Chapter 7
Production of Bioactive Secondary Metabolites

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Contents

Abstract This chapter includes information based on published literature on utilization of agro industrial residues for the production of bioactive compounds. Various approaches using microbial fermentation technology have been explored for the production of bioactive compounds which as secondary metabolites could be produced by selected microorganisms. Certain factors have been found to affect the

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productivity of these compounds, hence the yield of secondary metabolites may be manipulated by controlling these factors in fermentation system.

Keywords Secondary metabolite · Stationary phase · Idiophase · Biologically-active compounds · Antibiotics · Mycotoxins · Ergot-alkaloids

7.1 Reasons to Use Agro Residues as Starting Material

Despite the obvious problems that agricultural waste can create, the vast quantities of agricultural and agro-industrial residues that are generated as a result of diverse agricultural and industrial practices represent one of the most energy-rich resources on the planet. Accumulation of this biomass in large quantities every year results not only in the deterioration of the environment, but also in the loss of potentially valuable material which can be processed to yield a number of value added products, such as food, fuel, feed and a variety of chemicals. The agro-industrial residues are generated globally and a major portion is left unutilized and left over as wastes in surrounding environment. Such wastes produced annually can be used as a natural bioresource for the production of bioactive compounds such as secondary metabolites from various selected microorganisms.

Secondary metabolites are excreted by microbial cultures at the end of primary growth and during the stationary phase of growth. Secondary metabolites represent some of the most economically important industrial products and are of huge interest. The best known and most extensively studied secondary metabolites are the antibiotics, steroids and alkaloids. A variety of agricultural residues (Table 7.1) such as wheat straw, rice hulls, spent cereal grains, various brans such as wheat and rice bran, and corncobs, are available globally which can be considered the cheaper and often free of cost substrates for the commercial production of secondary metabolites.

7.2 What are Bioactive Compounds

Bioactive compounds are mostly secondary metabolites produced by microorganisms in an active culture cultivation process. Secondary metabolites usually accumulate during the later stage of microbial growth in process of fermentation, known as the "Idiophase". This later stage of microbial growth follows the active growth phase called "Trophophase". Compounds produced in the idiophase have no direct relationship to the synthesis of cell material and normal growth of the microoganisms. Secondary metabolites are formed in a fermentation medium after the microbial growth is completed. Filamentous fungi synthesize many secondary metabolites and are rich in genes encoding proteins involved in their biosynthesis. Genes from the same pathway are often clustered and co-expressed in particular conditions (Khaldi et al. 2008). Most common secondary metabolites are antibiotics

Table 7.1 Use of solid-state fermentation for the production of secondary metabolites and their applications (Adapted from Pandey et al. 2001)

Substrate used	Microorganism employed	Secondary metabolite produced	Application of metabolite
Wheat, oat, rice, maize, peanuts	*Aspergillus oryzae, A. panasitus*	Aflatoxin	Mycotoxin
Impregnated loam based compost	*B. subtillis*	Antifungal volatiles	Antifungal compounds
Coconut waste	*Bacillus thuringenisis*	Bacterial endotoxins	Insecticide
Barley	*Cephalosporium armonium*	Cephalosporin	Antibiotic
Wheat straw with cotton seed cake and sunflower cake	*Streptomyces clavuligerus*	Cephalosporin C	Antibiotic
Wheat straw with cotton seed cake and sunflower cake	*S. clavulingerus*	Clavulanic acid	β-Lactamase inhibitor, antibacterial
Wheat bran	*Tolypocladium infautum*	Cyclosporin A	Immuno suppressive drug
Rice, rice bran, rice husk	*Metarhizum anisopliae*	Destrucxins A and B	Cyclodesipeptides
Sugarcane bagasse	*Claviceps purpurea, C. fusiformis*	Ergot alkaloids	Disease treatment
Wheat bran, corn cob, cassava flour, sugarcane baggase	*Gibberella fujikuroi, Fusarium moniliforme*	Gibberellic acid	Plant growth harmone
Okara, wheat bran	*Bacillus subtilis*	Iturin	Antibiotic
Wheat bran	*P. brevicompactum*	Mycophenolic	–
Wheat, oat, rice, maize, peanuts	*oryzae A. Panasitus*	Ochratoxin	Mycotoxin
Corn cob	*S. rimosus*	Oxytetracycline	Antibiotic
Sugarcane bagasse	*Penicillium chrysogenum*	Penicillin	Antibiotic
Soyabean residue Okara	*B. Subtillis*	Surfactin	Antibiotic
Sweet potato residue	*S. viridifaciens*	Tetracycline, chlortetracycline	Antibiotic
Rice panicles	*Ustilaginoidea virens*	Ustiloxins	Antimitotic cyclic peptides
Corn	*Fusarium monoliforme*	Zearalenone	Growth promoter

