

Article

Industrial Catalytic Production Process of Erythromycin

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Abstract: The impact of COVID-19's unexpected outbreak forced the scientific community to seek alternative treatment methods in order to overcome the hindrance of traditional medicine in terms of alleviating the symptoms of this virus. Erythromycin, which was introduced in 1952, is an antibiotic that is reported to pose as an effective substitute medication for various ailments such as skin, respiratory, bone, and female reproductive conditions, and cancer, as well as the newly added COVID-19. The importance of both the erythromycin molecule and the catalyst of its production, namely P450eryF of the cytochrome P450 family, in many health-concerned and environmentally related applications, has led several countries, the World Health Organization (WHO) and the health industry to recruit and cooperate with numerous universities and institutions, in an attempt to tackle the demand for efficient antibiotics. The aim of this study is to discuss and further analyze the overall structure and catalytic mechanism of erythromycin's synthesis and industrial production, in order to gain a better comprehension of this molecule's significance and value in the pharmaceutical field. This was carried out through the citation of the current production rates per country and the latest statistics and published patents. As implied in this manuscript, the demand for an increase and improvement in the production of erythromycin and its antibiotic derivatives should be globally promoted to deliver more effective results against infectious diseases, such as COVID-19.

Keywords: erythromycin production; catalytic mechanism; cytochrome P450; COVID-19



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1. Introduction

Erythromycin is an important antibiotic in the healthcare system, as it has broadly been applied in clinical treatment over the last few years [1–4]. In 1949, a team of scientists led by Aguilar first isolated and identified erythromycin from a Philippine soil sample [3–6]. Erythromycin was produced by *Saccharopolyspora erythraea*, formerly called *Streptomyces erythraeus*, a Gram-positive bacterium, that produces antibiotic compounds and is isolated from soil [3,4,7–9]. It was later discovered that erythromycin could prevent the growth of bacteria and has since been used as an antibiotic agent [3,10,10–13]. Lately, it has been implied that erythromycin's modification improves its acidic stability and optimizes it for local drug delivery [12]. Recently, a transethosome-loaded, cinnamon oil-based emulgel has been identified as an effective alternative way to deliver erythromycin for the treatment of topical bacterial infections, culminating in the recognition of erythromycin's antimicrobial efficiency and potential in the pharmaceutical and medical field [13].

Erythromycin is synthesized by *Saccharopolyspora erythraea* via a multi-step enzymatic process involving the cytochromeP450 enzyme and P450eryF [3,14–17]. P450eryF, in fact, plays a significant role; it is the catalyst in every step of the synthetic reaction of erythromycin. More specifically, eryF is an enzyme that belongs to a group of cytochromes, the P450 enzymes, also known as P450eryF [16,18–21]. The catalytic reaction that occurs proceeds by using eryF to fabricate a group of compounds, and ultimately, via many intermediate reactions, erythromycin A is formed [16]. In addition to P450eryF's role in the

production of 6-DEB, the same enzyme is also vital for achieving the proper formation of the macrolide ring, thus making the molecule active as an antibiotic [17].

In the industrial production of erythromycin, various methods of crystallization are used to ensure a continuous line of production, which are associated with a high yield and an elimination of erythromycin B and C. Via the utilized crystallization technique, erythromycin could be commercially available and simultaneously remain economically profitable according to the global market's needs [18,22,23]. It is widely suggested that the production of erythromycin is of great importance because of its functions in day-to-day life [24–26]. Erythromycin displays significant clinical activity against a variety of bacteria, while it is utilized as a component in bone cement destined for infection-prevention purposes [9,27]. The group of P450 cytochromes, on the contrary, is involved in vital functions of the human body, including homeostasis, also contributing to the oxidation of xenobiotic chemicals in microorganisms, higher animals and plants [28]. Cytochromes' assistance in the metabolism of endogenous substances is indisputable, as such enzymes metabolize hosts of xenobiotics in hepatocytes, while they efficiently remove potentially toxic compounds [9,24–26,29,30].

From 2011 to 2021, the production of erythromycin mostly increased. However, in Europe and North America, their total production decreased [30]. Especially during the COVID-19 outbreak, Asia, South America and Africa exported erythromycin antibiotics and managed to profit. Hence, their erythromycin production skyrocketed while many other countries displayed little to no erythromycin import or export activity [9,10,30–34].

Even though erythromycin has been put aside the five last years, mostly because of the COVID-19 pandemic outbreak and the global need for its efficient medical treatment, recent studies have implied the crucial capability of erythromycin in terms of treating COVID-19 [35–37]. Notably, erythromycin successfully inhibited the SARS-CoV-2-induced cytopathic effect (CPE) in Vero cells dose-dependently, while the inhibitory concentration–50 (IC₅₀) for erythromycin was at the clinically achievable plasma concentration in humans, which suggested a possible role of this drug in COVID-19's management [35]. However, the antiviral effect of erythromycin against human coronavirus remains still not fully clarified, and it must be further studied [38]. The aim of this study is to elucidate the production of erythromycin, as well as to evaluate its vast potential concerning the treatment of globally spreading diseases, including the pandemic of COVID-19.

2. Historical Retrospective and Recent Applications

2.1. Reactions' Discovery

The dawn of the discovery of cytochrome P450 enzymes dates back to the 1940s, during experiments that dealt with a class of reactions involving molecular oxygen (O₂) [1,2]. As it was known that such enzymes further catalyze erythromycin production, it was ascertained that the antibiotic of erythromycin was a significant invention.

Erythromycin was only later declared an alternative drug for penicillin-allergic patients. Embarking on the original work organized by a group of scientists guided by Aguilar (1949) [3], McGuire et al. (1952) named this extraordinary compound “Erythromycin” [4]. On the other hand, the uncovering of cytochrome P450 enzymes is attributed to the work of Sato and Omura (1962), who isolated the erythromycin compound produced by the bacterium *Streptomyces erythreus*, which was in turn able to restrain the growth of bacteria. Then, it was confirmed that erythromycin's production was inhibited by the cytochrome P450 enzymes family. This discovery led to further research into the role of cytochrome P450 enzymes in drug metabolism and many other biochemical processes that involve mainly antibiotics [1,3,8,21].

All the above discoveries were considered milestones in the development of many erythromycin-based remedial products that predominantly aimed to ameliorate various infectious diseases and conditions, and they are currently trending in the medical field.

2.2. Reaction's Importance

The relevance of the erythromycin-producing reaction via the catalytic response of cytochrome P450 eryF has a notable impact on the pharmaceutical field. Cytochrome P450 enzymes form a group of molecules with many different structural variations, which provide an additional defense mechanism for the human body, alongside the immune system. Digging deeper in those structural variations of the erythromycin group, those originating from bacterial strains are eryA, B, C, D, E, F and G. Among all different erythromycin molecules, eryA, otherwise known as ilotycin, is the primary constituent, while eryE and F act as erythromycin metabolites. All the other compounds play the role of mediators, which are formed in the biosynthesis of eryA [10].

Generally, all cytochrome P450 enzymes have the power to metabolize hazardous substances, such as benzo[a]pyrene, constituents used in tobacco smoke, and many endogenous substances, including steroids and fatty acids. P450 enzymes are located in bacteria and in all types of cells, except those forming red blood and skeletal muscle cells—this indicates their great role in a variety of scientific medicinal pathways [11].

