gained increased importance due to its higher productivity through fermentation, eco-friendliness and lesser disadvantages when compared to SmF (Poonam Singh and Pandey 2009).

7.2.1 Secondary Metabolites and Growth

Metabolites are organic compounds produced by organisms using multitude of enzyme-catalysed biochemical reactions called metabolic pathways. Metabolites can be the initial, the intermediary or the end products of these biochemical reactions. A variety of metabolites and reactions combine and work together allowing an organism to sustain life. Primary metabolites are found in almost all species within broad phylogenetic groups, produced by nearly the same pathway and are normally involved in growth, development and reproduction. The secondary metabolites are often restricted to a very narrow set of species within the same phylogenetic group. Secondary metabolites secreted by organisms are the chemical components that do not involve or interact in normal growth and development, but usually have a potent function. The growth and metabolism of many microorganisms in fermentation generally implies a series of phases. In microorganisms, secondary metabolites are not usually produced during the log or exponential phase of a culture (trophophase), but are synthesised in culture medium after a period of complete consumption of key nutrients, such as carbon, nitrogen, phosphate and mineral sources. Although primary and secondary metabolism share the common transcriptional and translational machinery, the nutrient depletion initiates the actions of precursors to accumulate products other than primary metabolites called the secondary metabolites during a subsequent production stage (idiophase).

7.2.2 Biosynthesis of Microbial Secondary Metabolites

The evolution of exciting and new secondary metabolic pathways is likely to have been driven by the ecological robustness, abiotic and biotic stresses and survival in unique ecosystem. Microbial secondary metabolites show enormous diversity of chemical structures. The biosynthetic pathways have emerged from network of primary metabolisms at a relatively small number of points (Barrios-Gonzalez et al. 2003) and evolve later independently.

Most secondary metabolites are synthesised from one or a combination of different biosynthetic pathways (Fig. 7.1):

1. Metabolites derived from sugars (streptomycin, neomycin and kanamycin)
2. Metabolites derived from shikimic acid pathway; shikimic acid is one of the major source for the biosynthesis of antibiotics (ansamycin and rifamycin)
3. Metabolites derived from aliphatic amino acid pathway for the biosynthesis of β-lactam antibiotics (penicillin, cephalosporins and cephamycins)
4. Metabolites derived from chorismic acid pathway (candicidin, nystatin and chloramphenicol)
5. Metabolites derived from aromatic amino acid pathway (actinomycin, indolmycin, novobiocin, lincomycin and polymyxin)
6. Metabolites derived from the acetyl-CoA and malonyl-CoA (erythromycin, vancomycin and tetracyclines)

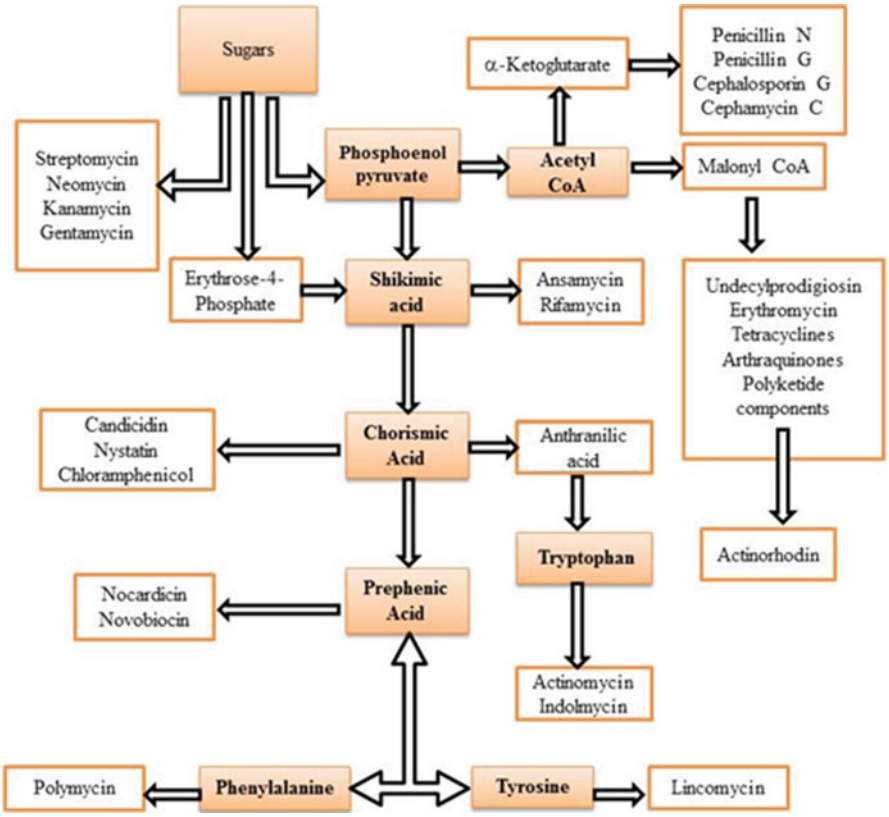

Fig. 7.1 Metabolic pathways leading to biosynthesis of secondary metabolite, antibiotics

7.3 Regulatory Mechanisms Involved in the Biosynthesis of Antibiotics

Antibiotic production is a multi-complex and highly regulated process, controlled by different physicochemical, biological and environmental factors. Although a wide range of primary and secondary metabolites from microorganisms has been identified, these products cannot be easily distinguished on the origin of precursors, chemical structures, functional analysis or its synthesis. In common, products of the primary metabolism serve as precursors of secondary metabolic pathways and increase its production.