and others include mycotoxins, ergot-alkaloids, the widely used immunosuppresant cyclosporin, and fumigillin, an inhibitor of angiogenesis and a suppresser of tumour growth.

7.2.1 Properties of Bioactive Secondary Metabolites

The desired product is released in fermentation medium as secondary metabolite of a particular microorganism grown for the purpose. These metabolites are usually not

derived from the primary growth substrate, but a product formed from the primary growth substrate acts as a substrate for the production of a secondary metabolite. Secondary metabolites have the following characteristics:

a. Secondary metabolites of choice can be produced only by few selected microorganisms
b. These compounds are not essential for the organism's own growth and reproduction
c. Growth conditions, especially the composition of medium within a fermentation system, control the formation of secondary metabolites.
d. These compounds are produced as a group of closely related structures.
e. Secondary metabolic compounds can be overproduced.

There are several hypotheses about the role of secondary metabolites. Besides the five phases of the cell's own metabolism i.e. intermediary metabolism, regulation, transport, differentiation and morphogenesis, secondary metabolism is the activity centre for the evolution of further biochemical development. This development can proceed without damaging primary metabolite production. Genetic changes leading to the modification of secondary metabolites would not be expected to have any major effect on normal cell function. If a genetic change leads to the formation of a compound that may be beneficial, and then this genetic change would be fixed in the cell's genome, and becomes an essential one. Now this secondary metabolite would be converted into a primary metabolite.

7.3 Biotechnology Used for Production of Secondary Metabolites from Agro-Industrial Residues

The most commonly used technology is microbial-biotechnology practiced in industry, where secondary metabolites are mostly produced in a microbial fermentation process. This fermentation is performed in liquid state growing culture under submerged (SmF) conditions. This is mainly because the processes associated with scale up are much simplified and easy to manipulate for control of factors associated with whole production process. Liquid state fermentation allows greater control of parameters, such as pH, heat, nutrient conditions etc.

But for using agro-industrial residues for the production of bioactive compounds, another type of technology of microbial culture cultivation process would be ideal. In this technology process is performed under solid state condition rather in liquid state. This biotechnology is solid state (or substrate) fermentation (SSF) and characterized by a fermentation process on a solid support, which has a low moisture content (lower limit $\approx$ 12%), and occurs in a non-septic and natural state. Such technology describes the microbial transformation of biological materials in their natural state. The process is carried out in absence or near absence of free flowing water in the system, and it mainly utilizes fungal species.

Fungal species are ideal for this type of cultivation as these are capable of growth at lower water activity while bacteria require the presence of free water in fermentation system. SSF presents a low-cost system, it utilizes naturally occurring substances such as agricultural residues and forestry as substrates. Fermenters are easily to construct often only incorporating a tray and microorganism can be natural in some processes. The low water volume in SSF has a large impact on the economy of the process mainly due to smaller fermenter-size, reduced downstream processing, obviated or reduced stirring and lower sterilization costs. SSF produces a high product concentration with a relatively low energy requirement. Due to all these advantages over submerged fermentations, SSF has been exploited for the production of primary metabolites as well as secondary metabolite production.

7.3.1 Reason for Selecting Solid State Technology for Bioactive Secondary Metabolites

The use of SSF technology for the production of secondary metabolites should not be discounted. The mycelial morphology associated with the microorganisms that are predominately used for secondary metabolite production is well suited to growth on a solid support. This can also have a detrimental effect on product formation in liquid media, as highly viscous liquid media is required for successful metabolite production and can interfere with oxygen transfer. The filamentous morphology of these microorganisms and the secretion of these metabolites into the growth media can increase viscosity further. Therefore, SSF, technology can be exploited as an alternative, allowing better oxygen circulation (Hesseltine 1977).