This macrolide antibiotic's spectrum is the same and maybe moderately wider than that of penicillin. From respiratory tract infections to antibacterial, antifungal and antimicrobial properties, erythromycin effectively combats various atypical organisms, including *Mycoplasma* and *Legionellosis*. It was first marketed by Eli Lilly & Company, and as of today, it is commonly known as EES (erythromycin ethyl-succinate), an ester-based prodrug, commonly administered in modern treatment [1,3,8,38].

2.3. Uses of Erythromycin—Recent Applications

2.3.1. Erythromycin's Profile and Applications

Erythromycin encloses vital anti-inflammatory properties and the ability to suspend osteoclasts formation, and as a component in bone cement, it is able to halt possible infections [10,27,31]. Furthermore, it functions as a motilin receptor agonist and is used as effective treatment for the acceleration of gastric emptying, or for many lung-targeted diseases, like the chronic obstructive pulmonary disease [10,33]. Infectious diseases caused by pathogens, such as *Staphylococcus aureus*, *Neisseria*, and many more, have also been considered treatable under the prescription of erythromycin and/or its derivatives [10].

Studies have been conducted on the neuroprotective effects of erythromycin, concerning cerebral ischemia, reperfusion injury, and cell viability, following the oxygen–glucose destitution in cultured neuronal cells [32]. Additionally, it is supported as a potent treatment for chest or lung infections (pneumonia, skin conditions such as acne, rosacea, dental abscesses or even sexually transmitted diseases (STDs), like syphilis). Regarding children, erythromycin is often considered as a remedy for ear or chest infections [10,38]. Erythromycin is in fact a potent threat against cancer, as it hinders proliferation and induces the apoptosis of cancer cells with high HERG K⁺ (human ether-a-go-go-related gene potassium) channel expression. A combination of erythromycin with other anticancer agents leads to a more comprehensive anti-cancer approach [39].

As of the latest decades, a plethora of scientific projects have been conducted that involve the exploration of erythromycin's potential. For instance, lately, it was claimed that metoclopramide was inferior to erythromycin concerning its ability to lower the need for a second-look gastroscopy in patients suffering from upper gastrointestinal disease (UGID) [40]. Plus, modified erythromycin has improved local drug delivery [12,13], has been successfully impregnated in materials ranging from bone cement [27] to drug delivery systems like emulgels [13], and has been proclaimed as a potent candidate for the restoration of osteoblast differentiation and osteogenesis [41]. Various respiratory, intestinal and skin infections, STDs, and pelvic inflammatory disease (PID) [5], as well as serious bacterial infections, are nowadays treated via the use of erythromycin and its derivatives [10].

Recent scientific studies have implied erythromycin's hidden potential in fighting COVID-19's current negative impact on human health. HCoV are the main pathogenic viruses that induced the pandemic of COVID-19, with its outbreak causing millions of peo-

ple's suffering or death globally. Therefore, scientists all over the globe have collaborated in order to search for and develop effective anti-HCoV drugs. Since this outbreak, drug repurposing has been the main focus in the process of antiviral agents' development [35,38]. Unfortunately, the declared lab biosafety level 3 (BSL-3) implies that the needed laboratory facilities that specialize in the conduction of high-risk experimental procedures are inadequate and require a lot of improvement, prior to such development.

Ery-Est, otherwise known as erythromycin estolate, is the lauryl sulfate ester of propionyl erythromycin, a macrolide antibiotic, which shows a broad antibacterial activity spectrum. Erythromycin estolate manages to diffuse inside the bacterial cell membrane and reversibly bind to the 50S subunit of the bacterial ribosome. This ability allows Ery-Est to inhibit infections of ZIKV (Zika virus) and other flaviviruses via drug repurposing and implies its hidden potential towards COVID-19's treatment [35,38].

The constrained activity of Ery-Est against the HCoV-OC43 strain was thoroughly investigated. Later, it was discovered that Ery-Est may efficiently hinder HCoV-OC43 infection in BHK-21 (Baby Hamster Kidney Fibroblast Cells), as revealed by a plaque reduction assay, and that the viral concentration in a supernatant of HCoV-OC43-infected RD and HCT-8 cells was crucially reduced using only 2.5 μ M of Ery-Est [38]. This work indicated that Ery-Est was able to accomplish an effective antiviral concentration in many contaminated patients, as it displayed an inhibitory activity against highly pathogenic HCoVs, such as SARS-CoV, MERS-CoV and the newly emerging SARS-CoV-2—a virus highly connected to COVID-19's development and resistance to conventional virus treatment ways [38]. Ery-Est basically directly disrupted HCoV-OC43, probably by causing damage in its viral lipid envelope, and accelerated the release of viral genomic RNA and the generation of the irreversible loss of HCoV-OC43 infectivity [38].

Recently, the identification of erythromycin and other drugs as therapeutic agents against COVID-19's progression has been revealed. Not as a single unit, but via a combination of such drugs, a full or close-to-full effect is now an attainable target of drug therapy [35]. However, even though Ery-Est is a promising candidate for HCoV-OC43 infection, more controlled clinical trials and research are needed so as to further elucidate upon erythromycin's potential towards COVID-19's advancement [35,38]. Many clinicians are already focusing on macrolides as "remedies" for COVID-19 off-label, but without sturdy evidence of the appropriate safety measures or effectiveness. Consequently, although the utilization of macrolides in COVID-19 reception is still questionable, this drug's reported potential highlights an urgent need for well-conducted clinical trials, so as to ensure its safety, treatment validity and effectiveness [35].

2.3.2. Cytochromes' P450 Profile and Applications

Cytochrome P450 enzymes exhibit a variety of functions both in the human body and in the environment. Specifically, it is worthwhile to remark on their ability to remove foreign chemicals from the body and their role in the metabolism of endogenous substances [1,21,42]. The number of active P450 cytochromes in the human body alters the individual's response to drugs due to the different side effects individuals may experience when being exposed to a kind of medication. Statistically, 10% of the population has a deficiency of these particular enzymes, resulting in the accumulation of the drug.

Great outcomes follow the existence of such enzymes in the human body. For instance, it was discovered that cytochrome P4502D6 was responsible for the riddance of debrisoquine, an antihypertensive drug, from the body [1,43]. Moreover, cytochrome P450 reactions participate in the formation of tumors in response to a carcinogen, with either positive or negative effects. The examination of the account of the human cytochrome P4501A2 towards the carcinogenic effects of cigarette smoke and burned foods is followed by the realization that heterocyclic amines are produced by the burning of these substances, while amino acids and carbohydrates are altered. These altered amine-based derivatives' carcinogenic tendencies are not activated as long as P450 enzymes do not interfere [1,43]. P450 enzymes also catalyze the synthesis of two alkaloids, morphine and codeine, which are

produced mainly in the brain and are crucial for our well-being [1,27,43,44]. Additionally, these enzymes and especially P450eryF engage in the synthesis of 20 carbon eicosanoids, which consist of signaling molecules like prostaglandins, thus acting as a remedy against neuroinflammatory diseases. They also take part in the synthesis of poly-eicosatrienes that modify the transport of molecules, Na^+ and K^+ ions, water absorption and the degree of vasoconstriction in the kidneys [1,45,46].

