

Chapter 1

Introduction and literature review

1. General introduction

The word antibiotic was derived from the Greek *anti* (against) and *bios* (life). Paul Vuillemin, a pupil of Louis Pasteur, coined the term "antibiosis" in 1889 to describe the process by which life could be used to destroy life. However, it was Selman Waksman who coined the term "antibiotic" in 1945. Originally the term only applied to compounds derived from living organisms capable of killing or inhibiting bacterial growth. The term has now been expanded to include synthetic antimicrobial drugs as well. Natural antibiotics belong to the group of compounds known as secondary metabolites. They are generally produced at low specific rates, and are not considered essential to the organism producing them, at least in pure culture. In their natural environment these antibiotics are essential to the organisms that produce them. Antibiotics are considered as the chemical weapons of the microbial world, where they are used for the survival and competitive advantage within the microenvironment (Demain and Fang, 2000).

When antibiotics, such as penicillin, were first introduced in the 1940's a victory was declared against infectious diseases. A panacea had been found, which would change the way humans thought about infectious disease. The free love movement of the "swinging sixties" might not have been so free if it weren't for the relatively simple treatment of many sexually transmitted diseases with antibiotics. Unfortunately the euphoria of the antibiotic "magic bullet" has dissipated. The huge consumption of antibiotics, and their widespread use, and abuse, has led to the concomitant emergence of bacterial resistance to antibiotics. Over-optimism and complacency in the pharmaceutical industry, even as recently as the 1980's, saw little development towards new antibiotic drugs. At the same time the spread of antibiotic resistance has accelerated. This has led to the recurrence of diseases which were once controlled, are once again, posing greatest threats to human health (Cohen, 2000).

1.1 Antibiotic Classes

Antibiotics can be organized into different classes according to their effect and mode of action. Their effect is either bactericidal or bacteriostatic, allowing the immune system to deal with the infection. Antibiotics can be described as those possessing "large spectrum of activity", or "broad spectrum" antibiotics are effective against a

large number of infectious bacterial species. The other type is narrow-spectrum antibiotics, which are only active against a specific family, or genus of bacteria (Calderon and Sabundayo, 2007; Finberg, 2004; Cunha, 2009). The different classes of antibiotics, their effect on bacteria and principal mechanisms have been summarized in Table 1.

Table 1.1 Representative classes of antibiotics, their effect, and mode of action.

Class	Effect	Action
Aminocyclitols	b.s	Protein synthesis
Aminoglycosides	b.c	Protein synthesis
β -lactams	b.c	Cell wall
Fluoroquinolones	b.c	Genetic replication
Lincosamides	b.s	Protein synthesis
Macrolides	b.s	Protein synthesis
Sulfonamides	b.s	Metabolic processes
Tetracyclines	b.s	Protein synthesis
Phenicol	b.c	Protein synthesis

The effect of antibiotics defined as either bacteriostatic (b.s) or bactericidal (b.c).

1.2 Antibiotics and the mode of action

All these antibiotics have different mode of action by which they act as therapeutic agents. Some of the modes of action by different antibiotics are mentioned below:

1.3 Cell wall synthesis inhibitors

Bacteria contain murein or peptidoglycan that is highly essential in maintaining the cell wall structure. Cell wall synthesis inhibitors such as β lactams, cephalosporins and glycopeptides block the ability of microorganisms to synthesize their cell wall by inhibiting the synthesis of peptidoglycan (Britta and Ralf, 1997).

1.4 Interfering with protein synthesis

These classes of antibiotics inhibit the protein synthesis machinery in the cell. Some examples include tetracyclines(Slover *et al.*, 2007), chloramphenicol (Jardetzky, 1963), aminoglycosides (Levison and Matthew,2009) and macrolides(Tenson *et al.*, 2003)

1.5 Cell membrane inhibitors

Antibiotics such as polymyxins disrupt the integrity and structure of cell membranes, thereby killing them. These set of antibiotics are mostly effective on gram negative bacteria because these are the bacteria that contain a definite cell membrane (Tran *et al.*, 2005).

1.6 Effect on nucleic acids

DNA and RNA are extremely essential nucleic acids present in every living cell. Antibiotics such as quinolones and rifamycins bind to the proteins that are required for the processing of DNA and RNA, thus blocking their synthesis and thereby affecting the growth of the cells (Robinson *et al.*, 1992).

1.7 Competitive inhibitors

Also referred to as anti-metabolites or growth factor analogs, these are antibiotics that competitively inhibit the important metabolic pathways occurring inside the bacterial cell. Important ones in this class are sulfonamides such as Gantrisin and Trimethoprim(Case *et al.*, 1993).

1.8 Antibiotic resistance

Antibiotic resistance was defined as the increased tolerance of specific type of bacteria against specific types of antibiotics. This means that the microorganism can withstand the therapeutic dose of the antibiotic without showing any effect on the microorganism (Levy, 1994).

Antibiotic resistance occurs through what is called natural selection and mutation. The simple explanation for this process is that when an antibiotic is administered to combat a certain species of microorganisms it may cause a mutation in the genetic code of some microorganisms of this specific species causing them to

survive the course of antibiotic so, some of these microorganisms will die and the mutant microorganism will tend to replicate and spread the mutant gene between them horizontally, and the colony became fully resistant to that type of antibiotic(Witte, 2004).

1.9 Side effects of antibiotics

Sometimes the adverse effect of the antibiotic was related to the dose administered and this require precise dose calculation before administering the antibiotic to the patient especially in case of injection because the amount that reach the blood was higher than oral administered antibiotics. Calculating the antibiotic dose per kilogram of patient body weight is the best solution to avoid this problem(Slama *et al.*, 2005).

The other type of adverse effect of antibiotics was due to the amplification of the body reaction against some antibiotic substances. Some patients may develop allergic reactions against certain types of antibiotics and this side effect could be treated easily through performing a simple allergy test before starting the course of treatment to find out whether the body can tolerate the antibiotic substance or not. If the patient develops skin rashes then the antibiotic should be replaced with another to avoid more serious allergic reactions (Pirodda and Garland, 2006).

