1. Introduction

The study of microorganisms in the technological and scientific fields have been used from ancient times to improve living conditions and to increase survival opportunities by transforming risks into challenges.

The scientific phenomenon involved in microbial processes started to be understood and well known. Around 1860 Pasteur discovered the major role of yeast in food and beverage industries and became the “Father of Modern Microbiology. The idea of using microorganisms as living material, that could be used as elementary chemical reactors, became widely accepted. Thus, industrial microbiologist could address the same concepts as engineering chemists (energy and mass balances). The capability to control and make these processes much more profitable has been increased. This capability is derived by recognizing the conditions at which microbial “machines” gives an optimal yield and controlled, by external ones (pH, temperature, pressure, feed flow) that do not affect the machine itself: the microorganisms used, to grow also naturally in the environment. Furthermore, all the processes focus on maintaining and try to avoid variations that might affect product yield. Even today some of the regulations for whisky and wine production are related to the to use of endogamic yeast. Therefore, mutation degradation or contamination of the working microbial species are the risks to be avoided.

During the past few decades, molecular biology and subsequent genetic engineering have contributed to a better understanding of both the ultimate mechanisms involved in industrial microbial processes and the main conditions influencing them. For the first time, it has been possible to modify selectively and efficiently these microbiological machines; their natural abilities have been improved or new ones derived from other microorganisms (either microbes or still plants) have been added to them. As a consequence, these living machines have turned out to be more productive and more resistant against changes, and can thus be used for other new biochemical reactions. Over the last 120 years, new or more efficient industrial processes involving microorganisms have been launched, yielding highly pure, less expensive products or substances that are not available using classical chemical methods.
In general, it is now possible to:

- Convert secondary metabolites into main metabolic pathways
- Optimize productivity and yields
- Alter the original metabolic pathways to allow the use of less expensive raw materials, or to obtain previously unknown molecules
- Use the enantiomeric properties of enzymes to obtain new chiral molecules
- Obtain immunosppressive agents, including cyclosporine A, from *Tolypocladium nivenum*, or mycophenolate mofetil, from several *Penicillium* species (Isaac *et al.*, 1990; Anjum *et al.*, 2012).
- Produce antitumor agents such as Taxol which was first discovered in plants but later transferred and produced by *Taxomyces andreanae* (Staniek *et al.*, 2009) in 2000 it comprised 10 % total sales for Bristol Myer-Squibb, reaching US $1 billion.
- Use pigments including, carotenoid astaxanthin from *Phaffia rhodozyma*, and β-carotene from *Blakeslea trispora* in food and textile industries (Naziri and Tsimidou, 2008; Bhatt *et al.*, 2013).
- Produce polyunsaturated fatty acids such as including gamma-linoleic acid from *Mucor circinelloides* and arachidonic acid from *Mortierella isabellina* (du-Preez *et al.*, 1995; Lina *et al.*, 2011).
- Make recombinant DNA such as human interferon, epidermal growth factor and haemoglobin, antigens for hepatitis-B virus, stabilizers for erythropoietin and human chorionic gonadotropin obtained from different microorganisms like *Saccharomyces cerevisiae, Pichia pastoris, Hansenula polymorpha* or *Agrobacterium tumefaciens* (Chang *et al.*, 1986; Chiruvolu *et al.*, 1997).
- Obtain recombinant enzymes for industrial processes obtained from microorganisms. The industrial enzymes market for non-therapeutic uses, such as food, detergents, textiles, leather, pulp and paper industry has reached US $2 billion in 2000 (Carlsen, 1990).
- Produce antibiotics including biosynthetic penicillin V and natural penicillin G of the 12,000 antibiotics known in 1995, out of which more than 20 % can be produced by filamentous fungi.
This journey is only at its beginning, and still faces many challenges. As for example, the technology suitable to synthesize all these molecules in a less expensive and more efficient way, is needed which uses CO$_2$ as the carbon source, water as the electron acceptor, and sunlight as driving energy. Many scientists have tried to solve these problems for years, unaware that such a process namely, photosynthesis, already exists and has been used by photosynthetic organisms (many microorganisms and all plants) for millions of years. As a final example, the previously mentioned switch from petroleum based fuels to bio-ethanol will mean a new revolution in the energy market, and will change significantly international commercial relationship. Nevertheless, this is only the first step towards the final goal: the hydrogen based battery. Microbiology no doubt will take part in this task as well.