Our specific P450 enzyme group of interest, P450eryF enzymes, is involved in a plethora of reactions. P450eryF is a bacterial P450 enzyme that shows not only excellent solubility but also great aggregation properties and the cooperativity of substrate binding [47]. Regarding the modification of endogenous substances, P450 enzymes and especially P450eryF [48] are also involved in the hydroxylation of long-chain-fatty-acid steroids, bile acids, and vitamins A and D and their metabolites. These enzymes assist in the synthesis of NO, which acts as a neurotransmitter, a mediator of blood pressure, and a toxin that kills invading pathogens. Concurrently, P450eryF possesses a great structural role since it catalyzes the 6S-hydroxylation of 6-DEB in a multistep pathway that leads to the conversion of 6-DEB to erythromycin [16].

Furthermore, P450eryF's enlarged substrate binding pocket enables this enzyme to bind to certain steroid compounds and azole-based steroid hydroxylase inhibitors [16]. More specifically, in the P450eryF/ketoconazole structure, the azole moiety and the nearby rings of ketoconazole were placed in the active site in a way that this binding led to unexpected conformational changes in the I-helix, which in turn induced its flexibility and ability to adopt many conformations [49]. The azole-based P450eryF inhibitor ketoconazole is used to treat fungal infections and functions by blocking ergosterol's biosynthesis in yeast [49]. Recently, regarding the ferric P450eryF structure, it was suggested that a well-ordered active-site water molecule that formed a hydrogen bond with a substrate OH group could serve as a direct proton donor to the iron-linked dioxygen, since its implications for its participation in O_2 binding and cleavage were confirmed [50].

3. Catalytic System Characterization

Generally, macrolides refer to secondary metabolites regarding the genus *Saccharopolyspora erythraea* and are natural products that include a large macrocyclic lactone ring with attached amino-deoxy sugars. Erythromycin (ery) is a macrolide compound consisted of rings containing 12, 14, or 16 atoms [9], with a broad clinical activity and crucial antimicrobial role in treating a huge number of respiratory, skin, intestine, bone and other infections originating from different bacteria. This antibiotic's popularity arose after its discovery in 1952 and stems from its effective therapeutic properties against dangerous pathogens that are resistant to many other drugs known and used at the time [10].

The biosynthesis of erythromycin involves a complex catalytic system, primarily facilitated by a large enzyme complex widely known as erythromycin polyketide synthetase (PKS), which plays a significant role in the assembly of the erythromycin molecule [51,52]. Erythromycin PKS's key components are modules and enzyme domains, as well as tailoring enzymes including glycotransferases, hydroxylases, methyltransferases and oxidases, which are able to further modify erythromycin's structure and thus enhance its pharmacological properties [51–54].

In order to obtain a more detailed image of erythromycin's structure and catalytic system, many characterization techniques have been employed, including many genetic and molecular biology studies that involve cloning and sequencing the genes encoding erythromycin, biochemical assays, X-ray crystallography and NMR spectroscopy. The characterization of erythromycin's catalytic system is indeed a multifaceted process that entails genetic, biochemical and structural techniques, which are crucial in order to fully elucidate upon erythromycin's biosynthesis [51–54].

3.1. Proenzyme

The genome that produces erythromycin is circular. *Saccharopolyspora erythraea* “NRRL 2338”, with its white and less-pigmented form, is excessively used, because it results in the production of more erythromycin than its red counterpart [14,22]. The bacteria of the genus *Saccharopolyspora* are members of *Pseudocardiaceae* and in general assist in the production of erythromycin and other polyketide macrolide antibiotics [14,22]. The specific strain used in the production of erythromycin is called “*Saccharopolyspora erythraea* HOE107” [15]. The first macrolide to be characterized was erythromycin A, which inhibits the protein synthesis inside bacteria cells, thus showing bacteriostatic activity [14,34]. The enzymic precursor of the catalytic production of erythromycin is in fact encoded by the bacteria *Saccharopolyspora erythraea*, namely eryF. In addition, the enzyme P450eryF has experienced neither any modifications nor maturation, so it is used in the production process of erythromycin as is. Erythromycin’s molecular structure is depicted in Figure 1.

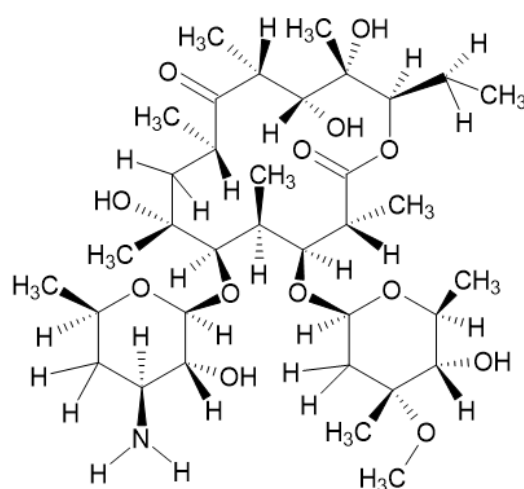


Figure 1. Chemical structure of erythromycin.

3.2. Catalytic System

As is widely accepted, cytochrome P450eryF is a type of cytochrome P450 enzyme, which contributes to the conversion of 6-deoxyerythronolide B (6-DEB) into erythromycin. Explicitly, the conversion of 6-DEB to erythronolide B is catalyzed by P450eryF, which specifically catalyzes the 6S-hydroxylation of 6-deoxyerythronolide B. This catalytic process acts as the initial reaction of the conversion of 6-DEB into erythromycin (Figure 2). Generally, the above-mentioned *Saccharopolyspora erythraea* is the bacterium responsible for the production of the desirable product of erythromycin, erythromycin A, through the utilization of a metabolic pathway in which 6-DEB is converted to erythromycin as a result of hydroxylation reactions in positions 6 and 12 on the macrolide ring [16,17,22].

3.2.1. Structure

Cytochrome P450eryF, as well as the other P450 cytochromes that belong in this enzyme family, has an alpha/beta structure with an alpha-helical domain and a domain with coils and beta-sheets [1,16,43]. Most of the residues, 51% of them specifically, are implicated in an alpha-helical configuration, and only a small percentage (17%) of them are involved in beta-sheets. When a substrate is present, the haem group exists in a high-spin state and is penta-coordinated, with the haem iron bound to the sulphur of Cys351. In general, the haem group is found between the I and L helices (Figure 3A,B) [1,16].

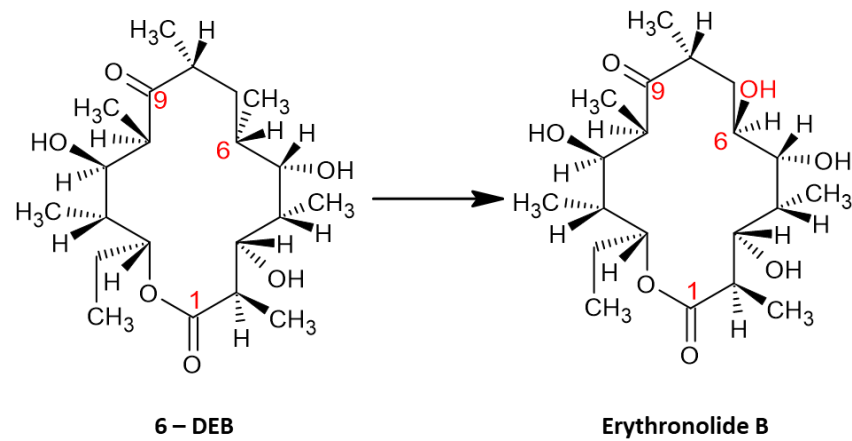


Figure 2. The conversion of 6-DEB to erythronolide B, which is catalyzed by P450eryF. P450eryF catalyzes the 6S-hydroxylation of 6-deoxyerythronolide B, which finally leads to the production of erythromycin. The transformation–hydroxylation that took place in the 6S is marked in red.