Solid-state fermentation (SSF) is an important area of biotechnology, since the last decade has witnessed an unprecedented increase in interest in this technology (Nigam and Singh 1996a,b). This culture-technique is increasingly being used in the development of various bioprocesses in pharmaceutical, industrial and environmental sectors. Solid State (substrate) Fermentation (SSF) can be used successfully for the production of secondary metabolites (Robinson et al. 2001). These products (Nigam and Singh 2000) associate with the stationary phase of microbial growth and can be produced on an industrial scale for use in agriculture and the treatment of disease. Many of these secondary metabolites are still produced by submerged liquid fermentation (Nigam and Singh 1999), although production by this method has been shown less efficient in comparison to SSF. As large-scale production increases further, so does the cost and the growing energy demands. SSF has been shown to produce a more stable product, requiring less energy, in smaller fermenters with easier downstream processing measures. SSF technology has several advantages over submerged fermentation; primarily it represents a low-cost and easy to operate user-friendly system (Nigam and Singh 1994).

Concerning the production of secondary metabolites, SSF have ability to produce higher yields and productivities in certain cases. If the quality of the products could be guaranteed and the process-variables such as temperature, and pH could be controlled, SSF production of secondary metabolites would be very attractive

(Robinson et al. 2003). If these problems could be overcome, SSF technology could reduce the production-cost. It would enable third world countries cheaper access to secondary metabolites. SSF has found important applications particularly in the production of value-added products, such as biologically active secondary metabolites. Some of the important secondary metabolites produced in SSF are antibiotics, alkaloids, and plant growth factors (Balakrishnan and Pandey 1996). SSF systems, which during the previous two decades were termed as "low-technology" systems, appear to be a promising one for the production of value-added "low-volume and high-cost" products such as bio-pharmaceuticals (Pandey et al. 2000a,b). The recent evidence indicates that bacteria and fungi, growing under SSF conditions, are more than capable of supplying the growing global demand for secondary metabolites.

Though there are certain advantages of SSF-production process over conventional SmF systems but many practical advantages have been attributed to the production of biologically-active secondary metabolites through SSF route. Due to the lack of free water smaller fermenters are required for SSF and therefore less effort is required for downstream processing of secondary metabolites. Wild type strains of bacteria and fungi tend to perform better in SSF conditions than genetically modified microorganisms reducing energy and cost requirements further.

Different strategies and processes have been developed utilizing SSF technology for the production of biopharmaceuticals. Potential applications of SSF systems have been realized to produce high value bioactive secondary metabolites. Various secondary metabolites such as mycotoxins, bacterial endotoxins, plant growth factors, antibiotics, immuno-suppressive drugs and alkaloids etc. are among the important group of bioactive compound which can be produced by SSF technology (Table 7.1).

7.4 Biosynthesis of Secondary-Metabolites

Secondary metabolites comprise a diverse range of compounds synthesised by various fungal cultures (Nigam and Singh 2000) and some bacteria such as *Streptomyces*. Fungal secondary metabolites are an important source of bioactive compounds for agro chemistry and pharmacology. Over the past decade, many studies have been undertaken to characterize the biosynthetic pathways of fungal secondary metabolites. This effort has led to the discovery of new compounds, gene clusters, and key enzymes, and has been greatly supported by the recent releases of fungal genome sequences (Collemare et al. 2008). These secondary metabolites are of great commercial importance. Some are beneficial to life such as antibiotics and growth-promoters and some metabolites are mycotoxins, a threat to human and animal life. Various fermentation systems (Nigam and Singh 1999) such as surface-liquid, submerged, batch or fed-batch processes have been used for the production of different secondary metabolites. The use of certain liquid fermentation processes is established industrial practice, there are following reasons for this practice: the relative ease of scaling up liquid culture process; the greater homogeneity of liquid systems and the use of soluble starch; the superior monitoring for the precise

regulation of process-parameters which particularly control the biosynthesis of secondary metabolites. As described above due to many other advantages solid state fermentation have been considered for certain secondary metabolite production.