7.3.1 Inducer

Microorganisms have evolved the ability to survive and proliferate to a constantly changing physical and chemical environment. The ability of microbial cells to live, function and replicate in a suitable environment depends on the presence of a

specific compound. These compounds, the inducer, initiate the production of biochemical metabolites or intermediates based on their requirements. In certain pathways, amino acids stimulate the production of secondary metabolites either by increasing the quantity of a limiting precursor or by inducing a biosynthetic enzyme. Inducers include valine for valine dehydrogenase of the tylosin process in *Streptomyces fradiae* (Nguyen et al. 1995), lysine for lysine-ε-aminotransferase in the cephamycin pathway of *Streptomyces clavuligerus* (Rius et al. 1996) and methionine for δ-(L-α-aminoadipyl)-L-cysteinyl-D-valine (ACV) synthetase, isopenicillin-N and deacetylcephalosporin-C synthase in the cephalosporin pathway of *Acremonium chrysogenum* (Juan and Arnold 2002). In the production of cephamycin C by *Nocardia lactamdurans* using SSF, addition of 1,3-diaminopropane acted as an inducer having a beneficial effect on production and increased the yield to 27.64 mg/g dry substrate (Kagliwal et al. 2009).

7.3.2 Autoregulator

Autoinducers are one of the best-known regulators of secondary metabolism in bacteria (Recio et al. 2006) and fungi (Martin et al. 2011). There are different types of autoregulatory molecules having ability to trigger wide ranges of antibiotic production (Recio et al. 2006). The regulatory factors of antibiotic biosynthesis are of great interest. One of the most established family of autoregulators consists of γ-butyrolactones, which are active even at nano-level concentrations and elicit antibiotic production by modulating the DNA-binding activity of cognate receptor proteins. Thus, the γ-butyrolactones have been referred to as bacterial hormones (Takano 2006). In several *Streptomyces* species, γ-butyrolactone autoregulator-receptor systems are well known to regulate antibiotic production (Arakawa et al. 2007). In *Streptomyces coelicolor*, a furan-type autoregulator, methyl furan was seen to induce antibiotic production (Corre et al. 2008). In *Amycolatopsis mediterranei*, B-factor [3′-(1-butylphosphoryl)adenosine] induced rifamycin production (Kawaguchi et al. 1988). In certain cases, modified peptides (Kleerebezem et al. 1997) and other small molecules, such as 2,3-diamino-2, 3-bis(hydroxymethyl)-1,4-butanediol (Recio et al. 2006) and 1,3-diaminopropane (Martin et al. 2011), serve as autoregulators. In *Pseudomonas fluorescens*, pyoluteorin serves as an autoregulator, positively influencing its own production. In addition to its autoregulatory role, pyoluteorin influenced the production of another secondary metabolite, 2,4-diacetylphloroglucinol. This findings elucidate that pyoluteorin establishes its contribution to regulation of at least two metabolic pathways within the bacterial cell (Brodhagen et al. 2004).

7.3.3 Carbon Catabolite Repression

The concentrations of carbon, nitrogen and phosphate imply an important regulatory effect on primary and secondary metabolism in different microorganisms. Antibiotic production rate can be influenced by manipulating the type and concentration of

nutrients formulating the production medium. Carbon sources, such as glucose and other carbohydrates, are excellent sources for growth and metabolism in microorganisms, but they interfere with antibiotic synthesis and this effect depends on the rapid utilisation of the preferred carbon source. Once the preferred carbon source is completely utilised, the next available carbon source is used for the production phase, known as the "idiophase". The readily available carbon source exerts some negative effect on the production of antibiotics. Different mechanisms have been described in bacteria and fungi to explain the negative effects of carbon catabolites on antibiotic production. This carbon catabolite regulation mechanism is widely distributed among microbial systems. In this regulation, the microorganisms catabolise the readily assimilable carbon source through biochemical pathways suppressing the secondary metabolite biosynthesis. In the filamentous fungus *Aspergillus nidulans* and *A. chrysogenum*, the biosynthesis of the β-lactam antibiotic penicillin and cephalosporin is repressed by glucose, respectively (Espeso et al. 1995; Jekosch and Kuck 2000).

7.3.4 Nitrogen Regulation

Many secondary metabolic pathways are influenced by nitrogen sources available for growth of microorganisms. Various nitrogen sources, inorganic (ammonium and nitrate) and organic (different amino acids), are used to enhance the production of secondary metabolites. The complex media used in fermentation often include a protein source (soybean meal) and the defined media usually contain a slowly assimilated amino acid (proline) as the nitrogen source for production of antibiotics (Gupte and Kulkarni 2002). Production of some aminoglycoside antibiotics is unfavourably affected by ammonium, e.g. neomycin and kanamycin (Shapiro 1989), whereas nitrate and certain amino acids possess stimulatory effect. Doull and Vining (1990) observed the nitrogen catabolite regulation during the actinorhodin production in *S. coelicolor*. Ammonium, either directly supplied as a nitrogen source or originating from the breakdown of amino acids, plays a vital role in nitrogen catabolic repression of pristinamycin production by *Streptomyces pristinaespiralis* (Voelker and Altaba 2001).