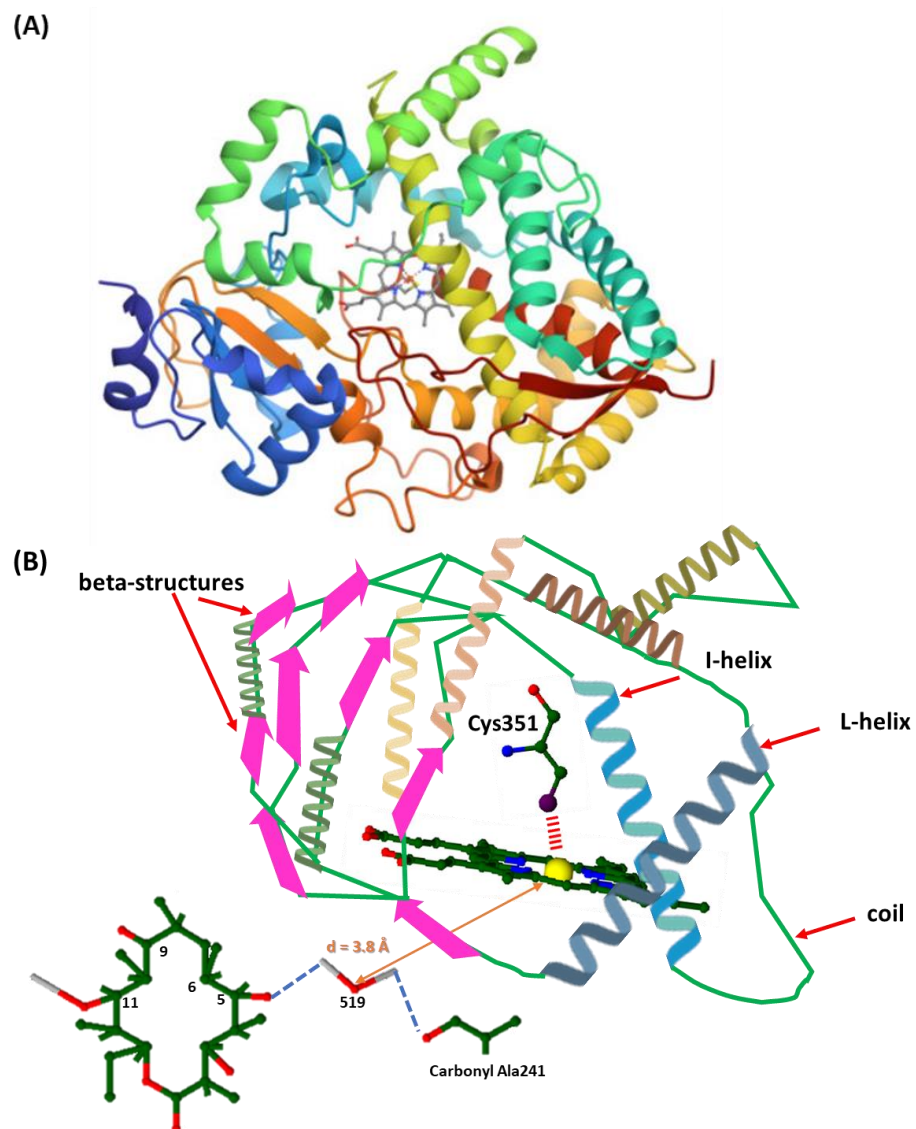


Figure 3. (A): Cytochrome P450eryF general 3D structure (downloaded by the RCSB PDB (Protein Data Bank)). (B): The alpha/beta structure of cytochrome P450eryF in detail. In figure (A), alpha-helices are represented by ribbons of different colors on the right side, while beta-structures with coils

on the left are depicted with orange arrows. In figure (B), the alpha/beta structure involves alpha-helical domains represented by ribbons of different colors, while all coils are represented by green lines and beta-structures (beta-sheets) are depicted as purple–fuchsia broad arrows. The color coding consists of red as oxygen, blue as nitrogen, grey as hydrogen, green as carbon (as well as carbon/carbon bonds), sulfur as deep purple and yellow as iron (II). The cyan dashed lines correspond to all hydrogen bonds formed between amino acids, water molecules, etc., and the red dashed line corresponds to the electrostatic repulsion formed between the iron of the haem group and the sulfur of Cys351. The water molecule Wat519 is close to the iron of the haem group by 3.8 Å. The haem prosthetic group is in a high-spin state, as well as penta-coordinate, with the haem iron bound to the tethered to the sulfur of Cys351 and embedded between the I and L helices.

3.2.2. Substrate-Binding Site

The substrate of P450eryF 6-DEB is placed over the A and D pyrrole rings, while the macrolide plane is oriented around the haem plane. The upper part of the F helix and the haem group form altogether the “floor” of the substrate. Concurrently, the binding pocket and its sides are formed by the synergistic work of the B helix, the I helix and the beta-structure with residues of 290–296 [17,55,56].

In the substrate-binding pocket of P450eryF, several ordered water molecules exist, which form a hydrogen-bonding network and interact with the peptide backbone, amino acid side chains, the substrate and the haem prosthetic group. As a result, three ordered water molecules create hydrogen-bonding bridges between the protein and the substrate. More specifically, the C15 and C16 of 6-DEB contact the side chains of the Ile174 and Leu175 of the F helix. With the aim of placing the previous residues near the substrate-binding pocket, the F helix is repositioned [55,56].

Different hydrophobic interactions occur between Ala 74 and C14, Thr 92 and C20, Val237 and C19, Ala241 and C19 and lastly Leu391 and C21. The substrate-binding pocket consists of more hydrophobic residues that are greater than 4 Å in size and therefore aid in isolating and binding the substrate. Moreover, the keto group C9 of 6-DEB and hydroxyl groups of C5 and C11 are within the radius of solvent molecules, making hydrogen bond formation possible with the peptide backbone and amino-acid chains of Tyr75 and Asn89 (Figure 4A,B) [17,55,56].

3.2.3. Helix I

The keto group C9 of 6-DEB and hydroxyl groups of C5 and C11 are within the radius of solvent molecules, making hydrogen bond formation possible with the peptide backbone and amino-acid chains of Tyr75 and Asn89. P450 cytochromes bear a unique structure of an extended I helix, whose residues around the haem group play a crucial role in this catalytic system [16,17]. This helix displays a different hydrogen-bonding sequence alongside a gap between the aforementioned region and the iron protoporphyrin IX [16,17,55]. Under normal circumstances, the hydrogen bond system of the alpha-helix would consist of bonds formed between the carbonyl oxygen of Ala241 and Gly242 and the amide nitrogen of Ala245 and Ser246. Poulos et al. (1995) [16] suggest that Ser246 forms hydrogen bonds through the amine group with Wat564, which is also bonded similarly with the -NH of the peptide group of Ser246. Likewise, hydrogen bonds are formed between Glu360 and Wat515. Therefore, this hydrogen bond system stabilizes the I helix’s local distortion as it substitutes the missing alpha-helical hydrogen bonds. In actuality, the water molecule Wat519 is close to the iron of the haem group by 3.8 Å, and it is also suggested that it may be the proton donor of the O₂ cleavage reaction—hence, its position is vital for the haem’s functionality (Figure 5) [16,55].