7.4.1 Utilization of Agro-Industrial Residues as Substrate

Following points are worth consideration for the application and suitability of solid agricultural residues in the biosynthesis of secondary metabolites:

1. In several productions, the product formation has been found superior using solid insoluble substrates.
2. The most commonly used microorganisms in the production of secondary metabolites are fungi and *Actinomycetes*; and the mycelial morphology of such organisms is ideal for their invasive growth on solid and insoluble substrates.
3. The fungal morphology is responsible for considerable difficulties in large-scale-submerged processes. These include highly viscous, non-Newtonian broths and foam production. This results in very high power requirements for mixing and oxygen transfer. The presence of chemical antifoam in fermentation broths reduces oxygen transfer efficiency and can lead to problems in the product recovery.
4. In some processes, the final product is required in form of solid consistency, such as antibiotics present in animal feed.
5. The capital cost of overall production process using solid substrates is claimed to be significantly less.
6. The yields of certain secondary metabolites as aflatoxin B_1 and ochratoxin A obtained from liquid culture were found to be very poor. This led to the use of solid substrates and subsequently, a higher yield of 100 g. Similarly the production of the cyclic pentapeptide mycotoxin, malformin C was performed using *Aspergillus niger* in solid culture and a higher yield of 369 mg/kg was obtained compared to the yield of 15–200 mg/kg from liquid fermentations (Kobbe et al. 1977).

The production of extremely toxic mycotoxins by fungi has attracted attention, due to their importance in human and animal food chain. The aflatoxins have considerable economic impact, the poultry industry in U.S. lost US$100 million per year from aflotoxin poisoning in 1970s. Solid state cultivation has been used to produce sufficient quantities of these compounds for toxicity studies and these cultivations have been performed to study the conditions that promote toxin formation on cereal grains (Greenhalgh et al. 1983). The production of gibberellic acid in SSF has been adopted to eliminate the need of cell-removal in downstream-processing after submerged culture process, which contributes a significant part in the production cost. Another concept is the growth of antibiotic producing microorganisms on animal-feed for two purposes, firstly to enrich the protein-content in nutrient-poor feed and secondly to produce antibiotics, such as cephalosporins, tylosin and monensin.

7.4.2 Process Operation for Secondary Metabolites

The biotechnology based process for the production of secondary metabolites can be performed in batch, fed-batch, continuous or plug-flow bioreactor operation. These modes of operation are well suited to solid state process, but a well-mixed fed-batch bioreactor is difficult to operate on a large scale. A plug-flow mode process operating in a continuous system could be more straightforward on industrial-scale compared to a submerged fermentation process. The productivity in a continuous fermentation is higher in such process compared to batch system. Mostly the secondary metabolites are produced in batch reactor system. The productivity of compounds is low in batch process because the time required to achieve the phase for secondary metabolite synthesis is longer, occurring after the primary metabolite production stage and after culture-growth has happened. While a continuous process runs for a longer time with a continuous yield of secondary metabolites once the phase of synthesis has started. Genes responsible for biosynthesis of fungal secondary metabolites are usually tightly clustered in the genome and co-regulated with metabolite production (Patron et al. 2007).

The substrate addition in the fermentation-medium also affects the process-operation. The addition of soluble starch or glucose in the initial wheat-bran medium of *Gibberella fugikuroi* affected the synthesis and a reduced yield of gibberellic acid was obtained. A fed-batch process with intermittent feeding of soluble starch instead of including starch in the beginning increased the yield of gibberellic acid by 18% compared to the lower yield obtained in batch process. As catabolite regulation by glucose is common to the synthesis of many secondary metabolites, solid-state fed-batch operation is likely to be superior to batch systems for most. For example, a 47% of increase in product yield has been obtained using feeds of cornstarch intermittently. A study of the hydrolytic enzymes secreted by *G. fugikuroi* during batch and fed-batch solid-substrate fermentation demonstrated that the quantity and rate of production of enzymes were higher in batch cultures. This was suggested that the glucose levels in the medium would be increased over fed-batch cultures leading to catabolite regulation of gibberellic production by glucose and which resulted in lower yields of the product in batch process.

7.5 Process Control in Synthesis of Desired Metabolite

The production-times for many secondary metabolites have been found similar time-periods required in many submerged state and solid state fermentation using solid substrates. SSF processes performed for the synthesis of secondary metabolites show similar process-kinetic patterns such as microbial-growth, substrate-utilisation and bioconversion, and product-synthesis, similar to the characteristics of a submerged fermentation. Similar patterns have been observed for the production of aflatoxin B1, ochratoxin A (Lindenfelser and Ciegler 1975), trichothecene mycotoxins (Greenhalgh et al. 1983), polyketide pigments (Lin and Lizuka 1982) and

gibberellic acid (Kumar and Lonsane 1987a,b,c). The kinetics of spore formation by *Penicillium roqueforti* is similar to those of secondary metabolite production. Process using agro-residues as starting substrates is controlled by a number of process – regulating factors such as: the initial moisture content of the substrate; rate of aeration; mixing of the fermenting solid medium; substrate-type, composition and structure of the substrate, and the constitution of fermenting medium; temperature; and the choice of microorganism.