7.3.5 Phosphate Regulation

Phosphate, an essential component of the energy dynamics of cells, regulates the biosynthesis of many different types of antibiotics and other secondary metabolites. High concentration of phosphate had a negative effect on the biosynthesis of streptomycin, oxytetracycline, clavulanic acid, tylosin, echinomycin, cephalosporin, cephamycin and thienamycin (Juan 2004). In certain cases, negative phosphate control is exerted at the transcriptional level. Recently, it was shown that phosphate control of antibiotic biosynthesis in *Streptomyces lividans* and *S. coelicolor* is

mediated by the two-component PhoR–PhoP system that also controls the (*phoA*) alkaline phosphatase gene (Juan 2004). Phosphate limiting nutritional condition regulates biosynthesis of two antibiotic secondary metabolites, prodigiosin and carbapenem, through a multiple biochemical pathways, incorporating transcriptional control mediated by three regulators, PhoB, SmaR and Rap, in *Serratia* 39006 (Gristwood et al. 2009). Several mechanisms have been proposed and illustrated to explain the effect of phosphate on the biosynthesis of antibiotic, such as phosphate stimulating primary metabolic pathway, phosphate transferring carbohydrate catabolic pathways and phosphate inhibiting the formation of precursors required for antibiotic synthesis (Juan 2004).

7.3.6 Feedback Regulation

Many secondary metabolites inhibit or repress their own biosynthetic and catabolic pathways. Secondary metabolites can regulate and/or operate the activity of preexisting enzymes (feedback inhibition) or stop their synthesis (feedback repression). The role of feedback regulation in controlling primary and secondary metabolism is well established. In tylosin synthesis, feedback control of polyketide metabolism is observed (Butler et al. 2001). Bacitracin represses enzyme bacitracin synthetase involved in its biosynthetic process (Froyshov et al. 1980). In tetracycline synthesis, enzyme anhydrotetracycline oxygenase is inhibited by tetracycline, chlortetracycline and oxytetracycline (Behal et al. 1983).

7.4 Genetic Regulation of Antibiotic Production

The genetic regulation of secondary metabolite biosynthesis includes multitude layers of cellular control. These genetic elements, though poorly understood, are competent of influencing rates of biosynthesis significantly in microorganisms.

Malik (1979) groups the genes controlling antibiotic production into five classes. These are as follows:

1. Structural genes coding for enzymes that specify the biosynthesis of the secondary metabolite
2. Regulatory genes that determine the onset and extent of repression of the structural genes for biosynthesis
3. Genes that determine the resistance of the producing organism to the product (product toxicity)
4. Genes controlling the permeability to the compound (transport/excrete complex metabolites)
5. Regulatory genes that control primary pathways (precursors and cofactors) needed for antibiotic synthesis

7.4.1 Gene Clusters

Details of the pathways involved in the biosynthesis of antibiotics have been widely studied in microorganisms. Furthermore, sufficient information is available on the regulation of genes, important for antibiotic production. Many researches showed that the genes encoding specialised and myriad functions for the biosynthesis of many antibiotics are often coded by clustered genes on chromosomal DNA and less frequently on plasmid DNA (Brakhage and Schroeckh 2011). In operonic organisation of genes, the groups of functional and closely related genes are expressed as a single polycistronic mRNA, regulating the process of transcription and subsequent translation (Koonin et al. 2001). This is the governing principle of genomic organisation and expression in most microorganisms.

Karray et al. (2007) analysed the biosynthetic gene cluster for spiramycin production in *Streptomyces ambofaciens* and demonstrated the origin of precursors used by polyketide synthase. The 12-membered macrolide antibiotics, namely, methymycin and neomethymycin, and 14-membered macrolide antibiotics, namely, narbomycin and pikromycin, produced by *Streptomyces venezuelae* have gene cluster which is a polyketide synthase that encodes a six-module enzyme system (Xue et al. 1998). Gutierrez et al. (1999) elucidated the biosynthetic gene cluster in *Penicillium chrysogenum* consisting of three genes pcbAB, pcbC and penDE responsible for penicillin production. Brautaset et al. (2000) observed the biosynthetic gene cluster consisting of six genes coding for polyene antibiotic nystatin in *Streptomyces noursei*. The cloning and functional analysis of nucleoside antibiotic, polyoxin, and the biosynthetic gene cluster showed 20 different genes involved in antibiotic biosynthesis in *Streptomyces cacaoi* (Chen et al. 2009). Keller et al. (2010) identified a gene cluster encompassing 50 kb of contiguous DNA containing 28 genes with the biosynthetic functions on the chromosome of *Streptomyces chrysomallus* involved in the biosynthesis of actinomycin. Gene cluster, gene order representation for the biosynthesis of antibiotic and manipulation of the genes identified may potentially lead to the generation of novel antibiotics as well as yield enhancements in the microbial strains. Expression of the secondary metabolite gene clusters in wild-type microbial strains in natural habitats is frequently very low and is modulated in response to different physical, chemical or environmental stimuli (Laich et al. 1999).