Microorganisms are used in many industrial processes, such as the production of enzymes, vitamins, polysaccharides, polyhydric alcohols, pigments, lipids and glycolipids. Some of these products are produced commercially while others are potentially valuable in biotechnology. Microbial secondary metabolites are extremely important to our health and nutrition and have tremendous economic impact. In addition to the multiple reaction sequences of fermentation, fungi are extremely useful in carrying out biotransformation processes. These are becoming essential to the fine-chemical industry in the production of single-isomer intermediates.

Many microorganisms are useful to human and have been exploited both industrially and commercially. The microorganisms have been utilized for centuries in a wide variety of ways by capitalizing on the metabolism and metabolites (chemicals made from metabolism) produced. The oldest and best example is the use of yeast performing fermentation in a brewing, food processing, biocontrol agents, enzyme biotechnology, as well as research and development.

Large scale production of microbial products is a multibillion dollar industry. Genetic engineering has now made possible the directed construction of microorganisms that will do almost anything, and new products are being announced almost regularly. The core microbial product manufacturing is “Fermentation Technology” which is a process for the production of products by means of mass culture of microorganisms. Pasteur for the first time, reported about the involvement of microorganisms in the fermentation.
1.1 Terpenoids as Natural Product and Secondary Metabolite

The story of pentaecyclic triterpenoid glycosides based drugs provides an interesting imminent into the discovery and development of modern pharmaceuticals as well as looking at the complexity of the technology and science involved. Various saponin secondary metabolites had been discovered during the 1950s and 1970s. However, most of them are with unwanted side effects. In chemistry, a glycoside is a molecule in which a sugar is bound to a non-carbohydrate moiety, usually a small organic molecule. Glycosides play numerous important roles in living organisms. Many plants store chemicals in the form of inactive glycosides. These can be activated by enzyme hydrolysis (Marco, 2007) which causes the sugar part to be broken off, making the chemical available for use. Many such plant glycosides are used as medications. In animals and human, poison is often bind to sugar molecules as part of their elimination from the body. In formal terms, a glycoside is any molecule in which a sugar group is bonded through its anomeric carbon to another group via a glycosidic bond. Glycosides can be linked by an O (an O-glycoside), N- (a glycosylamine), S- (a thioglycoside), or C- (a C-glycoside) glycosidic bond. The given definition is the one used by IUPAC, which recommends the Haworth projection to correctly assign stereochemical configurations (Lindhorst, 2007). In addition many authors suggested that the sugar should be bonded to a non-sugar for the molecule to be classified as a glycoside, thus excluding polysaccharides. The sugar group is then known as the glycone and the non-sugar group as the aglycone or genin part of the glycoside. The glycone may consists of a single sugar group (monosaccharide) or several sugar groups (oligosaccharide). In 1830, the first glycoside ever identified was amygdaline by the French chemists Pierre Robiquet and Antoine Boutron-Charlard.

Secondary metabolites are natural products that often have an ecological role in regulating the interactions between plants and their environment. They can be defensive substances such as phytoalexins and phytoanticipins, anti-feedants, attractants and pheromones (Hanson, 2003). The importance of plant secondary metabolites in medicine, agriculture and industry has led to numerous studies on the synthesis, biosynthesis and biological activity of these substances. It has been estimated that over 40 % of medicines have their origin in these active natural
products (Gershenzon and Kreis, 1999). A prominent group of natural products are the terpenes and derivitized terpenoids.