7.5.1 Preparation of Agro Residues for SM-Production

A variety of solid substrates for secondary metabolites production have been tried in solid state fermentation (Table 7.1). These substrates derived from various sources vary in their nature, structure, and composition. Substrates coming from different origins have ability to provide a range of easily to poorly metabolizable nutritional sources and therefore, various substrates have been utilized as single carbon source or in combination with others, and also some substrates have been used as inert solid-supports for fungal colonization required for secondary metabolites production. Ultimate choice of a substrate for a particular metabolite is made after extensive trials with various types of substrates. Spent cereal grains such as wheat and rice have predominated as substrates for secondary metabolites. Aflatoxin has been produced using corn (Silman et al. 1979), rice (Shotwell et al. 1966), peanuts, corn meal and crushed wheat (Chang et al. 1963). Many brans such as wheat and rice brans are used singly or in combination with grains.

Mostly the production yields of secondary metabolites can be improved with a right choice of substrate or mixture of substrates with appropriate nutrients. A single-selected substrate performs in a different way changing the overall fermentation efficiency of the process; if the substrate is used in its different forms; for example pieces, fibres, particles or flour of a same substrate are metabolized in a different way. Though smaller particle size of a solid substrate has larger surface area for microbial action, but at the same time small particles have tendency of the increasing packing density. Densely packed fermentation system results in higher heat output per unit area of fermenter or output per unit space of fermenting solid medium. A column and other large size bioreactors would have a problem of poor aeration if used with smaller particle size substrates. In a process of gibberellic acid production using *Gibberella fuzikuroi*, higher yields have been achieved with larger particle size of wheat bran such as 0.3 to 0.4 cm (Kumar and Lonsane 1987a,b,c) compared to smaller particles of wheat bran.

7.5.2 Control of SM Production by Temperature

Normally the incubation temperature in a cultivation process is the optimum growth temperature of the particular microorganism used for secondary metabolite

production, and this optimal temperature for any secondary metabolite biosynthesis is similar in solid substrate fermentation to that for liquid fermentation. Each process has its range of temperature over which secondary metabolite production occurs, since temperature can not be precisely controlled at all times in all layers of solid substrates and within same system the temperature can vary by few degrees due to metabolic heat generation. Excess temperature due to poor heat transfer in fermenting system may adversely affect the yields of secondary metabolite. Therefore, the temperature regulation is achieved with the mixing of fermenting solid substrates by rotation or agitation and aeration. In a production process of aflatoxin production, the effect of temperature was studied over the range of 27°C to 40°C using flasks and small column fermenters (Silman et al. 1979). Aflatoxin formation was achieved over this range, but it ceased above 40°C; the production of toxin could be restored if the temperature was lowered and controlled within the range of 27°C to 40°C. Hence, it is possible to control the production of secondary metabolites by simply controlling the temperature of fermenting agro-industrial substrates in fermentation system.

7.5.3 Control of SM Production by Agitation of Substrates

The mixing of contents such as solid substrate, nutrient medium and seed-culture is very important to start any process in effective way, but also the mixing is required during fermentation to aid the aeration and to facilitate the heat-transfer in some processes. The extent of mixing or the rate of agitation in system depends primarily on the type and design of the bioreactor used in that particular process of secondary metabolite production. Mixing of fermenting contents in a production process where solid substrates are being used, has generally been found to increase the productivity of the secondary metabolites, whereas lower yields were obtained in a similar process run without agitation or mixing. The only disadvantages noticed of mixing in fermentation are shear damage to the growing microorganism and the extra power requirements to run the agitator. In some processes agitation has increased the products-yield considerably. A possible physiological explanation for the increase or improvement in yields through agitation is that the mixing of fermenting solid insoluble substrates suppresses the process of sporulation. Sporulation occurs simultaneously in system with the production of secondary metabolites and it may compete for common intermediates and substrates. Suppression of this competition has been suggested as the only reason for the increased yield of the desired product.