In some cases most of the genes required for the secondary metabolite biosynthesis remain dormant, although it is likely that they may be expressed under unknown conditions (Scherlach and Hertweck 2009). The expression levels of several genes involved directly or indirectly in cephalosporin C (CPC) biosynthesis in *A. chrysogenum* are studied under a variable pH in SmF and SSF. Differences in intermediate and certain biosynthetic gene expression levels are observed predominantly and they evidence the relationships between physiological features and gene expression that open important advancement perspectives for fermentation systems (Lopez-Calleja et al. 2012).

7.5 Solid-State Fermentation Production Systems

Antibiotics are usually produced, using SmF, which require high energy and capital investment. Continuous requirements for huge amounts of antibiotics with minimum production costs are the need of the hour for the constant fight against dreadful diseases and multidrug-resistant pathogens. Recently, SSF has gained much importance due to higher productivity, shorter production time, lower energy and less capital investment using agricultural wastes as low cost carbohydrate sources and with much lesser disadvantages when compared with SmF (Table 7.2).

In the SSF process, the solid substrate supports the growth of microorganisms by providing nutrients and essential cofactors. The substrate that provides all the essential nutrient sources to the microorganisms should be considered as the ideal substrate for process optimisation. However, in certain cases, single natural substrate can provide the nutrient and it is essential to provide them externally. In general, SSF can be distinguished into two types, depending on the nature of solid phase matrix (Barrios-Gonzalez and Mejia 1996).

1. Solid culture of single support-substrate phase: solid phase addressing the functions of support and nutrient source. Examples include agriculture residues or animal wastes as support substrate.
2. Solid culture of dual substrate-support phase: solid phase is constituted by an inert matrix impregnated with a liquid medium, which serves as a reservoir for nutrients, water and additional supplements. Examples include polyurethane as inert support.

In both cases, the success of the fermentation and antibiotic production process is related to the physical characteristics (particle size and shape, porosity and consistency) of the support, which favours gases, nutrients and metabolites diffusion.

7.5.1 SSF on Natural Support Systems

Cultivation on natural substrates, SSF system uses natural materials that could serve both as a support matrix and as a nutrient source. Agricultural residues are one of the ideal substrates for the cultivation of the culture in SSF. These materials are typically starch or lignocellulose-based agricultural products or agro-industrial sources such as grains and grain by-products, cassava, soybean, sugar beet, sweet potato, potato and sweet sorghum; crop residues such as bran and straw of wheat and rice, bran of maize, ragi, green gram, black gram and red gram, hull of soy, corn and rice, sago and bagasse of sugarcane and cassava; residues of the coffee processing industry such as coffee pulp, coffee husk, coffee spent ground; residues of fruit-processing industries such as pomace of apple and grapes, wastes of pineapple and carrot processing, banana waste, waste of oil-processing mills such as coconut cake, soybean cake, peanut cake, canola meal and palm oil mill waste; and others such as sawdust, corncobs, carob pods, tea waste, chicory roots and bread.

Table 7.2 Application of agro-industrial residues as a substrate for antibiotic production

Antibiotic	Species	SSF substrate	Reference
Bacitracin	*Bacillus licheniformis*	Soya bean meal, sunflower meal, wheat bran	Farzana et al. (2005)
Cephalosporin C	*Cephalosporium* sp.	Wheat bran, wheat grains, rice grains, barley and rice bran	Ellaiah et al. (2002)
	Acremonium chrysogenum	Wheat bran and rawa, bombay rawa, barley, rice bran	Adinarayana et al. (2003)
	Acremonium chrysogenum	Sugarcane bagasse, sugarcane molasses, corn steep waste	Cuadra et al. (2008)
Cephamycin C	*Nocardia lactamdurans*	Soybean flour	Kagliwal et al. (2009)
	Streptomyces clavuligerus	Soybean meal	Bussari et al. (2008)
	Streptomyces clavuligerus	Wheat rawa, cotton seed deoiled cake, sunflower cake	Kota and Sridhar (1999)
Compactin	*Penicillium brevicompactum*	Groundnut oil cake, wheat bran, soybean meal	Shaligram et al. (2009)
Cyclosporin A	*Tolypocladium* sp.	Wheat bran	Sekar et al. (1997)
	Tolypocladium inflatum	Wheat bran flour and coconut oil cake	Survase et al. (2009)
Iturin A	*Bacillus subtilis*	Soya bean curd residue, okara	Mizumoto et al. (2006)
Griseofulvin	*Penicillium griseofulvum*	Rice bran	Saykhedkar and Singhal (2004)
Meroparamycin	*Streptomyces* sp.	Rice, wheat bran quaker, bread, ground corn	El-Naggar et al. (2009)
Mevastatin	*Penicillium citrinum*	Wheat bran	Ahamad et al. (2006)
Neomycin	*Streptomyces fradiae*	Apple pomace, cotton seed meal, soy bean powder, wheat bran	Vastrad and Neelagund (2011)
	Streptomyces marinensis	Wheat, rice, maize and ragi bran, red, green and black gram bran, wheat and rice rawa, rice husk rice straw, corn and jowar flour, sago and sugar cane bagasse	Ellaiah et al. (2004)

(continued)

Table 7.2 (continued)