1.10 Production of antibiotics

Since the first pioneering efforts of Florey and Chain in 1939, the importance of antibiotics, including antibacterial, to medicine has led to intense research into producing antibacterial at large scales. Following screening of antibacterial against a wide range of bacteria, production of the active compounds was carried out using fermentation, usually in strongly aerobic conditions(Madigan *et al.*, 2005).

1.11 Fermentation technology

The term fermentation was derived from the Latin word *Fermentum* that stands for boiling. Fermentation was the process of digesting certain substances that leads to chemical conversion of organic substance into simpler compounds. Fermentation technology began with sweet substances (vegetable or animal) in different parts of world. The process of fermentation was probably discovered by observing the

changes in the juices of several fruits and other substances that had been kept for a day or more. It appears that fermentation technology started simultaneously with settled agriculture during the neolithic period (Stanburry *et al.*, 1997).

Bio-fermentation technology makes it possible to grow a number of production strains in large tanks and, in a matter of days, large quantity of product can be produced. Microbial fermentation is necessary for manufacturing such as pharmaceutical and biopharmaceutical products. Product diversity makes fermentation technology a multi-disciplinary expertise associated with microbiology, organic chemistry, biochemistry and molecular biology (Purves *et al.*, 2004)

Fermentation processes can be in either submerged or solid state forms. Submerged liquid process involves the growth of microorganisms in liquid medium with dissolved or suspended nutrients. Solid state process uses solid substrates or nutrients with the addition of certain amount of water. Although submerged process creates a more homogeneous growing environment, solid state fermentation is closer to the natural habitat for microorganism to grow (Dirand *et al.*, 1997). Studies show that solid state process could promote the production of antibiotic, and the antibiotic produced are more concentrated and more substrate specific (Barrios-González J and Mejía, 2007).

1.12 Solid substrate fermentation for antibiotic production

Solid substrate fermentation (SSF) refers to the growth of microbes on solid substrates without the presence of free flowing liquid (Rahardjo *et al.*, 2006). There are many examples of solid substrate fermentation occurring in nature. Food fermentation such as cheese making, composting etc. was an example of natural solid substrate fermentation (Paredes-López and Harry, 1988). Different terms used by different workers like semi solid fermentation, surface fermentation, koji fermentation, solid state fermentation and solid substrate fermentation (Hesseltine, 1972; Ralph, 1976; Hesseltine, 1977 a & b; Kannap and Howell, 1980; Onians *et al.*, 1981; Loanesane *et al.*, 1985; Lonsane and Ghildyal, 1993). The solid substrate fermentation for the production of commercial useful substance could be performed by using solid substrates such as wheat bran, rice bran, maize bran, peanut meal etc (Deschamps *et al.*, 1985). All these solid substrates were easily available and cheap. The other of employing SSF in the fermentation industry simplicity of the technique, low capital investment, low recurring expenses, reduced energy requirement for

running the process and the low waste water output which reduces the disposal problem of large volume liquids. In addition, the product can be obtained in a more concentrated form from the solid biomass by extracting the product from the fermented solids in small volumes of solvent (Babu and Satyanarayana, 1995). The extensive centrifugation required in the case of submerged liquid fermentation (SLF) can be avoided in this method. All these advantages contribute greatly to the overall economy of the production process. On the other hand, limitations also exist there which include chance for contamination (Lu *et al.*, 1997), heat buildup in the fermentation solid, critical influence of moisture content etc. But since the merits of SSF outweighs demerits, it could be supposed to be an applicable method for the small scale and large scale production of secondary metabolites (antibiotics) (Wang *et al.*, 1984; Jermini and Demain, 1989; Barrios-Gonzalez *et al.*, 1993; Robinson *et al.*, 2001; Farzana *et al.*, 2005).

Solid substrate used in SSF were agricultural by products or agro-industrial wastes or residues which were chosen because of economical advantage and also to overcome environmental pollution. The solid substrates meet the vital requirements of water by microorganism by having water absorbing capacity (Cannel and Moo-Young, 1980 a& b). Bacteria and yeast grow by adhering on to the solid surfaces where as fungi will penetrate deep into the solid substrate (Loanesane *et al.*, 1985). Various microorganisms grow on solid substrate depending upon water activity of the substrate (Corry, 1973).

1.13 Specific advantage of SSF technique for microbial antibiotic production

The listing of an array of advantages of SSF may not be sufficient enough to indicate better economics of solid substrate over SLF. Direct and indirect comparative data available in the literature can be used as an index to determine the economic advantage of SSF over SLF (Ghildyal *et al.*, 1985). The comparison based on product titres in fermentation medium indicated higher product titres in SSF (Lekha *et al.*, 1993; Srinivas and Loanesane, 1993). The high product concentrated in the fermented solid in the case of SSF leads to lower expansion on downstream processing and effluent treatment. The absence of undesirable compounds in SSF compared to SLF, not only allows higher selling cost but also attracts more demand

for the product (Xavier *et al.*, 1993). On the whole, SSF appears to be comparatively economical than the conventional SLF processes for the production of some antibiotics.

Though no extensive comparisons are made between SLF and SSF with respect to antibiotics, a few inferences could be drawn to highlight the advantages of the later over former. The major drawback in developing biotechnological processes in recent times is the larger quantities of water that has to be disposed during downstream processing operations (Hahn-Hagerdal, 1986). Therefore, an alternative fermentation technique such as SSF, which offers reduction fermentation broth that has to be processed, will have far-reaching effects on economics. This was in fact proved in case of amyloglucosidase (Ramakrishanna *et al.*, 1982), gibberelic acid (Kumar and Lonsane, 1987) and bacterial alpha amylase (Ramesh and Lonsane, 1990). In case of amyloglucosidase production, *A. niger* CFTRI produces 600 units of enzyme/ml in SLF, as against 6000 units of enzyme/ml extract obtainable for 1 g dry mouldy bran under SSF technique (Ramakrishanna *et al.*, 1982). Thus, it will be necessary to handle a ten times more volume in case of SLF, as compared to that in SSF. It was also reported that there was saving of 50 to 60% cost in downstream processing of gibberelic acid produced in SSF as compared to that in SLF (Kumar and Lonsane, 1987). These advantages were due to higher product titer under SSF technique (Mitchell and Lonsane, 1992). Despite extensive efforts on optimization of medium components in SLF, the concentration of product still low (Hahn-Hagerdal, 1986). The high concentration of product formation in SSF and involvement of less liquid for isolation of the metabolite, as compared to those in SLF processes, also bring down the production cost due to lower investment on waste disposal (Ramakrishanna *et al.*, 1982).

