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"Antibiotic-Producing Microorganism from Agro-Industrial Wastes"

Thesis
Submitted for the degree of Ph.D. of philosophy in Science
In Biochemistry and Nutrition

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2006

Aim of the work

The main objective of the present study was concerned with:

(1) The isolation of microorganisms producing antibiotics from soil and production of antibiotics under solid state fermentation (SSF) conditions.

(2) Controlling the optimal antibiotic production under SSF and carrying out the optimum parameters under large scale in 50L fermentor SSF.

(3) Purification and determination of the chemical formulae and antimicrobial tests of the produced antibiotics.

(4) The characterization, and biological activities of antibiotics produced by microorganisms under investigation were also undertaken

Solid State Fermentation

Solid state fermentation (SSF) has usually exploited the production of value added products (antibiotics, alkaloids, plant growth factors, etc…), biofuel, enzymes, organic acids, aroma compounds and also for bioremediation of hazardous compounds, biological detoxification of agro-industrial residues, nutritional enrichment, biopulping, biopharmaceutical products, etc. This technology has gained renewed attention from industry because it has become a more attractive alternative to liquid fermentation for many productions. Thus, SSF was found to produce a more stable product with less energy requirements, in smaller fermenters and smaller volumes of polluting effluents**, *Guerra et al., (2003).***

Solid state fermentation (SSF) processes can be identified as the growth of microorganisms (mainly fungi) on moist solid materials in the absence of free-flowing water. Substrates that have been traditionally fermented by solid state include a variety of agricultural products such as rice, wheat, millet barley, grains, beans, corn and soy beans. However, non-traditional substrates which may also be of interest in industrial process development include an abundant supply of agricultural, forest and food processing wastes such as wheat bran and soy grits (flakes remaining after extraction of oil), ***Guerra et al., (2003).***

The ability of the microorganisms for growing on a solid state substrate is a function of their requirements of water activity, their capacity of adherence and penetration into the substrate and their ability to assimilate mixtures of different polysaccharides due to the nature, often complex of the substrate used**, *Guerra et al., (2003).***

The filamentous fungi are the best adapted microorganisms for SSF owing to their physiological, enzymological and biochemical properties.The hyphal mode of fungal growth gives the filamentous

fungi the power to penetrate into the solid substrates. This also gives those major advantages over unicellular microorganisms for the colonization of the substrate and the utilization of the available nutrients. In addition, their ability to grow at low activity (a_w) and high osmotic pressure conditions (high nutrient concentration) makes fungi efficient and competitive in natural microflora for bioconversion of solid substrates, ***Mitchell, (1992).***

However, bacteria and yeasts have also been used in traditional cultivation in SSF processes, ***Mitchell and Lonsane, (1992).***Bacteria have been used for enzymes production, composting, ensiling, and some food processes (e.g.: sausages, Japanese natto, fermented soybean paste, Chinese vinegar) ***(Mitchell, 1992; Ramesh and Lonsane, 1992 and Prabhu and Chandrasekaran, 1995).***Yeasts have been mainly used for ethanol production and protein enrichment of agricultural residues, ***Robinson and Nigam, (2003).***

Environmental factors such as water activity, moisture content, temperature, pH, oxygen levels and concentrations of nutrients and products significantly affect microbial growth and product formation, ***Guerra et al., (2003).***

The role of water of the substrate has been widely described and reviewed by different authors ***(Krishna and Chandrasekaran, 1996; Gervais and Molin, 2003 and Bellon-Maurel et al., 2003)***. Moisture content is a critical factor on the SSF processes because this variable has influence on growth and biosynthesis and secretion of different metabolites***, Ellaiah et al.,(2002)***.

The increase of temperature on SSF is a consequence of the metabolic activity when the heat removal is not enough. These effects directly spore germination, growth and product formation. The temperature level reached is a function of the type of microorganism, the porosity, the particular diameter and the depth

of the substrate ***(Raimbault, 1998***; ***Gervais and Molin, 2003 and Raghavarao et al., 2003)***.

The measurement and control of pH in SSF is very difficult. Nevertheless, the substrate employed in SSF usually has buffering effect due to their complex chemical composition. In these cases, the control of pH is not necessary. When this variable must be controlled, buffering solutions are added as liquid phase, but this strategy can be inadequate when the process is scaled-up. Another possibility to control the evolution of the pH consists on adding a mixture of sources of nitrogen with opposite influence on the evolution of the pH in such a way than ones counteract the effect of the others. In this sense, ammonium salts have been used in SSF combination with urea or nitrate salts due to the respective effects of acidification and alkalization of the former and the later, ***Raimbaul, (1998) and Torrado et al.,(1998)***.