1.2 Chemical Diversity of Terpenoids

Several thousand terpenes and terpenoids occur in many genera of higher plants and organisms (Devon and Scott, 1972; Darnley, 1974) and although often the structures of the various classes seem to be unrelated, but detailed biochemical studies have revealed their biosynthesis patterns (Bell and Charlwood, 1980). The basic skeleton of terpenes are derived from isoprene (2-methylbutadiene) units (Ruzika, 1953; Gershenzon and Kreis, 1999). The isoprenes units (C5H8) polymerizes and subsequently fix the number and position of the double bonds. The basic molecular formula of terpene is thus (C5H8)n. Most terpenes have cyclic structures and are classified by the number of C5 isoprene units that they contain. The classes are: hemiterpenes consisting of a single C5 isoprene unit, monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), sesterterpenes (C25), triterpenes (C30), carotenoids (C40) and polyterpenes consisting of long chains of many isoprene units. The triterpene group of compounds includes sterols and triterpenes, which can accumulate as glycosides (saponins) in extensive amounts in plants (Sparg et al., 2004). Saponins are glycosylated (aglycone = sapogenin) secondary metabolites found in a variety of plant species (Papadopoulou et al., 1999). Their surface active properties are what distinguished these compounds from other glycosides (Sparg et al., 2004). Due to the fact that some of these saponins are the starting points for the semi-synthesis of steroidal drugs, these metabolites are highly sought after by the pharmaceutical industry (Liu et al., 2002). Saponins are classified according to their aglycone skeleton. The first group consists of non-steroidal saponins, which are the most common and occur mainly in the dicotyledonous angiosperms. The second group consists of the steroidal saponins which are derived from the tetracyclic triterpenoids and isoprene units and are almost exclusively present in monocotyledonous angiosperms. Some claim a third class called steroidal amines, which are also referred to as steroidal alkaloids (Bruneton, 1995).
1.3 Biotransformation of Triterpenes

Microbial transformation of xenobiotics (triterpenoids) is a very useful approach to expand the chemical diversity of natural products (Cheng et al., 2004). Advantages often associated with biocatalysis include region selective, stereo selective and environmental friendly reactions. Moreover, microbial transformation is often the only rational way to convert a precursor molecule to a desired compounds. In the past decades, several studies have demonstrated that microbial transformation is a versatile tool to enlarge the structural diversity of triterpenoids (Qian et al., 2009). A great challenge for the realization of a desired biotransformation reaction is finding the appropriate microorganism. Thus, classical screening of a series of microbial strains is still the most widely used technique. Very currently, a comprehensive review on the microbial transformation of triterpenoids was published (Parra et al., 2009) which focuses on the selection of microbial strains frequently cited in the respective literature and the corresponding biotransformation reactions exemplified with tetra and pentacyclic triterpenoids.

1.4 Microorganism Used for the Production of GA

Among all the important phytoconstituents of Glycyrrhiza glabra i.e. liquiritic acid, glabranins A and B, glycyrrhetol, glabrolide, formononetin, liquiritin, isoliquiritin and other phenolic compounds (Ali et al., 2009), the pharmacological activity is due to its triterpene aglycone 18β-glycyrrhetinic acid (GA) and in lesser measures, to its glycoside glycyrrhizin (GL) (Hansen et al., 1999). After oral absorption, GL is hydrolyzed to GA and glucuronic acid within the gastrointestinal tract by enzymes/microorganism through an unknown metabolic pathway. GA is absorbed into the systemic circulation and produces pharmacological action. Further, it is metabolized to 3β-monoglucuronyl-GA (3-MGA) in the liver and excreted out from the body in the urine. Because of lower absorption, GL molecule is not adequately transported into liver while GA is rapidly absorbed and transported via carrier molecules to the liver. Therefore hydrolysis of glycoside molecule GL acid yield a more potent and easily absorbable aglycone fraction, GA (Hattori et al., 1985; Akao et al., 1991). Various fungi and bacteria have been used for the commercial production of GA from GL in the root of G. glabra. Using different strategies for
improving production levels, yields have been increased. β-glucuronidase enzymes are the members of the glycosidase family II that hydrolyze the glycosidic bond between two or more carbohydrate or between a carbohydrate and a non-carbohydrate moiety. The enzyme β-glucuronidase catalyzes hydrolysis of β-D-glucuronic acid residues from the non-reducing end of mucopolysaccharides (Amin et al., 2011). It is known to be present in different groups of organisms including archaebacteria, eubacteria, fungi, invertebrates and vertebrates (Arul et al., 2008). β-glucuronidase from both Escherichia coli and bovine liver cleaved the prodrugs efficiently to release O\textsuperscript{6}-benzylguanine and O\textsuperscript{6}-benzyl-2'-deoxyguanosine, respectively. These prodrugs may be useful for prodrug monotherapy of necrotic tumors that liberate β-glucuronidase or for antibody-directed enzyme prodrug therapy with antibodies that can deliver β-glucuronidase to target tumor cells (Wei et al., 2005).