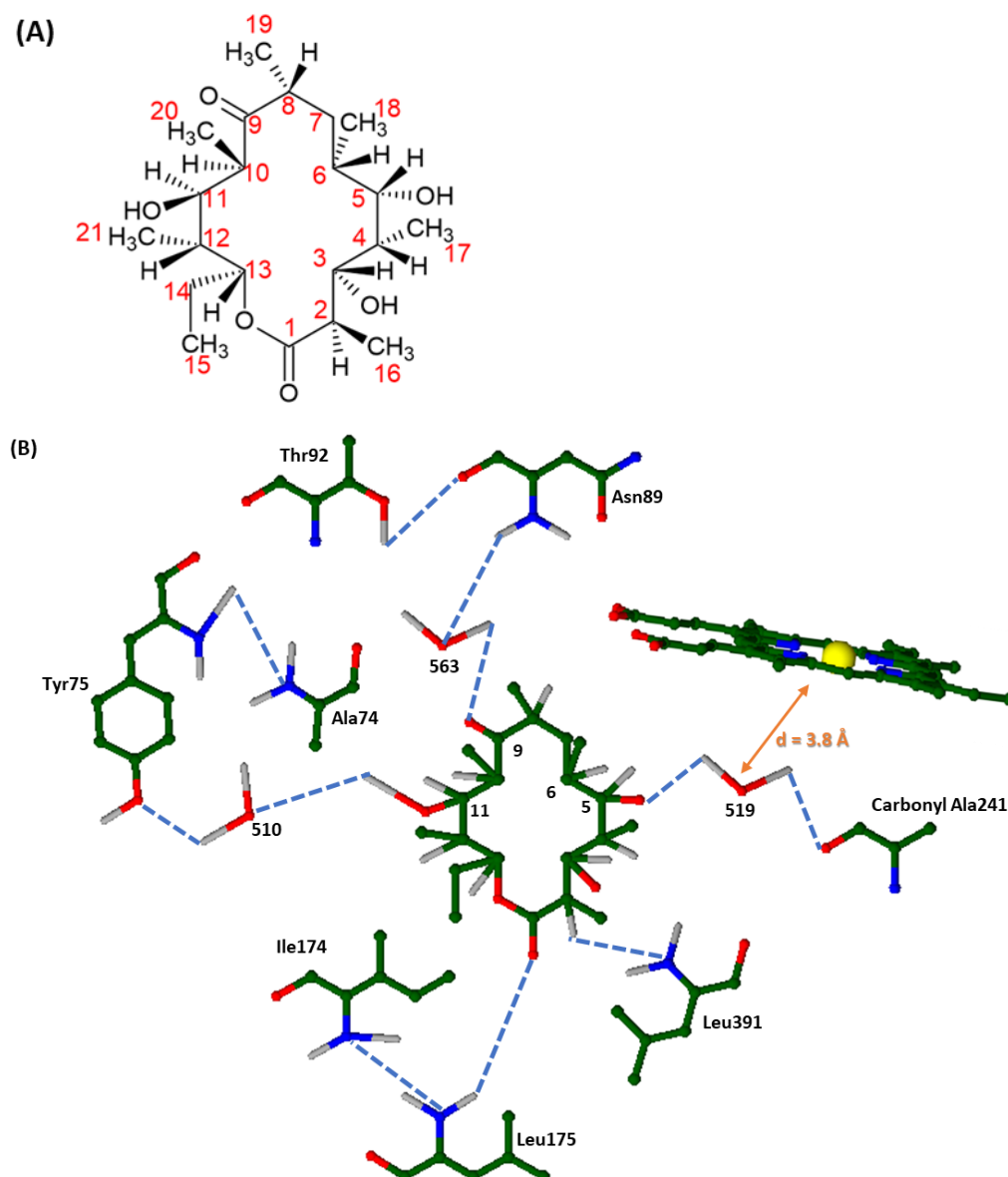


Figure 4. (A): The substrate of P450eryF, 6-deoxyerythronolide and the numbering system used. (B): 6-DEB in the active site of P450eryF. The color coding consists of red as oxygen, blue as nitrogen, grey as hydrogen, green as carbon (as well as carbon/carbon bonds) and yellow as iron (II). The cyan dashed lines correspond to all hydrogen bonds formed between amino acids, water molecules, etc. Not connected to the actual structure but vital for P450eryF's functionality is the haem structure. All water molecules that participate in the reaction are named 510, 519 and 563. In actuality, the water molecule Wat519 is close to the iron of the haem group by $3.8 \text{ \AA}$, and it is also suggested that it may be the proton donor of the O_2 cleavage reaction (orange arrow shows this distance).

3.2.4. Active Site

The substrate 6-DEB connects with the active site in the P450eryF with the aid of seven hydrophobic residues and three water molecules that provide hydrogen-bonding bridges between the protein and the substrate. To estimate the significance of the hydrophobic and hydrophilic interactions to the substrate-binding's specificity and hydroxylation site's stereospecificity, it is essential to examine the binding and hydroxylation of altered substrates in this condition [5,16,17,22].

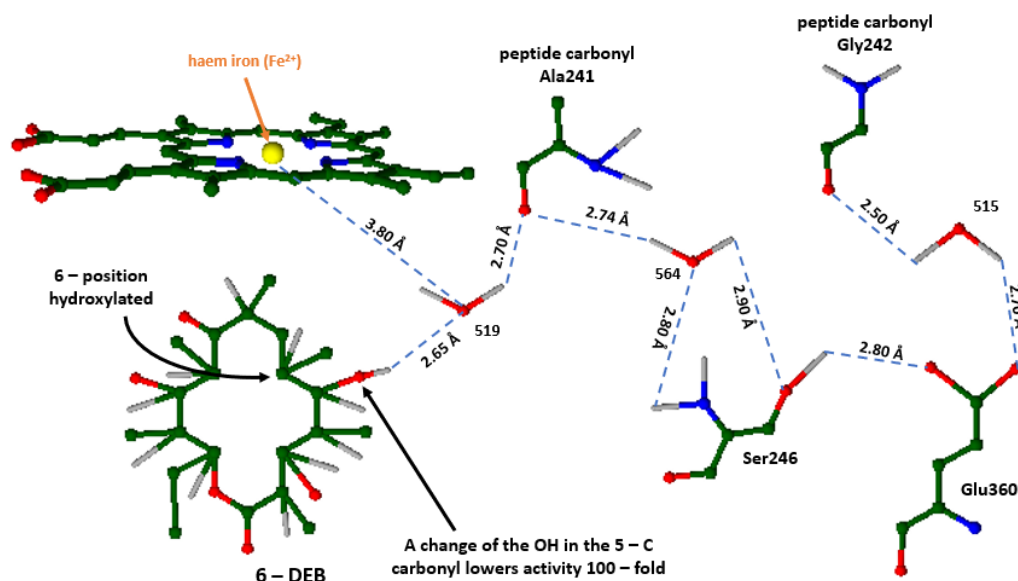


Figure 5. Schematic representation of the hydrogen bonded system—the deformation of the I-helix in the P450eryF enzyme. The color coding consists of red as oxygen, blue as nitrogen, grey as hydrogen, green as carbon (as well as carbon/carbon bonds) and yellow as iron (II) (depicted via an orange arrow). The cyan dashed lines correspond to all hydrogen bonds formed between amino acids, water molecules, etc. The bond's length is measured in Angstroms. All water molecules that participate in the reaction are named 515, 519, and 564.