In the last years, new applications of SSF in the environmental control have been developed including bioremediation and biodegradation of hazardous compounds and the detoxification of agro-industrial residues. Table (1) shows some examples of SSF process in economic sectors of agro-industrial, environmental control and fermentation industry, ***Raimbault,(1998) and Pandey et al.,(2000)***.

Table (1): Some economic applications of SSF.

Sector	Application	Examples	References
Agro-food Industry	Biotransformation of crop residues	Traditional food, Fermented protein enrichment and single cell protein production, Mushrooms production.	***(Pandey et al.,2000)***
	Food additives	Aroma compounds dyestuffs, Essential fat and organic acids	***(Christen et al., 1997 and Pandey et al.,2000)***
Environmental control		Caffeinated residues, Pesticides, Polychlorinated biphenyls(PCBS)	***(Hang and Woodams,1998 and Pandey et al.,2000)***
		Coffee pulp, Cassava peels, Canola meal, Coffee husk	***(Guerra et al.,2003 and Pandey et al.,2000)***
Industrial fermentation	Enzymes production	Amylase, Aminoglycosides, Cellulases, Proteases, Pectinases, Xylanases, Glucoamylases	***(Pandey et al.,2000 and Tengerdy and Szakacs,2003)***

(Table 1)… Continued.

Sector	Application	Examples	References
Industrial fermentation	Bioactive products	Mycotoxins, Gibberellins, Alkaloids, Antibiotics, Hormones	***(Pandey et al.,2000 and Tengerdy and Szakacs,2003)***
	Organic acids production	Citric acid, Fumaric acid Itaconic acid, Lactic acid	***(Pandey et al.,2000 and Tengerdy and Szakacs,2003)***
	Biofuel	Ethanol production	***(Pandey et al.,2000 and Tengerdy and Szakacs,2003)***
	Miscellaneous compounds	Pigments, Biosurfactants, Vitamins, Xanthin	***(Pandey et al.,2000 and Robinson et al.,2001)***

Antibiotics:

Antimicrobial agents produced by microorganisms that kill or inhibit other microorganisms. This is the microbiologist's definition. A more broadened definition of an antibiotic includes any chemical of natural origin (from any type of cell) which has the effect to kill or inhibit the growth of other types of cells. The most clinically-useful antibiotics are produced by microorganisms and are used to kill or inhibit infectious bacteria, ***Toddar, (2003).***

Antibiotics are low molecular weight (non-protein) molecules produced as secondary metabolites, mainly by microorganisms that live in the soil. Most of these microorganisms from some type of a spore or other dormant cell, and there is thought to be some relationship between antibiotic production and the process of sporulation. Among the molds, the notable antibiotic produced are Penicillium and Cephalosporium, which are the main source of the beta-lactam antibiotics (Penicillium and its relatives).In the bacteria, the actinomycetes notably *Streptomyces* species, produce a variety of types of antibiotics including the aminoglycosides (eg.streptomycin), macrolides (e.g. erythromycin) and the tetracyclines. Endospore-forming Bacillus species produce polypeptide antibiotics such as polymyxin and bacteria***, Toddar, (2003).***

The most important property of a clinically-useful antimicrobial agent, especially from the patient's point of view, is its selective toxicity,i.e. that the agent acts in some way that, inhibits or kill bacterial pathogens but has little or no toxic effect on the animal taking the drug. This implies that the biochemical processes in the bacteria are in some way different from those in the animal cells, and that the advantages of this difference can be taken in chemotherapy. Antibiotics may have a cidal (killing) effect or a static (inhibitory) effect on a range of microbes. The range of

bacteria or other microorganisms that are affected by a certain antibiotic is expressed as its spectrum of action. Antibiotics effective against prokaryotes which kill or inhibit a wide range of Gram-positive and Gram negative bacteria are said to be broad spectrum. If effective mainly against Gram-positive or Gram-negative bacteria, they are narrow spectrum. If effective against a single organism or disease, they are referred to as limited spectrum, ***Toddar, (2003).***

The aerobic actinomycetes are soil-inhabiting microorganisms that occurs worldwide. According to ***McNiel and Brown, (1994),*** Nocard (1888) was the first to recognize the pathogenic potential of this group of microorganisms. Since then, several aerobic actinomycetes have been a major source of interest for the commercial drug industry and have proved to be extremely useful microorganisms for producing novel antimicrobial agents. They have also been well known as potential veterinary pathogens affecting many different animal species. The medically important aerobic actinomycetes may cause significant morbidity and mortality, in particular in highly susceptible severely immuno-compromised patients, including transplant recipients and patients infected with human immunodeficiency virus.