Generally, a large volume of the fermented broth was concentrated either by flash evaporation or ultra filtration (Meyarath and Volvasek, 1975). Centrifugation and microfiltration were used for harvesting the cells (Wanget *et al.*, 1979; Bell *et al.*, 1983; Yarmush and Colton, 1985; Belter *et al.*, 1988). These unit operations added to 48-76% of the total production cost of the metabolites (Dater, 1986). The ratio of product recovery to fermentation cost in antibiotic production was stated to be 1.0 (Barrios-Gonzalez *et al.*, 1993b).

The availability of cellulosic byproduct or residues and the environmental pollution caused by them if not disposed properly, dictate renewed efforts for their

efficient and economic utilization. Mostly SSF processes have been employed to use cellulosic byproduct or residues to produce the antibiotics. As these were cost intensive processes, a need exist to develop an economically viable SSF system for antibiotic production (Barrios-Gonzalez *et al.*, 1988).

Antibiotics are formed in the presence of cellulosic solid substrate and its derivatives. Thus, induction of antibiotic biosynthesis requires cost intensive pure substrates which can act as inducer and solid media for supporting the growth of production strains. This can be overcome by using agro-industrial waste or residue as substrate for the production of antibiotics by SSF system. Almost all the agro-industrial waste or residue contains celluloses, which could induce the biosynthesis of antibiotics in production strains (Yang, 1996).

The antibiotic and most other biological products were produced within limited pH and temperature ranges and any changes beyond the range may lead to the product formation was affected (Bull *et al.*, 1982). This instability impose a constrain on the choice of unit operation that can be used. The product formation affected stream handling at low temperature and with reasonable speed (Bull *et al.*, 1982). Consequently, the need exists to employ comparatively higher capacity infrastructure for faster recovery operation (Stanbary and Whiteker, 1984). The selection of most appropriate cost intensive infrastructure depends on the product concentration in the particular operation. Comparatively higher concentration of metabolite in SSF technique obviates these difficulties to a larger extent (Hahn-Hagerdel, 1986).

The recovery of antibiotics from fermented solid substrate to an extent about 75% was possible by using five to ten volumes of water (Yang and Ling, 1989). By employing multiple-contact counter current leaching, it is possible to get highly concentrated antibiotic solution from fermented solid substrate (Yang and Sweii, 1996). When the antibiotic is produced by in SLF, the concentrated of the antibiotics (Sasek and Gupta, 1981), and need exists for concentrating antibiotic solution before applying further down streaming processing unit operation (Barberet *et al.*, 2004).

The difficulty in sterility maintenance in large stirred tank reactors is well known (Tanaka *et al.*, 1986). Most of the media used in SLF, including those for production of microbial antibiotics, were favorable to many contaminant microorganisms (Elander, 2003). Though the vulnerability of SSF technique to contamination was expressed by many workers (Gibbons *et al.*, 1986; Yang, 1988),

it was reported that contamination problem was easily controlled in SSF technique (Loanesane *et al.*, 1985). The low water activity of system prevents the proliferation of the microorganisms, which were the main contaminants in SLF (Raimbault and Alazard, 1980). Moreover, the high density of inoculum generally used in SSF also aids in overcoming the contamination problem (Mudgetti, 1986). If the contaminated, the entire SLFmedium need to be discarded. In SSF, there was still possibility to use the medium by removing the patches, where the contamination occurred (Lockwood, 1952).

In antibiotic production by SLF, normally 8-10% batch processes. This results in the requirement of large volume fermenters, which in turn reflects higher capital investment, whereas it was possible to use 30-35% solid at much capital investment in SSF process. The foam problem which is frequently encountered in SLF system is totally absent in SSF.

The production of antibiotics under SLFrequires, in general, fermentation time of 96-144hr(Okami and Oomura, 1979). On the other hand, it takes only 30-96hr in SSF system (Venkateshwarlu *et al.*, 2000; Murthy *et al.*, 1999;Yang and Ling, 1989). Thus with reduced batch time, the use of SSF technique for microbial antibiotic production will lead to considerable reduction in capital and recurring investment (Farzana *et al.*,2005). It also offers many economical technological and advantages, which are not available when antibiotics were produced under SLF (Rainbauet, 1998).

Based on all above points, it could be concluded that the production of antibiotics using SSF process is highly potential, economical and feasible.

Table 1.2 Differences between solid-state fermentation and submerged liquid fermentation

Solid-State Fermentation (SSF)	Submerged Liquid Fermentation (SLF)
<ol style="list-style-type: none"> 1. Organisms requiring less water for growth are preferred such as filamentous microorganisms. 2. Inert support (natural or artificial), containing all components for growth in the form of solution. 3. Less chances of contamination because of low availability of water. 4. Small size bioreactors can be used. 5. Less consumption of energy for aeration and gas transfer. 6. Limiting factor for growth is diffusion of nutrients. 7. Lots of difficulties in measuring the quantity of biomass present and other online processes. 8. Downstream processing is easy, cheaper and less time consuming. 9. Liquid waste was not produced. 	<ol style="list-style-type: none"> 1. Media concentration is very much lower as compared to water content. 2. Required processed ingredients are expensive. 3. Higher water activity becomes the major cause of contamination in SLF. 4. Large-scale bioreactors are required because media is very much diluted. 5. High air pressure consumes more power and there is poor transfer of gas in SLF. 6. Vigorous mixing makes diffusion easy. 7. Online sensors are available and sampling is easy for biomass measurement. 8. Water makes downstream process difficult and very expensive. 9. High quantity of liquid waste was produced, causes difficulties in dumping.

1.14 Growth characteristics of the organisms in SSF

The complexity of the SSF in terms of heterogeneity (Kannap and Howell, 1980) and direct measurement of biomass (Mithel *et al.*, 1986) make the correlation of growth characteristics to antibiotic production difficult (Carrizales and Jaffe, 1986). In SSF, the basic mode of growth of filamentous microorganisms was combination of apical extension of hyphal extension tips, while generating new hyphal tips through branching (Edelstein and Segel, 1983; Hesseltine, 1981). The hyphal mode of growth also gives filamentous microorganisms the power to penetrate into solid substrates (Niladevi *et al.*, 2007). Thus the production of cephamycin C depends both on the nature and the physical state of the solid substrate (Kagliwal *et al.*, 2009).