Antibiotic	Species	SSF substrate	Reference
Tetracycline	*Streptomyces rimosus* *Streptomyces alboflavus* *Streptomyces aureofaciens* *Streptomyces vendagensis*	Peanut shells, corncob, corn pomace, cassava peels	Asagbra et al. (2005a)
Oxytetracycline	*Streptomyces rimosus*	Corncob	Yang and Swei (1996)
	Streptomyces rimosus *Streptomyces alboflavus* *Streptomyces aureofaciens* *Streptomyces vendagensis*	Peanut shells, corncob, corn pomace, cassava peels	Asagbra et al. (2005b)
Penicillin	*Penicillium chrysogenum*	Sugarcane bagasse	Barrios-Gonzalez et al. (1993)
Rifamycin B	*Amycolatopsis* sp.	Cornhusk corncob and wheat bran	Mahalaxmi et al. (2010)
	Amycolatopsis mediterranean	Coconut oil cake, groundnut oil cake, groundnut shell and rice husk	Vastrad and Neelagund (2012)
	Amycolatopsis mediterranei	Wheat bran	Venkateswarlu et al. (2000)
Surfactin	*Bacillus subtilis*	Soybean curd residue (okara)	Ohno et al. (1995)

The classical method of optimisation of fermentation medium involves changing one independent variable (nutrient, pH, temperature, etc.) while maintaining all others at a constant level. This is a time-consuming and expensive process for analysing a large number of fermentation variables. In order to overcome this difficulty, the fermentation parameters were further optimised by experimental factor design (EFD) and response surface methodology (RSM). The nutritional parameters for neomycin production by *Streptomyces marinensis* under SSF is optimised using EFD and RSM. The maximum productivity of antibiotic was 17,150 mg/kg of wheat rawa under optimum conditions of dextrin 14.1 g/kg, raspberry seed powder 64.91 g/kg and mineral salt solution 172.6 ml/kg (Adinarayana et al. 2003).

RSM was employed to optimise the cultivation conditions of *Bacillus subtilis* S3 in SSF for the enhancement of iturin A, a lipopeptide antibiotic. Maximum production of iturin A reached 11.44 mg/g when *B. subtilis* S3 was cultivated at 25 °C for 5 days in SSF containing high gluten flour and rice bran (Shih et al. 2008). Soybean flour was used as substrate in SSF for the production of cephamycin C by using *N. lactamdurans* and fermentation parameters were optimised by RSM. Under optimal SSF conditions, maximum production of 15.75 ± 0.27 mg/g of cephamycin C was

observed when compared to 8.37 ± 0.23 mg/g dry substrate before optimisation (Kagliwal et al. 2009).

7.5.2 SSF on Inert Support Systems

One of the negative factors of SSF processes, which utilise agricultural materials as substrate, is the impurity of the product. These impurities complicate the downstream processing when an end product of high purity is demanded. One of the potential alternatives to tackle these problems is to use an inert carrier as a supporting system. Recent results also show that the artificial inert support matrix enhanced the production of antibiotics. The use of inert supports for SSF resulted in the production of novel antibiotics, pyrrocidins A and B from *Cylindrocarpon* sp. and acremonidins A–E from *Acremonium* sp. In these experiments, the *Cylindrocarpon* sp. was cultured on a polyester-cellulose support on malt extract agar wherein pyrrocidin, which contains an unusual 13-membered macrocyclic ring, was produced. In contrast, a simple liquid version of the same medium failed to support the synthesis of the antibiotic. In the second case, an *Acremonium* strain produced the polyketides acremonidins A–E when cultured on a polyester-cellulosic support in malt extract medium, significantly in elevated levels over those produced in culture without the support (Bigelis et al. 2006).

7.6 Optimisation of Fermentation

7.6.1 Selection of Supplements

Research studies have proven that the optimisation of supplements has played a vital role in increasing the yields from different metabolites and solid substrates. The big advantage of selection of supplements is their uniqueness, since they often provide some variations in the secondary metabolite synthesis. Apart from usage of C and N sources and minerals in the substrate, the additional supplements can act as inducers or precursors in the process of synthesis.

In the process of cephamycin C production in SSF, wheat rawa enhanced the growth of *S. clavuligerus* and gave the highest titre value. Supplementation of sunflower deoiled cake, cotton seed deoiled cake and corn steep liquor enhanced production and at 0.5, 1.0 and 50 % weight of support, respectively, gave highest titres of (10 mg/g) cephamycin C (Kota and Sridhar 1999). External carbon sources like glycerol addition to the solid substrate resulted in the maximum Cyclosporin A (CyA) production of 4,659 ± 58 mg/kg followed by the sugars, dextrin and maltose. Addition of nitrogen sources like ammonium sulphate resulted in maximum production of 5,014 ± 65 mg/kg followed by 4,858 ± 45 mg/kg bacto-peptone and 4,827 ± 47 mg/kg casein peptone, respectively. The combination of glycerol (1 %)

and ammonium sulphate (1 %) gave a remarkable CyA production of 5,454 ± 44 mg/kg (Survase et al. 2009).

Yang and Swei (1996) utilised corncob, as SSF substrate in the production of oxytetracycline by *Streptomyces rimosus*, supplemented with 20 % (w/w) rice bran or 1.5–2.5 % ammonium sulphate and showed a yield of 10–11 mg/g substrate. Sircar et al. (1998) used supplementary sources including soya flour, KH_2PO_4 and sunflower oil cake and improved the production of clavulanic acid by *S. clavuligerus*. The maximum productivity of cephalosporin C (22,281 μg/g) employing *A. chrysogenum* ATCC 48272 was achieved by utilising wheat rawa with optimised process parameters including 1 % w/w soluble starch and 1 % w/w yeast extract as additives (Adinarayana et al. 2003).