Isolation of actinomycetes from soil:

Abussaud, (1996) studied the characteristics of *Streptomyces* strains isolated from soils in two landfill areas in north Jordan (Al-Akider and AlKafeer). Thirty–six percent of Al-Akider strains showed, in vitro activity against *Staphylococcus aureus* followed by *Aspergillus niger* (26%),*Bacillus cereus* and *Sacchromyces cerevisiae* (25% each), *Fusarium moniliforme* (21%), *Candida albicans* (15%), *Escherichia coli* (8%) and *Pseudomonas aeruginosa* (4%), while 51,49,46,34,25,15,7, and 2% of the Al Kafeer strains exhibited activity against *A. niger, S. cerevisiae, B.cereus, S. aureus, F.moniliforme, C.albicans, P.aeruginosa* and *E. coli*, respectively.

Species identification and antagonistic properties of 55 actinomycetes strains isolated from Syrian soil were studied by ***Wieczorek et al., (1997)***.They showed that, actinomycetes belong to 16 species exhibited antagonism in relation to Gram-positive and Gram-negative test bacteria, fungi and neoplastic cells.

Whilst ***Taddei et al., (1998)*** tested eighty nine actinomycetes strains for their viability, morphological and physiological characteristics after being kept under paraffin oil overlay and distilled water for a period between 10-30 years. Most of the studied strains belong to the "Lorenzo De Montemayor" collection. The study demonstrated that, *Streptomyces violaceusruber* produced its characteristic pigment even after 28 years under these conditions. All of the recovered strains were tested for their biological activity, but only *Streptomyces lavendulae* showed growth inhibition against *Staphylococcus aureus* and *Bacillus subtilis.*

One hundred soil samples from different parts of Zahedan region, south-east of Iran, were collected by ***Pirouz et al., (1999)*** to isolate and identify aerobic actinomycetes which could be considered as a source of actinomycetoma and nocardiosis, or constitute sources for production of new antibiotics.

In a study by ***Saadoun et al., (1999a)***, a total of 90 different Streptomyces isolates were recovered from 36 soil samples and assessed for their antibacterial activity. Nine isolates were identified by the absence of an aerial mycelium. The rest were grouped into six color series, namely grey, white, yellow, green, red and polymorphic colors (pink, orange or violet). The antibiotic activity against a wide range of bacteria was exhibited by 54% of the isolates which were effective against Gram-positive and Gram-negative test bacteria. The lowest activity (8%) was exhibited against *Pseudomonas* species and *Salmonella* Spp. The antibacterial activity of the isolates was divided into four groups according to the diameter of the inhibition zone produced. Groups 3 and 4 with larger inhibition zones indicated their potential as possible sources of novel antibiotics.

Wherein ***Lee and Hwang,(2002)*** summarized study on diversity of actinomycetes and their antifungal activities against some plant pathogenic fungi in various vegetative soils from 14 different sites in the Western part of Korea. A total of 1510 actinomycetes were isolated from the soil samples. *Streptomyces* was predominant in soils with a pH range of 5.1-6.5, 9.1-13% moisture, and 9.1-11% organic matter. Populations of *Streptomyces* were predominant in all the soils, but were highest in grassland and lowest in mountain-forest soils. Antifungal actinomycetes were abundant in orchard soil and lake mud. More than 50% of antifungal isolates from most soils were classified as genus *Streptomyces.*

On the other hand, ***Basilio et al.,(2003)*** evaluated the patterns of the production of antimicrobial compounds by diverse collection of actinomycetes isolated from different geographies under alternative conditions of pH and salinity in the media. Actinomycetes were grouped based on their method of isolation and their phenotype diversity was determined by total fatty acids analysis. A total of 335 representative isolates, including 235 *Streptomyces* species and 100 actinomycetes from other taxa, were screened for the production of antimicrobial activities against a panel of bacteria, filamentous fungi and yeasts, including some of clinical relevance. The results of this study support the idea that species of actinomycetes isolated in alternative selective conditions of pH and salinity present a significant capacity to produce compounds with antibacterial or antifungal activity. The best group of isolates in terms of production of active secondary metabolites was the one isolated in saline conditions.

In another study by ***Adeleye et al., (2004)*** compost samples obtained from different locations within the premises of the university of Lagos were analyzed to determine the presence and

types of antibiotic-producing bacteria, fungi and actinomycetes using nutrient agar, Potato dextrose agar and starch casein nitrate agar respectively as culture media. When these organisms were screened for antibiotics, the following species were found to be antibiotic producers: *B.licheniformis*, *B.subtilis*, *Penicillium chrysogenum Streptomyces reticuli*, *S.hygroscopicus* and *Micromonospora* species. The fungus *Penicillium chrysogenum* had the highest rate of antibiotic production with an inhibitory zone width of 17mm while *Trichoderma viridae* produced toxins lytic to other fungal hyphae.