When *Nocardia culyure* culture were grown on coconut oil cake in a tap water for overnight to effect fermentation, or on steamed coconut oil cake and examined by visual observation for degree of growth, it was found that the growth was faster and more profuse on steamed coconut oil cake medium (Kagliwal *et al.*, 2009). In fact, increase in cephalosporin C with increase in the growth was also observed by them. Packing density of the substrate also play a role in mycelia growth and cephalosporin C production (Cuadra *et al.*, 2008). Baburao *et al.*, (2008) correlated the biomass formation with that of carbon dioxide evolution and effect of carbon dioxide on cephamycin C production. It was observed that cephamycin C production in mycelia reached stationary phase.

1.15 Production strains for solid substrate fermentation

The development of highly producing strains was a prerequisite for efficient biotechnological processes. One major positive aspect of SSF was that antibiotics were, in manycases, produced at much higher yields than in liquid culture, also called SLF. Shankarananda *et al.*, (1992) observed discrepancies in the levels of α -amylase produced by different of *Bacillus* in SSF in relation to SLF. They concluded that cultures (strains), which were good producer in submerged liquid fermentation, cannot be relied upon to perform will in SSF. Working with *Penicillium chrysogenum*, our group showed that high penicillin producing strains (developed for submerged liquid fermentation) tend to be higher producers in SSF. However, it was determined that lower producing strains (closer to wild type) tend to more efficient to produce in SSF, therefore these strains produced several time more in

SSF than in SLF up to 17 time more (or relative production of 17) in this study (Barrios-González *et al.*, 1993a). Conversely, the relative production of high antibiotic producing strains ranged from 0.4 to 4 times more in SSF antibiotic production at 72 h in a 2-phase system (Barrios-González *et al.*, 2004a).

1.16 Culture vessel

Most of the work reported in the literature was limited to laboratory scale fermentation. It involved in growing organism on 10 g (Farzana *et al.*, 2005; Ellaiah *et al.*, 2004; Shazia *et al.*, 2009; Adinarayana *et al.*, 2003), 5 g (Kagliwal *et al.*, 2009; Baburao *et al.*, 2008), 2 g (Mahalaxmi *et al.*, 2010) solid substrate taken in a 250 ml flask. Yang and Ling, (1989) and Agnes *et al.*, (2005) used 100 g solid substrate in 250 ml flask for tetracycline production. Shinji and Shoda, (2007) reported that 15 g solid substrate in 100 ml flask. Ohno *et al.*, (1992) reported that 150 g solid substrate in petri dish for production of iturin. Scott *et al.*, (2007) reported that production of antifungal antibiotic in forced aeration batch micro reactors.

On medium scale, SSF were carried out mainly in column fermenter (Cuadra *et al.*, 2008; Barrios-González *et al.*, 1988). El-Naggar *et al.*, (2009) reported that production of meroparamycin in cylindrical percolation vessel.

El-Naggar *et al.*, (2009) used a cylindrical percolation vessel for monitoring the effect of carbon dioxide, oxygen and heat accumulation on the meroparamycin production. They reported that proper aeration needs be maintained to improve the antibiotic production. In this study, it was proved that the carbon dioxide and oxygen level can be maintained at optimum level for enhanced antibiotic production, by relying on forced aeration.

In recent years, various investigators had been found plastic bag or micro porous films as containment system for SSF (Cuero *et al.*, 1985; Concidine *et al.*, 1989). Plastic bag of 13 to 20 cm size and different thickness were evaluated by Battaglino *et al.*, (1991) to select 18 μm polyethylene films tied with a rubber tube filled with cotton to ensure gas diffusion. It was observed that the antibiotic production was reduced by 50% in case of heat sealed polyethylene bags, though the production was comparable to that in the culture flask with use of other two types of the method for tying of the bags (Battaglino *et al.*, 1991).

1.17 Autoclaving

In some cases, it was necessary to improve amenability of solid substrate to microbial attack and the property of solid substrate can be modified by pretreatment (autoclaving) to improve the performance of the process (Loanesane *et al.*, 1985).

The autoclaving time and temperature are known to have significant effect on fermentation in the case of SSF process involving microbial culture (Loanesane *et al.*, 1985). During autoclaving, the most solid substrate undergoes physical modification, partial hydrolysis of starch/ proteins present in the solid and killing of contaminating organisms. Ellaiah *et al.*, (2004) observed that the autoclaving time of 30 min was enough for proper cooking of the wheat rawa, as neomycin formed by *Streptomyces marinensis* was same when the substrate subjected to 30 or 60 min autoclaving time. However, less than 30 min autoclaving time lead to lower production of neomycin (Ellaiah *et al.*, 2004). The autoclaving temperature maintained at 100°C (Tanaka *et al.*, 1962).

1.18 Factor affecting antibiotic production in solid state fermentation

The efficiency, productivity and economy of SSF were affected by number of factors.

- Solid substrate
- Particle size of the substrate
- Moisture content and water activity
- Initial pH
- Incubation Temperature and time
- Inoculum concentration
- Nutritional factor

1.19 Solid substrate

The solid substrate used and its preparation can significantly affect the success of solid substrate fermentation. The substrate used for SSF were usually unprocessed agricultural products or byproducts because inexpensive. The solid substrates generally were lignocelluloses, cellulosic and starchy substrates (Yang, 1996; Ziffer, 1988).

The SSF, solid substrates not only supplies nutrients to the culture but also serve as an anchorage to the microbial cell (Kanap and Howell, 1980). Therefore, the particle size and chemical composition of the substrate were of critical importance (Loanesane *et al.*, 1985). The ideal solid substrate was the one which provides all the nutrients to the microorganisms for optimum function (Mitchell *et al.*, 1992a). The cost and availability were important consideration (Hesseltine, 1977). In some cases, extent of mechanical or chemical treatment necessary to improve the amenability of the solid substrate to microbial attack, and the changes that occur during sterilization of the solid substrate decides the suitability of the substrate (Loanesane *et al.*, 1985).