In some processes a clear advantage of mixing has been noticed such as the rotation at the speed of 16 rpm was found necessary for the production of high yields of ochratoxin A by *A. ochraceus* (Lindenfelser and Ciegler 1975). This culture was grown on wheat in a small-scale rotary drum bioreactor. This was confirmed with the low yields obtained if there was no rotation of the drum-bioreactor or the rotation was just brief and intermittent. Therefore, it was concluded that the superior performance of the rotary-bioreactor was due to the mixing. Similarly, in a utilization process of pearled barley cultivating a culture of *A. clavatus* NRRL 5890 (Demain

et al. 1977), the production of crude toxins was increased by 50% in agitated culture over static culture and the crude toxin was enriched in cyochalasin E. In contrast, the cephalosporin production was adversely affected by agitation of solid fermenting mass (Jermini and Demain 1989).

The production of most mycotoxins has been found to be improved in shaken cultures compared to stationary fermentations. Some of such improved-yield fermentations are: aflatoxin production by *A. flavus* NRRL 2999 using rice as substrate (Shotwell et al. 1966), ochratoxin A production by *A. ochraceus*, cytochalasin E and tremorgens production from culture of *A. clavatus*, cyclochorotine and simatoxin from *Penicillium islandicum* (Ghose et al. 1978), and cyclopiozonic acid by *A. flavus* (Luk et al. 1977). The yield of cyclopiozonic acid was obtained almost ten times (10×) in agitated fermentation of white wheat compared to the lower yield (1×) in static culture process (Luk et al. 1977).

7.5.4 Control of SM Production by Aeration of Substrates

Since the production of secondary metabolites has started utilizing agro industrial residues as the starting materials in fermentation process, the effect of aeration on the synthesis of various secondary metabolites has been investigated. The most comprehensive study has been performed on the production of aflatoxin B_1 in a corn storage bin with a capacity of 1266 bushels (Silman et al. 1979). The effect of aeration was studied by passing humidified air of 80–85% relative humidity through the bed of corn at flow rates between 0.001 to 0.04 l/kg corn per min and a recirculation rate of 1.5 l/kg per min. It was noticed that the rate of aflatoxin production and yields were directly proportional to the aeration rate. However, the direction of air-flow through the corn-bed had no apparent effect on aflatoxin yield or on the rate of production.

But the aeration may not be necessarily required in some cases, this needs to be confirmed before the running of process. In one case of secondary-metabolite production aeration proved to be the unnecessary where the production of ochratoxin A performed in a system using a rotating drum bioreactor was adversely affected by aeration (Lindenfelser and Ciegler 1975).

7.5.5 Control of SM Production by Moisture Content

The optimal moisture content of solid fermenting substrates varies for various metabolites production. The control of the water content present in solid substrate fermenting – medium or the maintenance of the initial moisture content in the system is very important factor. The moisture content of solid substrates greatly affects process of any secondary metabolite production. The optimal initial moisture contents have been found to be different according to the reactor-type used in the process. The initial moisture content may vary for the same fermentation process i.e. using same substrate and same microorganism for same metabolite production but

during the scaling up of the process it may vary using different designs of bioreactor, for example from flasks to tray type.

Production of another metabolite such as toxin, using substrate grains or corn is greatly favoured at low initial moisture contents. The most favourable moisture content for optimal toxin production has been found to be between 20–40%. The optimal range is characteristically broad, however outside this range the yield of the product and the rate of toxin-formation are severely affected. Aflatoxin B_1 production from corn by *Aspergillus flavus* is negligible at initial moisture content below 17% though the fungal growth takes place (Silman et al. 1979); at moisture contents between 18–20% toxin production occurs at a reduced rate and lower yield is obtained. There is a rapid fungal growth and aflatoxin production at the moisture contents of 20–30% with the optimal moisture content being 22.4% at 33°C in flask culture. Similarly, ochratoxin A production has been found affected due to the variation in initial moisture content using laboratory-scale rotary drum bioreactors. In these culturing processes, the most significant parameter identified has been the effect of initial moisture content that determines the toxin-yields. The optimal moisture content of rice for the production of cephalosporin C was found to be 49–51% using *Acremonium chrysogenum*. In such cultivation-systems, yields were severely affected (inhibited) below the optimal value, whereas at higher levels of moisture content the bacterial contamination became an unavoidable problem.