According to ***Augustine et al., (2004)*** about 312 actinomycetes were isolated from soil samples on chitin agar. All these isolates were purified and screened for their antifungal activity against pathogenic fungi. Out of these, 22% of the isolates exhibited activity against fungi. One promising isolate with strong antifungal activity against pathogenic fungi was selected. This isolate was from Pune, and was active against both yeasts and molds. Various fermentation parameters were optimized. Based on morphological and biochemical parameters, the isolate was identified as *Streptomycetes*. The correlation of antifungal activity with growth indicated, growth dependent production of antimetabolite.

Leiva et al., (2004) designed an experiment to isolate actinomycetes from sediments of Chilean rivers and lakes and to screen them for antimicrobial activity against reference bacterial strains and pathogenic fungi. Actinomycetes were isolated from sediment samples, using casein starch agar. The antimicrobial activity against 3 bacterial species and 7 fungal species was tested using the disc diffusion method. For the extraction of active metabolites, culture broths of antagonistic actinomycetes were

extracted with organic solvents followed by testing the antibiotic activity. A total of 62 strains of actinomycetes were isolated, mainly *Streptomyces* species (83.9%). Thirty six strains (58.1%) showed antimicrobial activity mainly against *Bacillus Subtilis* and *Candida albicans.* They concluded that, lakes and rivers of southern Chile are an important reservoir of antagonistic actinomycetes, a potential source of new drugs.

Screening of antibiotic-producing strains and antimicrobial spectrum of actinomycetes:-

(I) Streptomyces violaceusniger:

In a study by ***Haque et al., (1992)***, about 450 actinomycetes were isolated from nearly 100 soil samples collected from different parts of West Bengal. The isolates were screened on the basis of their inhibitory effect against test organisms. Finally two potent antibiotic producers were chosen having maximum inhibitory effect on both Gram-positive and Gram-negative test organisms.On the basis of morphological, structural, physiological and biochemical characters, the two potent antibiotic producers were identified as *Streptomyces violaceusniger* and *S.antibioticus.*

While ***Ubukata et al., (1995)*** reported that, three novel 36-membered macrolide antibiotics, RS-22A, B and C produced by *Streptomyces violaceusniger* have been isolated. These antibiotics were purified from an acetone extract of the mycelia followed by butanol extraction, centrifugal, partition chromatography and HPLC.RS-22A, B and C showed antimicrobial activity against fungus and Gram-positive bacteria.

Nine different isolates of aquatic actinomycetes identified as Streptomyces species were studied by ***Saadoun et al., (1999b)*** for their morphological and cultural characteristics. One of these isolates (*Streptomyces violaceusniger*) was extensively studied for

its inhibitory effect against a wide range of Gram-positive, Gram-negative test bacteria, *Mycobacterium vaccae* ATCC 29678, *Candida albicans* and several food associated filamentous fungi and yeasts. Most of these were characterized by flexous sporophore morphology and their inability to produce cultural pigments. Bioassay results indicated that, *Streptomyces violaceusniger* of 10 days culture age was highly active against Gram-positive cocci and bacilli with an inhibition zone of 16-25mm, and slightly active against *M.vaccae* ATCC 29678 with an inhibition zone of 5-10 mm. The inhibitory effect was slight against *Escherichia coli*, *Aspergillus niger* and *Candida albicans* with an inhibition zone of 8-10mm for each of them. There was no inhibitory effect of *Streptomyces violaceusniger* against other Gram-negative bacteria, filamentous fungi and yeast. The nature of the active molecule produced by *S. violaceusniger* showed a maximum absorption in the UV region at 210-260nm.

Large numbers of putatively novel Streptomyces were isolated from environmental samples collected from in and around the root system of the tropical angiosperm, Paraserianthes falcataria. Representative isolates were assigned to 37 multi-membered and 107 single membered color groups based on their ability to form pigments on oatmeal and peptone yeast extract iron agars. The largest taxon, color group 3, encompassed 94 isolates which had morphological properties typical of members of the *Streptomyces violaceusniger* clade. Twelve representatives of this taxon chosen on the basis of Curie-point pyrolysis mass spectrometric data were compared with representatives of the validly described species which constitute the *Streptomyces violaceusniger* clade, ***Sembiring et al., (2000).***

Hayakawa et al., (2004) carried an experiment to isolate members of the *Streptomyces violaceusniger* phenotypic cluster,