The substrate usually used include corn husk, wheat bran, corn cobs (Mahalaxmi *et al.*, 2010;Ellaiah *et al.*, 2004), almond seed cake, mustard seed cake, cotton seed cake, sesame seed cake, coconut cake, linseed cake and kalonji cake (Shazia *et al.*, 2009), Sugarcane bagasse (Cuadra *et al.*, 2008; Ellaiah *et al.*, 2004), Sweet potato residue (Shang- Yang and Ling, 1989), wheat rawa, rice bran, rice rawa, rice husk, rice straw, maize bran, ragi bran, green gram bran, black gram bran, red gram bran, corn flour, jowar flour and sago (Ellaiah *et al.*, 2004) etc., in order to make these type of cellulosic material easily accessible for antibiotic production.

1.20 Particle size of the substrate

Solid substrate used was unrefined material of agricultural origin, which not only supplies the nutrients to the culture but also serve as anchorage to the microbial cells (Kanap and Howell, 1980). Particle size and shape is extremely important since it affects surface area to volume ratio of the particle and packing density within substrate mass (Loanesane *et al.*, 1985; Barrios-Gonzalez *et al.*, 1993b). The availability of nutrients to the microorganism from the solid matrix was depends on the structure of solid substrate, porosity, shape, size, internal surface area and adsorption behavior of the solid substrate (Moo-Young *et al.*, 1983).In SSF, the mass transfer occurs in three phases. In gases phases, oxygen is taken up by the microorganism and the evolved CO₂ and other gases should be effectively removed. In the solid and liquid phases the diffusion of the nutrients and transport of the metabolite/product occur by intra and inter particle mass transfer. The rate at which exchange of gases take place in void space was called intra particular mass transfer

and was governed by particle size, porosity, gas flow rate and moisture level of the system (Zadrzil and Brunnert, 1989; Reid, 1985; Smith and Aidoo, 1988).

Ellaiah *et al.*, (2004) reported that the coarse size of wheat rawa was found to be optimal size of the substrate for higher neomycin production. Mahalaxmi *et al.*, (2010) reported that particles sizes the one designated as “C” the (6 X 4 mm) supported maximum rifamycin B. El-Naggar *et al.*, (2009) reported that particle size of wheat bran (0.3-0.4 cm) shows highest antibiotic productivity.

1.21 Moisture content and water activity

A good amount of work was done on this aspect (Moo-Young *et al.*, 1983; Oriol *et al.*, 1988; Laukevicset *et al.*, 1984), which was known to play a critical role to play in SSF (Ramesh and Lonsane, 1990b). The amount of moistening agent is known to influence the physical property of the moist solids (Fenicosava *et al.*, 1970). Lindenfelser and Ciegler (1975) and Gonzalez *et al.*, (1988) had shown that the initial moisture level was the most critical factor among all fermentation condition in case of *A. ochraceous* in SSF system. The level of moisture content in SSF usually varies between 30-85% (Oriol *et al.*, 1988; Reid, 1989). The moisture content of the medium during fermentation depends upon the relative humidity of the air in the fermenter with which it was in equilibrium. Too much of moistening agent was reported to make solid substrate to make less for microbial growth and also the antibiotic production (Tengerdy, 1985), due to reduction in the substrate porosity, loss of the structure of solid substrate, development of stickiness in the moist medium and difficulty in oxygen transfer, thereby making such medium difficult in oxygen transfer, thereby making such medium difficult to use especially on large scale (Moo-Young *et al.*, 1983; Lonsane *et al.*, 1985). On the other hand, less moisture content reduce the solubility of nutrients present in solid substrate during cooking/autoclaving (Feniksova *et al.*, 1970). The upper limit differ from substrate to substrate (Oriol *et al.*, 1988). From example, free water becomes apparent at 40% in maple bark, but only at about 75% level in case of straw (Cannel and Moo-Young, 1980).

Although cell growth in SSF was considerably influenced by moisture, it appears that the water activity was a more appropriate parameter than the moisture content for the growth of microorganisms (Narahara *et al.*, 1982). In this regard, Scott, (1975) had shown that the biological response to a particular water activity

was independent of the type of the solute and the total water content of the substrate. Water activity (a_w) gives the amount of unbounded water available in the immediate surroundings of the microorganisms (Scott, 1975). While related to the water content of the substrate (Griffin and Fogarty, 1973), it is not equivalent to moisture content (Narahara *et al.*, 1982). Water activity is defined as the ratio of the equilibrium vapour pressure of the substrate (P_s) to that of pure water (P_o) at the same temperature ($A_w = P_s / P_o$) (Gervais *et al.*, 1988). Water activity influences microbial stability, and each microorganism has its own minimum water activity level for metabolic activity (Troller, 1980). For example, optimal water activity of fungi was about 0.7, yeast was about 0.8 and bacteria was about 0.9 (Beuchat, 1981).

Moistening imparts suitable water activity value to the solid substrate for growth and swelling of the solid substrate (Iglesias and Chirife, 1982), and the later cases easier utilization of the substrate by mold (Wang *et al.*, 1974). Yang and Ling, (1989) and Agnes *et al.*, (2005) reported that water at 68% level improved the tetracycline titers at 72 hr fermentation, while higher water contentsuppressed the antibiotic production. It was interesting to note that growth of the microorganism was good at moisture level (Yang and Ling, 1989; Agnes *et al.*, 2005). Thus, it was suggested that the moisture should be kept at higher side during growth phase and then should be reduced during tetracycline formation level (Yang and Ling, 1989; Agnes *et al.*, 2005).

Different workers reported various moisture optimum ranging from 84.32(Venkata *et al.*, 2009),80% (Ellaiah *et al.*, 2004; Adinarayana *et al.*, 2003), 76.375% (Baburao *et al.*, 2008), 75%(Yang and Yuan, 1990), 70%(Shazia *et al.*, 2009; Barrios-González *et al.*, 1988), 69% (Ohno *et al.*, 1992), 65% (Kagliwalet *et al.*, 2009), 60% (El-Naggar *et al.*, 2009). It was apparent that the requirement of initial moisture content depends on the strain used for antibiotic production.