The use of ammonium oxalate as a supplementary nitrogen source for cephamycin C production using *S. clavuligerus* NT4, under the optimised conditions, yielded 21.68 ± 0.76 mg/g of cephamycin C as compared to 10.50 ± 1.04 mg/g dry substrate before optimisation (Bussari et al. 2008). Supplementing the solid substrate with 0.1 % of choline chloride served as precursor and produced a 76 % increase in the yield of griseofulvin in SSF (Saykhedkar and Singhal 2004). Haloduracin, a bacteriocin, was produced by *Bacillus halodurans* when cultivated on wheat bran as a solid-state substrate, at 245 AU per wheat bran. Under the optimum conditions, supplementation of the bran with 10 % (w/w) sodium carbonate, the organism produced about 3,000 AU per gram dry bran (Danesh et al. 2011). Compactin production validation studies by *Penicillium brevicompactum* under SSF using statistical model-defined conditions resulted in an improved yield of 1,250 μg/g. Further improvement in yield was obtained using carbon supplementation in fed-batch mode. The feeding of glycerol (20 % v/v) on day 3 resulted in the much improved compactin yield of 1,406 μg/g dry substrate. This demonstrates usage of statistical experiment design as an easy tool to improve the process conditions for secondary metabolites production (Shaligram et al. 2009).

7.6.2 Substrate Pretreatment

Natural substrates and even inert supports generally require some kind of physical or chemical pretreatments. This modulates the support to attain more accessibility to microbial colonisation and penetration through adhesion to more susceptible physical structure along with their chemical constituents. Moreover, it also contributes to the improvement of its moisture-holding capacity. Wheat bran supplemented at an initial moisture content of 55 % was pretreated in autoclave for 1 h at 121 °C and used for cyA production by *Tolypocladium inflatum* (Murthy et al. 1999). The commercial solid substrates such as rice bran, wheat bran and ground corns were used for the production of meroparamycin. These solid substrates were washed and soaked in starch-nitrate medium at room temperature overnight. The soaked substrate was sterilised and used for production of meroparamycin by *Streptomyces* sp. strain MAR01 in SSF (El-Naggar et al. 2009).

7.6.3 *Effect of Moisture Content*

Moisture level is not considered as a vital factor in all submerged fermentation as water occupies a major percentage of the medium. Whereas in SSF, where the process is carried out on a solid medium with low moisture content, it is a critical parameter vital for the microorganism to grow on the surface of the solid substrate particles. Moisture content has a predominant role in enhancing the diffusion of extracellular enzymes, nutrients and metabolic products through the solid matrix. In SSF, the initial moisture content value depends primarily on the water retention capacity of the substrate.

The initial moisture content of the substrate played an eminent role on cephamycin C production by SSF. The range of moisture content from 60 to 80 % in wheat rawa showed substantial growth of *S. clavuligerus* and cephamycin C production (7–10 mg/g substrate). Below 60 % or above 80 % moisture concentration, there was no appreciable growth and further the decrease of cephamycin C concentration was observed (Kota and Sridhar 1999). At low moisture levels of 1:1 (2 gds:2 ml), 1:1.5 (2 gds:3 ml) and 1:2 (2 gds:4 ml), no rifamycin B production by isolated *Amycolatopsis* strain was observed. A gradual increase in antibiotic production from 0.89 to 3.47 g/kgds is observed at moisture level increase from 1:2.5 (2 gds:5 ml) to 1:4.5 (2 gds:9 ml) and further increase in moisture levels reduced the antibiotic yield. Interestingly, the specific antibiotic production (44.91 mg/g biomass) remained constant at all moisture levels. The observed differences in antibiotic production values may be attributed to moisture dependent mass transfer and related variations during SSF (Mahalaxmi et al. 2010). It is reported that higher substrate moisture in SSF resulted in suboptimal product formation due to reduced mass transfer process such as diffusion of solutes and gas to cell during fermentation. Interestingly, some researchers have observed optimised antibiotic production at higher substrate moisture in SSF. The highest neomycin production (5,227 μg/g) was achieved at 80 % initial moisture content of wheat rawa (Ellaiah et al. 2004), and the high cephalosporin C antibiotic titre (4,445 μg/g) was attained when the initial moisture level was 80 % in comparison with that at low or high moisture levels (Adinarayana et al. 2003).

The critical importance of moisture level in SSF media and its influence on the biosynthesis and secretion of antibiotics can be attributed to the interference of moisture in the physical properties of the solid particles. Increase in moisture level reduce the porosity of the solid substrates, thus limiting oxygen transfer, and low moisture content causes reduction in the solubility of nutrients of the substrate and causes low degree of swelling, resulting in decreased secondary metabolites production.