Andersen et al. (1993) [16] revealed that the nature of the C14 side chain and the differences of the oxidation state of C5 and C9 may affect the P450eryF enzyme. While the crystal structure of both the C9 keto and the C5 hydroxyl groups was examined, it was found that they existed within the hydrogen-bonding distance of water molecules. Normally, altering the oxidation state of the previously mentioned groups should not be able to modify the hydrogen bonds [16,17], due to the fact that water molecules act both as an H-bond donor and an acceptor. However, it is possible that the alternation of the binding affinity observed after the change of the oxidation state is likely due to the transformation of the hybrid state of a carbon in the macrolide ring from sp^3 to sp^2 hybridization, or vice versa, that leads to the alternation of the ring's geometry. Furthermore, because of this change, the hydrogen bonding of 6-DEB with the protein through the water molecules may change as a result of the reposition of the oxygen substituents of C5 and C9.

Adding to their previous work, Andersen et al. (1993) [16] examined an extra derivative in which a hydrogen took the place of the methyl substituent in C14. As a result, the binding affinity was decreased by 50-fold. The fact that the Ile174 is positioned in such a way—so that it could interact with the C14 and the shortage in hydrophobic interactions—justifies the binding affinity decrease. Lastly, 6-DEB becomes more symmetrical by losing the C14 methyl group and might reorientate the active site [16,17,55].

3.3. Catalytic System Mechanism

The substrate 6-DEB is catalytically synthesized by one equivalent of propionyl CoA (coenzyme A) and six equivalents of methylmalonyl CoA, with the assistance of eryAI, eryAII and eryAIII. Propanol and glucose consumption leads to the accumulation of methylmalonyl-CoA and propionyl-CoA, whereas propanol conversion into propionate and transformation into propionyl-CoA are catalyzed by propionate kinase; otherwise, propionyl-CoA synthase is used. In addition, glucose is converted to succinyl-CoA, which also participates into the TCA (Tri-Carboxylic Acid) metabolic cycle [18–20]. The catalytic mechanism of erythromycin A consists of several modifying enzymes, such as eryBV (eryB5), eryCIII (eryC3), eryK, and eryG [18]. Lee et al. (2004) [18,19] reported that the gene cluster of *S. erythraea* consists of 20 genes translated into the aforementioned enzymes—a

state that allowed for the catalytic biosynthesis of the polyketide ring and mycarose that was attached to the macrolide ring alongside desosamine [19].

However, it should be noted that succinyl-CoA is utilized in both the synthesis of methylmalonyl-CoA and the TCA metabolic flux, which mostly indirectly decreases the overall yield of erythromycin. The enzyme eryF firstly modifies the substrate 6-DEB by adding a hydroxyl group to C6 [21]; hence, erythronolide B is produced (Figure 2). Following erythronolide B's production, the addition of mycarose to the C3 hydroxyl of erythronolide B is catalyzed by eryB5. Alpha-mycarosyl-erythronolide B is then modified by the eryC3 enzyme, which glycosylates the C5 hydroxyl, leading to the formation of erythromycin D [22]. In other words, eryB5 and eryC3 transfer two deoxysugar units on C5 and C3 of 6-deoxyerythronolide B [23]. EryK and eryG both catalyze erythromycin D into erythromycin B and erythromycin C. EryK catalyzes the hydroxylation of C12 of erythromycin D, in which erythromycin C is produced, whereas eryG catalyzes the methylation of erythromycin D; thus, erythromycin B is synthesized [18,24]. Erythromycin A is generated by erythromycin C's methylation and erythromycin B's C12 hydroxylation. It is also reported [23] that eryG acts competitively on the erythromycin D substrate, as it cannot be catalyzed into erythromycin A effectively; thus, it accumulates into the final product of erythromycin. Erythromycin B is not as toxic as the C counterpart; however, both substrates show low antibacterial activity and are less-effective antibiotics, unlike erythromycin A, which, on the contrary, is vastly effective (Figure 6) [25].

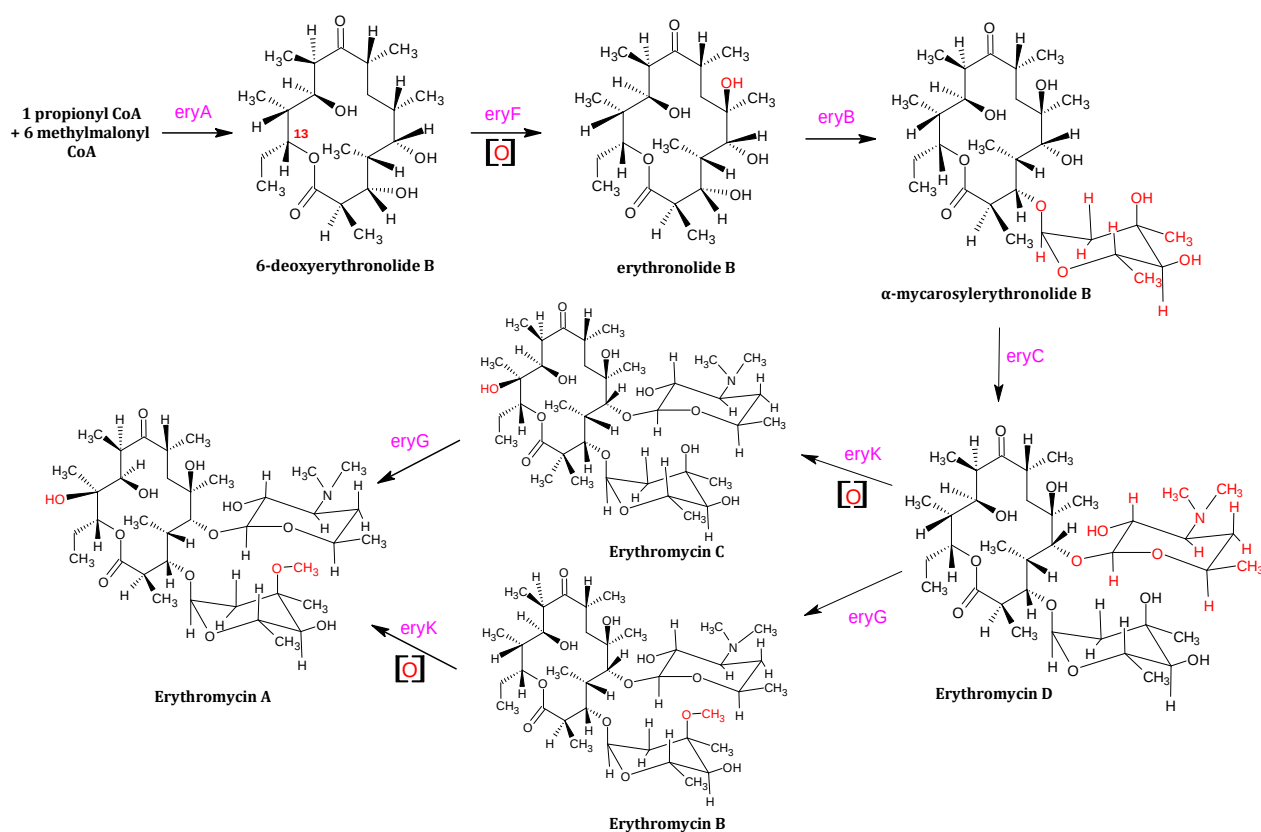


Figure 6. Catalytic process of erythromycin A via two different means (the oxidation reaction is displayed using [O]). The catalysts of all reactions are depicted in pink, and all transformations are marked in red.