1.22 Initial pH

The pH of the medium plays a significant role in the growth and metabolism of the organism which was difficult to control in SSF. Hence, it was desirable to maintain broad pH range and pH optima during the growth of the organism (Mitchell *et al.*, 1988). Some of the filamentous organisms grow well in the pH range 4-6 (Prior *et al.*, 1992).

It was a parameter in SSF which can be measured but very difficult to monitor. The microorganism should have a broad pH range and pH optima for growth in SSF (Mitchell *et al.*, 1986). Filamentous microorganisms grow well within a pH range with an optimum in the range of pH 3.8 to pH 6 (VanDemark and Batzing, 1987). The optimum pH range for microbial growth and product formation can differ considerable. For example, Cuadra *et al.*, 2008 reported that the optimal cephalosporin C production by *Acremonium chrysogenum* C10 on sugarcane bagasse residue in SSF occurred over much narrow pH range (pH 5.5-5.8) than mycelia growth pH (5.1-7.15).

Indirect control of pH in SSF can be affected and was done by manipulating the nitrogen concentrations (Prior *et al.*, 1992). A degree of pH control in SSF can be achieved by different ratio of ammonium salts and urea in the medium (Raimbult and Alazard, 1980; Mitchell *et al.*, 1986b) Yang and Chiu, (1986) showed that the use of ammonium sulphate results in sharp decline in pH during growth, whereas 1:1 ratio of ammonium sulphate to urea minimizes the pH change. Ammonium nitrate had stabilizing effect of pH (Prior *et al.*, 1992). Despite the enhancement of the production of tetracycline by *Streptomyces* sp. OXC1 (Agnes *et al.*, 2005). If a decrease in pH was anticipated during SSF, it could also be counter acted by adding calcium carbonate (Yang and Yuan 1980, Agnes *et al.*, 2005). In fungal SSF process, the buffering capacity of some medium constituents was useful to eliminate the need for pH control (Chahal, 1983). When the initial pH was increased from 6.9 to 7.2 by moistening the substrate with phosphate buffer and by adding 4% calcium carbonate to prevent a drop in pH during fermentation, the production of tetracycline in peanut shells increased by 5 times (Agnes *et al.*, 2005).

1.23 Incubation temperature and time

Biological processes characterized by the fact that they are developed in relatively narrow range of temperature. High temperature limits for development could be found in many cases at values not higher than 60-80°C and with particular strains at utmost at 120 °C. The significance of temperature in the development of biological processes was such that it could determine effects such important as protein denaturation, enzymatic inhibition promotion and inhibition on the production of a particular metabolites, cell death etc.

Microbial growth in SSF generates significant amount of metabolic heat. It has been reported that 100-300 kJ of heat per kg of cell mass was generated in a SSF process (Prior *et al.*, 1992). Establishment of temperature gradients and localized overheating of the substrate occurs because of inefficient removal of heat from the substrate. Heat transfer problem in SSF includes temperature gradients that may cause belated microbial activity, dehydration of the medium and undesirable metabolic deviations (Rathbun and Shuler 1983; Saucedo-Castaneda *et al.*, 1990). Heterogeneous materials make heat removal difficult; this was due to low heat transfer coefficient and low thermal conductivity (Narahara, 1984). Temperature can rise rapidly, because there is little water to absorb the heat or in other words mean specific heat capacity of the fermenting mass was much lower than that of water. Therefore, heat generated must be dissipated immediately as most of the microorganisms used in SSF were mesophilic, having optimal temperature for growth between 20 and 40°C and maximum growth below 50°C.

Temperature play crucial role in antibiotic production (Adinarayana *et al.*, 2003). Incubation at 26 to 35°C was reported to give better antibiotic yields (Yang and Yuan, 1990). *Acremonium chrysogenum* ATCC 48272 incubated in the 25 to 50°C in SSF. Short time increase in incubation temperature, followed by cooling to normal temperature has to improve the neomycin (Ellaiah *et al.*, 2004) Maximum production of rifamycin by *Amycolatopsis mediterranei* VA18 at 32°C (Venkateshwarlu *et al.*, 2000). In case of fungi, though growth occurs at 45°C, increase in temperature was found to decrease cephalosporin C production (Adinarayana *et al.*, 2003). Agnes *et al.*, (2005) reported good tetracycline production at 31°C. Shazia *et al.*, (2009) reported that 30°C was the optimum temperature for the production of tylosin by *Streptomyces fradiae* NRRL-2702 in SSF. Majority of reports, however, recommends the use of 30°C as optimum temperature for the growth and antibiotic production by antibiotic production strains (Mahalaxmi *et al.*, 2010; Yang and Ling, 1989; Ohno *et al.*, 1992; Barrios-González, 1988).

The incubation time varied from day 1 (Barrios-González, 1988; Cuadra *et al.*, 2008; Ohno *et al.*, 1992), day 2 (Yang and Yuan, 1990), day 3 (Agnes *et al.*, 2005; Baburao Bussari *et al.*, 2008; Mahalaxmi *et al.*, 2010), day 4 (Mizumoto *et al.*, 2006; Shazia *et al.*, 2009).

1.24 Inoculums concentration

Several methods have been used by different workers for preparation of inoculums. In case process by Ellaiah *et al.*, (2004), stock culture was maintained on jowar starch agar slants and organism transferred to fresh jowar starch agar slants and incubated at 30°C for seven days, before each experiment. The spore suspension for inoculums was prepared by adding 10ml of sterile distilled water to each slant and vigorously shaking the slant for 1 min. From this, 2 ml spore suspension (equivalent to 30 mg w/w dry cell mass) added to 10 g solid substrate as inoculum. Adinarayana *et al.*, (2003) reported that the 5% inoculum level (10^8 /ml) added to 10 g solid substrate for production of cephalosporin C.

The preparation of inoculums in the medium of essential the same composition that was employed for final culture to demonstrated to have a slightly beneficial effect (El-Naggaret *et al.*, (2009). Agnes *et al.*, (2005) used the inoculum from day 2 old culture by suspending in sterile water containing 0.05% Tween-80. The spore suspension containing about 10^8 spore/ml was used as inoculums for tetracycline production.