7.6.4 *Effect of Particle Size*

Among the several factors in SSF processes, Selection of proper particle size of substrates is one of the essential requirements for optimum production in SSF with different microbial strains. Generally, smaller substrate particles will provide a

larger surface area for microbial attachment and thus it should be considered as a desirable factor favouring SSF. However, too small substrate particles may result in substrate agglomeration in most cases, which may interfere with aeration and may result in poor growth rate. At the same time, larger particles provide better aeration efficiency but provide limited surface for microbial adherence. Thus, it may be necessary to provide an optimised particle size (Pandey et al. 2000). In rifamycin B production, a 30 % (3.55–4.53 g/kg) improvement was observed on optimised cornhusk particle size (6×4 mm) and further variation of substrate particle size resulted in reduction of antibiotic production (Mahalaxmi et al. 2010). In the neomycin production, wheat rawa of coarse size 0.84 mm gave the best results (4,478 μg/g) compared to the intermediate and fine size substrates, which yielded 4,043 and 3,427 μg/g substrate, respectively (Ellaiah et al. 2004). Some researchers used larger particle size in SSF for antibiotic synthesis; e.g. the sugarcane bagasse of particle size 14 mm increased the penicillin production by 37 %. However, this effect was due to higher sugar concentration in bagasse fraction (Barrios-Gonzalez et al. 1993).

7.6.5 Effect of pH and Temperature

The metabolic activities of the microorganisms are very much sensitive to the pH change, and antibiotic production by microorganism is found to be affected if pH level of the substrate is higher or lower compared with optimum value. Many researchers demonstrated the strain-dependent variation of pH for optimum antibiotic production in SSF. Cuadra et al. (2008) revealed pH as a key parameter in cephalosporin C production in solid-state fermentation by *A. chrysogenum* C10 using sugarcane bagasse as support. The production of cephalosporin C reached 3,200 μg/g of dry matter at an optimised pH between 6.4 and 7.8. In neomycin production by *S. marinensis*, when the initial pH was 6.0, there was less production and as the pH increased, its production reached the maximum (5,780 μg/g) at pH 7.5 (Ellaiah et al. 2004).

Strain-dependent variation of pH and temperature was reported for optimum rifamycin B production by *Amycolatopsis* sp. (Venkateswarlu et al. 2000) suggesting screening and determination of the optimum levels of fermentation parameters, which are very important for overall economic feasibility of the production process. Rifamycin B production by isolated *Amycolatopsis* sp. RSP 3 under SSF showed pH conditions pH 7–9 are favourable for production and maximal production (2.33 g/kgds or 40 mg/g biomass) was observed at pH 8.0. Maximum antibiotic yield (3.0 g/kgds or 41.3 mg/g biomass) is observed at 28 °C (Mahalaxmi et al. 2010). The optimal pH for tetracycline production by *Streptomyces viridifaciens* exactly matched the pH of sweet potato residue solid substrate, between pH 5.8 and 6.0. Each gram of dry substrate produced 1,570 μg total tetracycline equivalent potency (Yang and Ling 1989). In the course of cephamycin C production, when the initial pH was 5.0, there was very little growth in *S. clavuligerus* and no production of antibiotic is observed. Substantial increase of the pH increased the cephamycin C

production and reached the maximum (15 mg/g) at 6.5 pH. A further increase in pH resulted in a decrease in cephamycin C production and no production was observed at 8.0 pH. At 20 °C, cephamycin C production was 6 mg/g, at 30 °C production was 15 mg/g and it decreased to zero when the incubation temperature was 37 °C. The pH 6.5 and temperature 28 °C were found to be optimal both for SSF and SmF for cephamycin C production (Kota and Sridhar 1999). Ohno et al. (1995) elucidated temperature dependency for the production of lipopeptide antibiotics, namely, iturin and surfactin by *B. subtilis* RB14, in the solid-state fermentation of okara and observed the optimal temperature for iturin is 25 °C and for surfactin it is 37 °C. The highest neomycin production (5,760 μg/g) was attained at 30 °C, and a decrease in the yield of neomycin was observed when the incubation temperature was higher or lower than the optimum incubation temperature (Ellaiah et al. 2004). Higher temperatures were found to have adverse effects on the metabolic activities of the microorganism and it is also reported that the metabolic activities of the microorganisms become slower at lower temperature. Hence, incubation temperature and its control in SSF process are crucial as the heat evolved during SSF processes is accumulated due to poor heat dissipation in solid media.

7.6.6 Inoculum Level

Irrespective of the type of fermentation, whether it is SSF or SmF, inoculum level affects the yield of antibiotic. A progressive increase in rifamycin B yield is observed with increase in *Amycolatopsis* sp. RSP 3 starter inoculum from 2.4 to 7.2 %. Maximum antibiotic production of 4.84 g/kg dry substrate was observed with 7.2 % inoculum. However, higher inoculum level (12 %) resulted in more than 50 % reduced antibiotic production (Mahalaxmi et al. 2010). During the course of cephamycin C production, wheat rawa was found to be most suitable substrate in SSF for production by *S. clavuligerus*. When solid support was mixed with 1×10^2 cell per gram, cephamycin C production was very low (1 mg/g) but as the concentration reached 10^8 cells per gram, its concentration reached a maximum of 10 mg/g of substrate. It is important to note that further increase in cell concentration did not affect the concentration of cephamycin C (Kota and Sridhar 1999). Optimum neomycin production (6,880 mg/g) was observed at 0.5 % w/w dry cell mass of inoculum. At lower and higher inoculum levels, poor neomycin production was observed (Ellaiah et al. 2004). Inoculum level was also important factors for the production of cephalosporin C. High inoculum levels are inhibitory in nature. Higher antibiotic production (5,596 μg/g) was obtained at 10 % (v/w) inoculum level as compared to low or high inoculum levels (Adinarayana et al. 2003). It is important to provide an optimum inoculum level in fermentation process. A lower inoculum density may give insufficient biomass causing reduced product formation, whereas a higher inoculum may produce too much biomass and deplete the substrate of nutrients leading to poor product formation.