7.6 Recovery of Secondary Metabolites in Downstream Processing

The solid fermented mass obtained after the completion of the process is extracted using various solvents to recover the product. The extraction is usually performed using the aqueous or other solvents mixing in proper ratio with the solid fermented mass. The extracts obtained from the fermentation of solid substrates usually contain higher concentration of secondary metabolites compared to the concentrations present in the submerged culture medium (Kumar and Lonsane 1988). One advantage of using agro industrial residues as solid substrates in fermentation is that there is no need of cell removal prior to the extraction of metabolites. The fermented liquid medium from a submerged fermentation is subjected to downstream processing for the removal of microbial-cells or mycelial-biomass to obtain a clear supernatant for the extraction of secondary metabolites. The cost of cell-removal from the submerged culture broths prior to the extraction process is estimated to be between 48% to 76% of the total production cost of the final product (Datar 1986).

There could be certain problems associated with the recovery of metabolites from system. The recovery of secondary metabolites from the solid fermented mass offers less flexibility in the choice of the initial unit operation than submerged fermentation. The metabolites diffuse throughout the solid mass during the culturing, which requires longer extraction-times for complete recovery of metabolites. The extraction of larger amount of solids may increase the concentration of impurities in the extract. The cost of purification depends on the quality of extract. The presence and

concentration of inert compounds in the extract increase the cost of purification and therefore the cost of recovery is increased. Particularly those secondary metabolites which are used in bulk in the pharmaceutical and health industry and whose purity is governed by stringent regulations need to go through specific purification strategy. Now in antibiotic industry, the problem of culture-biomass in submerged process, is solved to some extent by the application of whole-broth processing. The extraction process uses the whole fermented broth including the cell-biomass. In such extraction the process is carried out using solvent extraction in multi-stage, centrifugal decanters.

The important variables in the extraction of an important secondary metabolite gibberellic acid from the fermented mouldy-bran (Kumar and Lonsane 1987) are the type of solvent to be used for an efficient extraction, concentration of solvent, the ratio of solvent to the solid, and pH. An 87% recovery of gibberellic acid in the extract of 0.9 mg/l was obtained using 2%, v/v ethanol as solvent in a multi-stage, counter-current extraction system. A considerable loss of solvent is common, with up to 68% of the initial solvent added to the bran, remaining absorbed into the solid on separation. In a particular production process the product is left on solid residue for use. Production of antibiotic monensin on various materials is carried out by *Streptomyces cinnamonensis* for use in poultry feeds to control coccidiosis. Tin such application no extraction cost would be involved.

7.7 Scaling-Up of Process For Secondary Metabolites

The elucidation of important scale-up criteria for SSF is very important to guarantee the successful large-scale operation of the processes. The scale-up of the each process results in mixed success for various processes employing different microorganisms and different solid substrates for a range of metabolites production. Since a particular individual process has its own characteristics due to the nature of organism and contents of the system such as type of agro residues used as substrate, each process behaves differently in scaling-up process. On the other hand the synthesis of secondary metabolites is very sensitive to the environmental factors and therefore the control of such factors becomes difficult in a similar process on large-scale, which is naturally expected.

Any process can be operated successfully regulating the various parameters on small-scale but the yield of the secondary-metabolites has been found adversely affected during large-scale cultivation. The synthesis of toxin has been found severely affected in a large-scale system, though the growth of employed microorganisms were always very obvious. In another process for the synthesis of aflatoxin B_1, the scaling-up using 75 g to 1266 bushels of corn have been successful producing good yields of aflatoxin even at larger scale (Silman et al. 1979).

Many of the important scale-up problems are generic to all solid state fermentation processes. The criteria of scale-up of systems utilising solid substrates are different to those established for submerged processes, such as volume-ratio, mixing time, k_1a, and foam-control etc.

7.7.1 Control of Temperature in Process Scale-Up

The precise control of incubation temperature in any bioreactor is a difficult problem, and this problem becomes a difficult engineering challenge with increase in scale of process for a large-scale production. Unlike submerged fermentation, where the dominant mechanism of heat-transfer is convection, the heat-transfer in a fermentation system using solid substrates occurs predominantly via two mechanisms i.e. convection and conduction. The convective medium in solid fermenting biomass is air and therefore the rate of heat-exchange is much smaller than in liquid submerged cultivations.