1.25 Nutritional factors

The uptake of available nutrient from solid substrates by microorganism depend on their capacity penetrate into the substrate particle (Loanesane *et al.*, 1985; Chahal, 1983). The nutrient present in the substrate at places, where the actinomycetes and fungal mycelium could not penetrate, were thus not available not available to the culture during fermentation. Some of the important nutrients necessary for growth and product formation for the microorganism under study may also be present at sub-optimal level in solid substrate. Hence, the supplementation of solid substrate with other solid or water soluble nutrients were found to lead to enhanced product formation in solid substrate fermentation processes (Kumar and Loanesane, 1987). The nature of the carbon source determines the effectiveness antibiotic production (Miranda *et al.*, 2003). Soluble carbon source were tried as an alternative carbon source because of their inexpensiveness and abundant availability for antibiotic production (Krishna *et al.*, 1998). The tetracycline productivity had found increase 1.4 fold when maltose used as carbon source (Yang and Yuan, 1990).

Like carbon source, nitrogen source was also an important factor for growth and production of antibiotics. The presence of external nitrogen source in growth

medium enhances the antibiotic by increasing permeability of the cell wall there by facilitating the entry of the nitrogen source and secretion of antibiotics (Abou-Zeid *et al.*, 1981). The nitrogen source like peptone, soybean meal, peanut meal and beef extract as an organic nitrogen source, while ammonium nitrate, ammonium sulphate, ammonium chloride and potassium nitrate as an inorganic nitrogen source were most commonly in production media. Peanut meal is the most commonly used organic nitrogen source and its presence in growth medium enhanced antibiotic productivity (Yang and Yuan, 1990). Increased titres of antibiotic had been observed when ammonium sulphate as an inorganic nitrogen source in the growth medium (Yang and Swei, 1996). These and other worker indicates that regulatory mechanisms were also active in solid substrate fermentation. A practical application of these finding was that, when a solid medium was designed, we must consider to by-pass these regulatory mechanisms by avoiding repressing carbon and nitrogen sources.

1.26 Isolation of antibiotic by solvent extraction

The water soluble antibiotics from fermented solid substrates can be extracted by magnetic shaker at room temperature with water or buffer solution (Yang and Ling, 1989; Ellaiah *et al.*, 2004; Shazia *et al.*, 2009; Yang and Swei, 1996; Adinarayana *et al.*, 2003), orbital shaker (Baburao *et al.*, 2008). In orbital shaker required based up on the weight of the solid substrate (Mahalaxmi *et al.*, 2010). High concentrated antibiotic extracted could be obtained by percolation technique (El-Naggar *et al.*, 2009).

Various solvents such as water, buffer solutions (Yang and Ling, 1989; Ellaiah *et al.*, 2004; Shazia *et al.*, 2009; Yang and Ling, 1989; Yang and Swei, 1996), methanol (Scott *et al.*, 2007; Ohno *et al.*, 1992; Shinji and Makoto, 2007), Ethyl acetate (Barrios-González *et al.*, 1988) was used for leaching of antibiotic from the fermented solid medium. The mouldy fermented solid were subjected to leaching immediately at the end of the fermentation run (Marvin JW and Wajman, 1978).

For cephalosporin C leaching from fermented wheat bran mixed with 50ml of water (Adinarayana *et al.*, 2003). Yang and Ling, 1989 leached the tetracycline from fermented sweet potato residue with 5 times volume of distilled water. It was reported that the bacitracin and rifamycin B leaching was complete when contact time was 1 hr (Farzana *et al.*, 2005) or 3hr (Mahalaxmi *et al.*, 2010). Though it was

claimed that the condition adopted by these worker completely leached the antibiotics, the retention of the antibiotics in the solvent which get adhered to the fermented solid medium was obviously ignored by them. It was interesting to note that the solvent, amounting to about two times the weight of the dry fermented solid, get adhere to solid during leaching process and that it cannot be recovered easily by normal pressing technique(Kumar and Loanesane, 1987).

1.27 Quantification of antibiotics

Several analytical methods have been reported to quantify the concentration of antibiotics. They can be classified as microbiological assays (bioassay), (Souza *et al.*, 2004; Hewitt, 2003) and turbidimetric method (Dwight and Margaret, 1950), automated techniques such as high performance liquid chromatography, (HPLC) and colorimetric determination (Joel, 2006).

The microbiological assay for antibiotics dates back to the demonstration of the agar diffusion assay for penicillin devised by Heatley, (1944). Successful application of growth inhibition techniques for quantitative determination of antibiotics relies on the sensitivity of the applied indicator microorganism to the antibiotic to which was exposed. However, information on indicator microorganisms performance and comparisons in antibiotics determination with bioassays was almost non-existing in the literature. The "indicator microorganism" in bioassays carried out on solid agar diffusion assay and liquid turbidometric assay substrates, applied in the quantification of the most studied antibiotics (Maria *et al.*, 2006).

Yang and Ling, (1989) reported that antimicrobial activity of culture extracts containing tetracycline was measured by the paper disc method with *B. subtilis* ATCC 6633 as the tested organism. Shazia *et al.*, 2009 reported that tylosin antibiotic determined by disc diffusion bioassay method with *Bacillus subtilis* was used as tested organism. Rifamycin B present in the extraction solution was estimated by colorimetrically (Mahalaxmi *et al.*, 2010). Cepharmycin C was estimated by HPLC method reported by Kota and Sridhar, (1999), Kagliwal *et al.*, (2009) and Bussari *et al.*, (2008) with some modifications. El-Naggar *et al.*, (2009) reported that the meroparamycin was quantified by both microbiological assay and HPLC method. Scott *et al.*, 2007 used 96-well microplate bioassay to determined iturin A with *Fusarium oxysporum f. sp. melonis* as the tested organism.

1.28 Statistical optimization approach

Traditional methods of optimization involve changing one independent variable while keeping the other variables fixed at certain levels. This one dimensional approach is laborious, time consuming, expensive and most importantly, incapable of providing the optimal conditions due to the lack of the interactions between different variables (Furuhashi and Takagi, 1984).