During the industrial production process, the broth emerging from the bioreactor is impure due to the presence of bacterial cells and other waste, like the by-products consisting mainly of erythromycin B and erythromycin C that derive from the aforementioned catalytic mechanism. Erythromycin C is highly toxic [25]; thus, the isolation process of erythromycin A must be arranged so that the purity of the final product does not exceed specific limits,

according to Pharmacopeia specifications of each continent, but also to limit the potential damage of the product caused by the high temperatures during all crystallization stages. There are three different purification pathways that erythromycin A can be obtained from: antisolvent, evaporative and reactive crystallization [26], following a pre-treatment plan consisted of isolation processes that include product extraction by filtration from resins, plate and membrane filters. The industrial production process is further analyzed in Section 3.4.

3.4. Industrial Production of Erythromycin

The fermentation broth of the industrial strain of *Saccharopolyspora Erythraea* HOE107 is added adjacent to glucose, which is considered a standard carbon source for the industrialized synthesis of erythromycin (Figure 7) [57]. The aforementioned method produces erythromycin A but also by-products of erythromycin B and erythromycin C. Nevertheless, it is important to mention that other bacterial colonies have been introduced to genetic tailoring in order to produce erythromycin in a higher yield or purity. *E. coli* can be used as a host due to its ability to convert D-glucose-1'-phosphate to TDP and thus introducing an alternative heterologous pathway, by altering its genome accordingly through plasmid insertion [58]. *Saccharopolyspora Erythraea*'s genome contains many regulatory factors that provide surviving mechanisms to inhibit the expression of several genes so as to conserve energy and secure its survival in a hostile environment. Many of these genes are attributed to the lesser production of erythromycin, even in nutrient-rich environments, and thus genetic tailoring takes place in some industrial colonies to deactivate these inhibiting factors [59]. Other carbon sources were also investigated, such as galactose, arabinose, fucose, starches and molasses, with the latter showing promise, as an increase in the erythromycin concentration in the broth and a decrease in the possible economic costs by utilizing cheaper carbon-source alternatives were observed. Other factors that contribute to erythromycin's production and cell growth, except the pH and temperature conditions inside the bioreactor, are the concentration of nitrogen and its supplementation form, n-propanol, and the ratio of carbon and nitrogen sources [60].

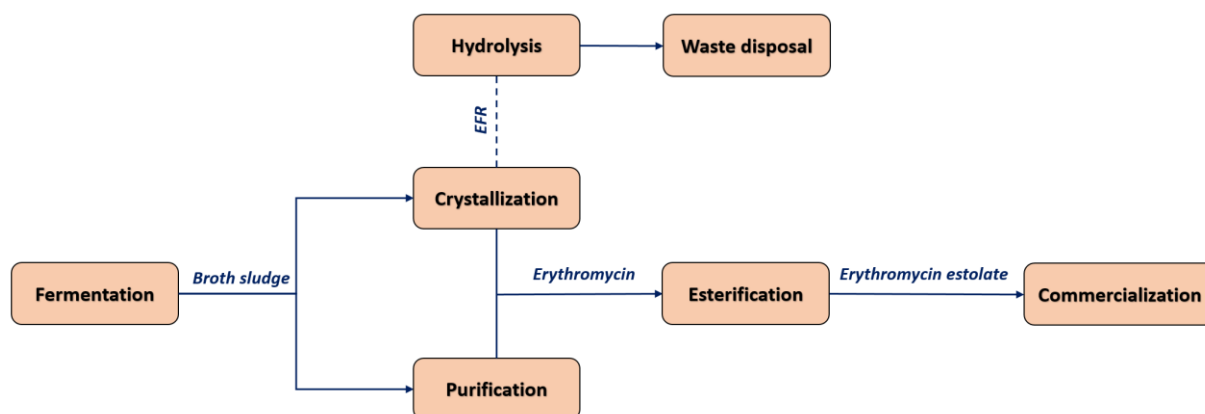


Figure 7. Flow chart of erythromycin production.

There are a variety of purification and isolation pathways from which EryA can be obtained, with Chen et al. suggesting antisolvent crystallization, evaporative crystallization and reactive crystallization methods [26]. The antisolvent method consists of several pre-treatment steps of the broth so that the erythromycin batch would be separated from any solid impurities by plate filters. Butyl acetate is added to the mixture once the purified broth enters the extraction chamber. Once the extraction of EryA during the broth filtration is completed, the pre-product is mixed with lactic acid or HSCN and is moved to the precipitation tank. The precipitate is moved to a double-wall crystallizer in which the antisolvent (water) is added to the alkaline solution, so crystallization takes place since erythromycin is less soluble in water than in acetone [33]. The target compound enters the

mixing section, where it interacts with the antisolvent in a microchannel by diffusive mixing in a laminar flow, to ensure decent mixing between the solvent and the final product [61]. Finally, the erythromycin thiocyanate salt is conserved in an alkaline environment and obtained in an erythromycin-free base form. The evaporative and reactive crystallization processes provide erythromycin and erythromycin thiocyanate, respectively. The broth is initially isolated by solid impurities such as mycelium, proteins, and other particles with the method of microfiltration. Resin filtration is necessary for the removal of un-ionized organic particles and pigment. Butyl acetate is also used in this method during the elution phase of the filtered broth to isolate erythromycin from the rest of the broth's impurities [26]. EryC shows high toxicity; thus, the isolation process of EryA must be arranged so that the purity of the final product can be maintained within acceptable limits. A separation process of EryA from the broth and EryC was outlined by Jin et al.: obtaining the absorbent from SP825 macroporous resin and preparing it accordingly so as to experiment on the absorption selectivity of EryA and EryC to various aqueous solutions [25].

The erythromycin fermentation residue (EFR) from the bioreactor is also an important part of the production waste, which is later hydrolyzed in order to prevent environmental damage from occurring during waste disposal, since it shows high chemical oxygen demand (COD) values. Erythromycin can be hydrolyzed under 85 °C in one hour, after the residues have been diluted and pre-treated in anaerobic bioreactors. This process also reduces the risk of bacteria developing resistance to antibiotics found in wastes [62,63]. Erythromycin, however, is introduced to the general public in its derivatives such as a base in tablets or capsules, salts, or esters in an oral suspension, since these forms offer an increased absorbability of the drug by the human body. This form of erythromycin estolate has been found to be the most easily absorbed by its counterparts and can be synthesized by the esterification of erythromycin. This drug is synthesized in the esterification of the hydroxyl group of amino sugar of the erythromycin molecule and the carboxyl group of propionic acid [64]. Moreover, its bitterness is reduced, while it is easily absorbed orally and is released as free erythromycin in the bloodstream. Nevertheless, this form shows a number of serious side effects such as the development of cholestatic hepatitis, especially in pregnant patients [65].

4. Industrial Applications

Production Per Country

The production of the antibiotic erythromycin and its derivatives (salts thereof) world-wide keeps expanding. More and more countries are partaking in the production of erythromycin. However, as expected, some countries' production "blossomed", while other countries' rates dropped. With a view to providing a more rounded overview, the comparison will include the years between 2011 and 2021—a decade of fluctuations [30]. The website "OEC" (Observatory of Economic Complexity) [30] provides the annual exports and imports of erythromycin by country. Over the 2011–2021 decade, any fluctuations in the countries' import and export percentages will be thoroughly examined.