7.7 Industrial Strain Development

Industrial strain development has been the main focus of research in the commercial development of microbial fermentation processes. Discoveries in mutation, protoplast fusion, genetic manipulations, recombinant DNA technology and operation of commercial large-scale fermenters have revolutionised the concept of microbial strain development. Although greater improvements in overproduction of metabolites and antibiotics of specific microbes have resulted from essentially random empirical approaches to mutation and strain development, future strain development technology will be supplemented by more knowledge-based scientific methods. With the advances in understanding biosynthetic pathways, elucidation of regulatory mechanisms related to induction and repression of genes and bioengineering design, it will be possible to apply new strategies and limitless combinations for isolating improved strains. Furthermore, tailoring genes through the avenue of in vitro DNA recombination techniques in both bacteria and fungi has been shown to be feasible. Perhaps these areas will facilitate new strategies and have higher impact on industrial strain improvement. Khaliq et al. (2009) developed SSF system for hyperproduction of tylosin using a mutant γ-1 of *S. fradiae* NRRL-2702 and its parent strain. Various types of agro-industrial wastes were screened to study their effect on tylosin production in SSF. Wheat bran is the ideal solid substrate giving highest production of 2,500 μg of tylosin per gram substrate by mutant γ-1 against parent strain which gave 300 μg tylosin per gram substrate. Fermentation optimisation (70 % moisture, 10 % inoculum (v/w), pH 9.2, 30 °C, supplemental lactose and sodium glutamate on day 9) further improved the tylosin yield to 4,500 μg/g substrate. Wild parent strain displayed less production of tylosin (655 μg/g substrate) in SSF even after fermentation optimisation. This study evidenced tylosin yield enhancement by γ-1 *S. fradiae* strain under solid-state fermentation system.

Production of lipopeptide antibiotic, namely, surfactin in SSF on okara (soybean curd residue) as a solid substrate was carried out using *B. subtilis* MI113 with a recombinant plasmid pC112, which contains lpa-14, a gene related to surfactin production cloned from a wild-type surfactin producer, *B. subtilis* RB14. The amount of surfactin produced by MI113 (pC112) was 2.0 g/kg wet weight, which was eight times as high as that of the original *B. subtilis* RB14 at the optimal fermentation conditions. Further, the stability of the plasmid studied both under SSF and SmF system showed a similar pattern; however, the production of surfactin in SSF was four to five times more efficient than in SMF (Ohno et al. 1995).

Clones of four industrial strains of *P. chrysogenum* producing higher penicillin were subjected to assess its capacity to produce high quantity of antibiotic in SSF (Barrios-Gonzalez et al. 1993). *S. rimosus* TM-55 is treated with 3 % EMS, and 29 auxotrophic mutants (AM-1 to AM-29) are isolated from 5,457 colonies. Three sets of the auxotrophic mutants were chosen for protoplast fusion with 50 % PEG 1000 for 30 min at 25 °C, and 25 fusants were isolated. In solid substrate tested for oxytetracycline production, 20 % of fusants production is higher than that of the wild strain (Yang and Kao 1991).

7.8 Future Prospects

Penicillin, the first commercial antibiotic produced by fermentation in large-scale process, has stimulated the development of the fermentation biotechnology significantly. To develop an economically viable SSF that can be scaled up to industrial level, production process must address the path integrated with the following two critical stages: upstream and downstream processing. The commercial scale production process development is much difficult, owing to limitations in control of operations (heat, mass transfer and cooling) and variable factors (temperature, nutrient concentration, pH and moisture) influencing fermentations, which are essential to the system.

Fermentation biotechnology plays a major role in development of both broad-spectrum and narrow-spectrum antibiotics. The production of antibiotics using SSF has addressed two important aspects. Firstly, the amounts of antibiotics obtained by SSF are manyfold higher than SmF. Secondly the products obtained have enhanced vital properties when produced in SSF. In addition, the SSF approaches provide an alternative technology platform for the production of potent metabolites, with the growth environment presenting different physiological challenges to the microorganisms inducing corresponding differences in the organism's biochemistry. Recent researches showed the use of inert supports for fungal SSF resulting in the production of novel antibiotics (pyrrocidins and acremonidins). Recently, the exploitation of marine microorganisms for the discovery of secondary metabolites has led to numerous new antibiotics discoveries. Future pertinence of marine microorganisms in SSF could revolutionise the production of novel antibiotics. Therefore, if SSF variables are well controlled at optimised culture conditions and the purity of the product is defined, this may be a lucrative technology for commercial production of antibiotics than any process available currently.

Acknowledgements The authors gratefully acknowledge the financial support given by the Earth System Science Organization, Ministry of Earth Sciences, Government of India. The authors are thankful to the Director, National Institute of Ocean Technology (NIOT), Ministry of Earth Sciences, Govt. of India, for his constant support and encouragement for preparation of this chapter. The authors are also thankful to all the scientific and supporting staffs of Marine Biotechnology, NIOT, Chennai, for their support.

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