Statistical experimental designs have been widely used for several decades (Plackett and Burman, 1946, Box and Hunter, 1957). Contour surfaces, central composite, Plackett-Burman and response surface methodology (RSM) are examples of statistical experiments designs that increase efficiency, improve products and decrease costs, therefore, they have received increasing attention. Statistical experimental designs can be applied at different aspects in process optimisation. The Plackett-Burman design is very useful for the screening of the most important factors from a lot of candidates (Plackett and Burman, 1946). This design does not consider the interaction effects between variables but the most important factors affecting the results. RSM was a factorial experiment design for examining the effect of test variables on measured responses. The RSM approach firstly requires an experimental design followed by fitting experimental data into an empirical model equation to determine the optimum conditions. With the development of bioprocess technologies like antibiotic production and enzyme production, more scientists were getting interested in adopting statistical experiment design to improve their biological processes and production by shortening time and increasing efficiencies (Rajasimman and Subathra, 2009; Tanyildizi *et al.*, 2005).

Reducing the costs of antibiotic production by optimising the fermentation medium was an essential aspect of basic research for industrial applications. Different statistical designs for medium optimization had been recently employed for gentamicin, actinomycin X2, pristinamycins and avermectin B1a production by *Streptomyces* cultures (Rajasimman and Subathra, 2010; Xiong *et al.*, 2008; Jia *et al.*, 2008; Gao *et al.*, 2009).

Parra and co-workers used an orthogonal design to optimise 13 medium components to enhance squalestatin production (Parra *et al.*, 2005). Castro and co-workers used a Plackett-Burman design to screen 20 different serum-free medium components to identify those important for cell growth and recombinant human

interferon (IFN)-gamma production (Castro *et al.*, 1992). The optimal medium composition of xylanase production by *Aspergillus terreus* was developed by Plackett-Burman design (Ghanem *et al.*, 2000). The effects of medium components, including glucose, asparagine, Tween-80, Cu^{2+} , Mn^{2+} and pH on laccase, manganese peroxidase and glyoxal oxidase production by *T. troglia* had been studied using Plackett-Burman experimental design (Levin *et al.*, 2005). The orthogonal and Plackett–Burman designs (PBD) were important methodologies that can reduce the number of runs to an absolute minimum. The principal objectives of these designs are to screen main factors for further optimization processes from a large number of process variables (Castro *et al.*, 1992, Parra *et al.*, 2005). This enables a better picture of the possible effects of each component in the medium. However, the main disadvantage of these designs was that they consider only first order effects and ignore the interactions between variables. On the other hand, a full factorial design provides almost every possible combination. Optimum performance has been determined using mathematical tools such as multiple regression of a partial or full factorial design to obtain a model of the production system, usually involving fitting of data to a polynomial equation, often using stepwise multiple regression. RSM has also been used to investigate the optimal regions of production of useful products (Prapulla *et al.*, 1992).

RSM, firstly described by Box and Wilson, 1951 (Box and Wilson, 1951), was a collection of mathematical and statistical techniques (Myers *et al.*, 2002), which was not only used to evaluate the relationship between a set of experimental factors and observed results but also to seek the optimum conditions for multivariable system. RSM has been established as a convenient method for developing optimal conditions for processes with reduced cost and efficient screening of parameters (Vohra and Satyanarayana, 2002). The models from RSM take into account the interactions of variables in generating a process response effectively. In many processes, the relationship between the response and the independent variables was usually unknown; therefore, the first step in RSM was to evaluate the function (response) in terms of analysing variables (independent variables). Usually, this process employs a low-order polynomial equation in a pre-determined region of the independent variables, which was later analysed to locate the optimum values of independent variables for the best response. RSM has been

successfully employed for many bioprocesses, particularly in optimisation of medium ingredients and operating parameters.

Thayer *et al.* (1987) and Oh *et al.* (1995) used response surface designs for rapid to optimisation of media and process conditions. Thayer and co-workers investigated the interactions of pH, temperature, air (aerobic versus anaerobic) and NaCl on the growth of *Salmonella typhimurium* ATCC 14028. RSM was used to develop equations that described the response of *S. typhimurium* to environmental changes (Thayer *et al.*, 1987). The method was applied to find optimum conditions of tryptone, yeast extract, glucose, Tween-80 and incubation temperature for the growth of *Lactobacillus casei* YIT 9018 and to evaluate the effects of these factors employing RSM (Oh *et al.*, 1995). RSM was also attempted to maximise lipid production by *Rhodotorula gracilis* (Prapulla *et al.*, 1992).

The growth medium for *Streptomyces marinensis* neomycin production was studied through RSM. The impact of three crucial variable on neomycin production, including dextrin, raspberry seed powder, and concentrated mineral medium was investigated thoroughly (Adinarayana *et al.* , 2003). A Box-Wilson central composite design was applied to optimize initial pH, initial water content, temperature, relative humidity, and volume of inoculum for lipopeptide antibiotic production in solid state fermentation of *Bacillus subtilis* RB14-CS (Mizumoto and Makoto, 2007). A five level Box-Wilson central composite design (CCD) was employed to optimize the production medium of *B. subtilis* S3 for improved lipopeptide antibiotic production (Ing *et al.*, 2008). Box-Wilson central composite design (CCD) was also applied in clavulanic acid production by *Streptomyces clavuligerus*. Four variables, including concentration of wheat rawa, soya flour, dipotassium hydrogen phosphate and sunflower oil cake, at five levels were studied to identify the correlation between those variables on clavulanic acid production (Anindya *et al.*, 1998).

The application of RSM requires the use of models which effectively describe the response quantitatively. The models generally were multinomial in nature, and the most adequate degree for each factor and interaction could be chosen in the final form of relationship obtained. This technique finds most utility in optimization of different processes to get the best performance under given constraints. Hence, we have under taken the researcher to investigate the optimization of antibiotic production on SSF using agro bio waste as substrate.

1.29 Objectives

1. Screening of various agro industrial residues for antibiotics production by SSF.
2. Study the impact of the physiological conditions as well as medium constituents, which provides the maximum yield of antibiotic.
3. Extraction and purification of the antibiotics from fermented solid substrates.
4. Quantification of antibiotics by using various methods.
5. Statistical optimization of antibiotic production (tetracycline, neomycin and rifamycin B) using Plackett–Burman designs (PBD) and central composite design (CCD).

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