The microbial-activity on agro residue substrates for the production of secondary-metabolites is similar and not greatly less than in liquid cultures; consequently the heat generation is also considerable. The increase in temperature due to heat generation during the production of amyloglucosidase has been found up to 56–57°C. The rise in temperature affects the rate of microbial-growth and the rate of moisture loss in that process and as a result the production yield of the secondary metabolite is also affected. Excessive heat production may result in the progressive drying of the solid substrate with time in a longer process which requires 10 or more days for the optimum synthesis of secondary metabolites. This is a serious problem, which may affect the yield of the products.

This is natural to face difficulty in maintaining the temperature control on large scale such as if the fermentation is carried in large 1266-bushel storage bins. In such fermenters the temperature up to 47°C have been measured, despite the continual circulation of cool air to regulate the temperature-rise. Even similar difficulties may be experienced on a small-scale process for the production of secondary metabolites. Using a small-scale rotary-drum bioreactor, the temperature increase may be normally noticed to be 1–2°C per day (Lindenfelser and Ciegler 1975).

7.7.2 Control of Factors Related to Substrates in Scale-Up

The yields of secondary metabolites are affected in scale-up due to few more factors other than temperature-rise, which are directly related to solid substrates. The importance of the moisture content of the fermenting solid substrates is very significant in determining the yield of metabolites. However, the control of the moisture content of the solid substrate can be difficult. There are certain reasons for this difficulty such as the combined effects of heat production and moisture release due to microbial-respiration. The excessive wetness may result in bacterial and yeast contamination and also in rotary bioreactors wetness promotes the clumping of the material. Therefore a purposely-built reactor-design is essential to prevent such problems. During the typical process using wheat grains as substrate for ochratoxin A production cycle of 10–12 days, the moisture content has been found to rise from 30–40% and as a result of this the volume of substrate used (wheat) increased by 60–70%. Such problems lead to the reduced yield of the secondary metabolite.

Another factor in scale-up of SSF is the amount of solid substrates used in bioreactors. The yields of secondary metabolites are superior in SSF to those obtained in submerged process based on weight of product obtained per gram substrate and per litre, respectively. Frequently considerable amounts of carbon remain unutilized in fermentation. The yields of secondary metabolites calculated on the basis of the weight of substrate consumed, needs to be ascertained, this is important to adequately compare the productivity in two types of processes. This measure of the productivity of secondary metabolites is important for a commercial operation.

7.8 Prospects of Agro Residues for Secondary Metabolites Production

The use of agro industrial residues for the production of commercially valuable metabolites is at present under-utilized, with a strong preference towards conventional and familiar liquid fermentations. This lack of adoption into industry seems strange, since research in this area clearly shows that SSF produces higher yields in a shorter time period. It is easy to see why a liquid state fermentation using simple sugars as substrate is still prevalent for the production of secondary metabolites; it is a familiar technique, scale-up from lab to industrial fermenter level is much simplified in comparison to SSF with parameters being easier to monitor and control. There are also problems associated with secondary metabolite production in liquid fermentation, such as shear forces, increasing viscosity due to metabolite secretion, and reduction in metabolite stability. There is an increasing demand on science in regards to antibiotic production, with a growing global demand and cases of antibiotic resistance. Therefore, the use of residual agro industrial wastes should be considered by industry, especially when large quantities of secondary metabolites are required in a shorter fermentation period, with minimal expenditure on media and downstream processing.

There is clear evidence that high concentrations of secondary metabolites can be achieved using a variety of cheaper and freely available agro industrial residual substrates, employing best performing microorganisms in suitable properly designed and purposely-built bioreactors for large-scale production. To achieve the high standard of this technology, a number of studies have been performed to elucidate the optimal conditions for the synthesis of various secondary metabolites. Some areas of further interest are the use of fed-batch and continuous plug-flow modes of operation; the study of broader range of secondary-metabolites; and analysis of bioreactors to identify criteria for successful scale-up and therefore to permit effective process control. This last criterion is particularly important for the lengthy nature of these culture-systems. The residual substrate fermentation process for the biosynthesis of secondary metabolites is more likely to be seriously considered for the industrial-scale production of some important secondary metabolites.

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