Figure 1: Bioconversion of GL to GA

Biocatalysis and biotransformation have many advantages, such as high substrate specificity and mild reaction conditions. It has been reported that GL was hydrolyzed directly into glycyrrhetinic acid monoglucuronide (GAMG) by an intracellular β-glucuronidase enzyme from Penicillium species Li-3 with high production (Feng et al., 2006). Different fungal strains are available which have shown tremendous biotransformation capability. Among the several fungal strains, Aspergillus niger, A. neveus, A. fumigates, A. flavus, A. terreus, A. ochraceous, A. versicolor, A. carneus, A. tamari, Penicillium auranticum, P. waksmanii, P. aurantigrism, P. chrysogenum, P. frangnatum, P. islandicum, P. cyclopium, and Fusarium solani have impending
capability to convert GL into GA mediated by β-glucuronidase. Aspergillus niger, A. tamari, A. terreus, Penicillium auranticum, P. cyclopium, P. frangnatum, and P. waksmani were found to be potential fungi for bioconversion (El-Refai et al., 2012). The highest GA percentage conversion (95 %) was obtained with Aspergillus parasiticus Speare BGB (Wang et al., 2010).

Aspergillus niger NRRL 595 showed a maximum total conversion value of 86.78 % with the production medium containing (% w v⁻¹), 1.75 GL, 0.5 glucose, 0.8 corn steep liquor; pH 6.5; with the consumption of about 90 % of the added GL. The medium was inoculated with 15 % (v v⁻¹) inoculum and incubated at 30 °C for 96 hr, produces GA three times higher than 3-oxo-GA (65 % and 22 %, respectively) and the cells bioconversion efficiency increased from 25.19 to 86.78 % (El-Refai et al., 2012). Bioconversion of GL into GA has already been carried out using enzymes derived from human intestinal bacteria (Morana et al., 2002).

By human gastro intestinal bacteria, GL (18-beta-glycyrrhetinic acid-3-O-[beta-D-glucuronopyranosyl-(1-->2) -beta-D-glucuronopyranoside]) was metabolized to GA (3β-hydroxy-11-oxoolean-12-en-30-olic acid). The main pathway of metabolism is by enzymes β-glucuronidases of Bacteroides and Eubacterium species at appropriate gastrointestinal pH between 5-6 (Kim et al., 1999). Moreover, different bacterial strains have been reported for biotransforming GL into GA acid which includes Bacillus sphaericus, B. cereus, B. megaterium, B. coagulans, B. subtilis, Mycobacterium luteus, Micrococcus, Staphylococcus aureus, Escherichia coli, Pumillus species (El-Refai et al., 2012). Certain yeast including Candida tropicalise and Saccharomyces cerevisiae are also reported to biotransform GL into GA (El-Refai et al., 2012).
References


Chapter 1

Introduction


Ph.D Thesis