In Figure 8, a comparison for the years 2011 and 2021 is displayed. During the ten-year span between 2011 and 2021, Asia, South America and Africa exhibited an increase in the imports of erythromycin, while all the other continents experienced a decrease. As for the exports of erythromycin in the same time interval, Europe and North America experienced a decrease in the export rates, while the rest of the continents' export percentages increased. Lastly, South America remained constant at 0%.

As illustrated in Figure 8, in 2021, the top exporters of erythromycin were China (USD 185 M), India (USD 77.9 M), United States USD 63.1 M), South Korea (USD 8.86 M), and Spain (USD 7.23 M). Meanwhile in 2021, the top importers of erythromycin were India (USD 104 M), Japan (USD 32.3 M), Croatia (USD 31.8 M), Pakistan (USD 11.5 M), and France (USD 10.7 M) [30].

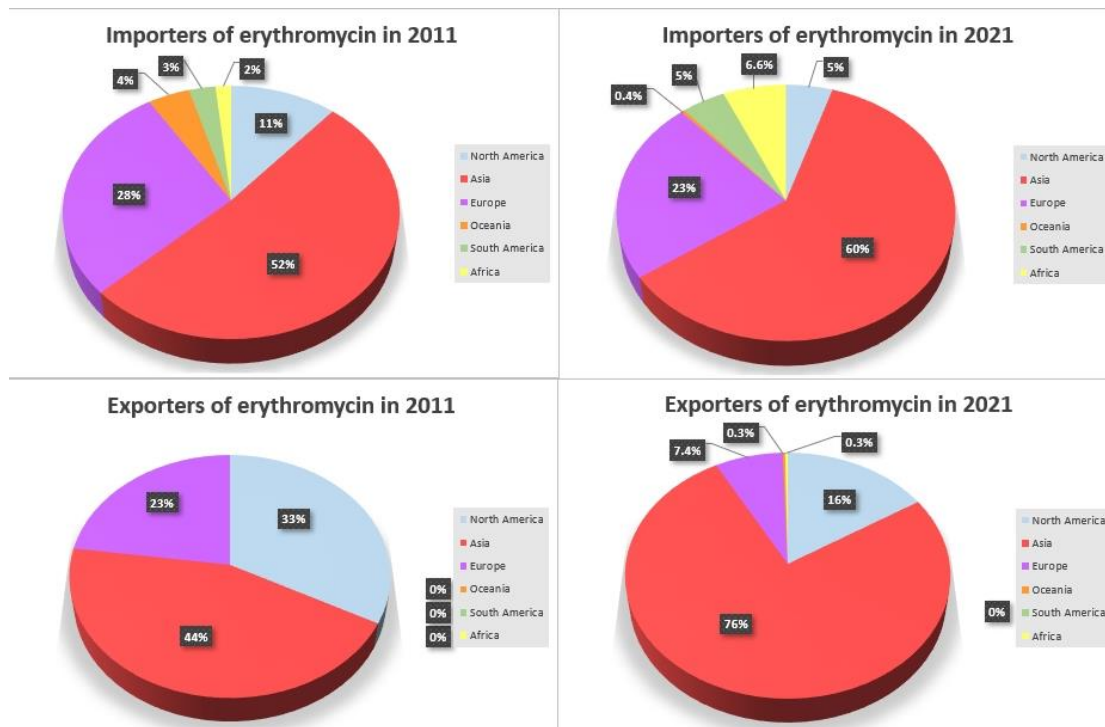


Figure 8. Importers and exporters of erythromycin throughout the years 2011–2021.

5. Statistics

According to the listed patents derived from “Espacenet” [66] by searching the key words “erythromycin”, “production”, “2011–2021”, full access to the 10-year span data was achieved. Statistical analysis that took place for the evaluation of all obtained information allowed for the comparison [66] of erythromycin production patents’ data between 2011 and 2021, which were categorized according to countries, organizations, applicants and inventors of erythromycin patents. A comparison of the publication and earliest priority patent dates also took place.

Figure 9 illustrates 17 countries that published patents about erythromycin in the year span 2011–2021. The total amount of patents filled out by countries and organizations in this 10-year period was 60,046 worldwide, of which 49,744 (82.84%) were published by individual countries and 10,302 (17.16%) were published by well-known global organizations. Out of all countries, the United States of America placed first in erythromycin patent publication, publishing 36.88% of the 49,744 overall patents issued by countries. China (23.40%) and Japan (11.30%) followed in second and third place, respectively, (Figure 9), while Australia, South Korea, Canada, Spain and Singapore came in this order, with their corresponding patent publication percentages being a little above 1% of the total publications. All the other countries participated in less than 1% of the total publications. Out of the total 10,302 patents published by universal organizations, the World Intellectual Property Organization (WO) filled out 61.68%, whilst the European Patent Office (EP) and the Eurasian Patent Organization (EA) published 36.72% and 1.60% of the total patents, respectively, as the statistical analysis results revealed [66].

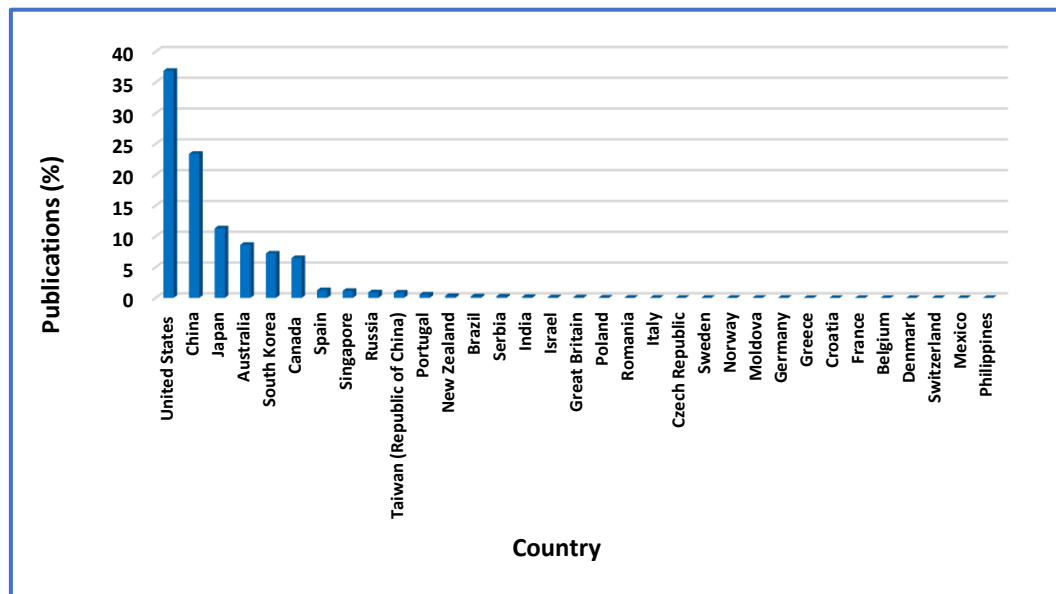


Figure 9. Publication of erythromycin patents per country.

According to the patent publications, every year between 2011 and 2021, in the total 60,046 documents, there were significant alterations in the number of published patents. The greatest amounts of erythromycin patents published were recorded in 2017 (10.10%) and 2018 (10.08%)—numbers that later dropped due to the shifting of global focus upon COVID-19's outbreak and the urgent need for its treatment (Figure 10) [66]. However, as is apparent in the graph listed below, the drop seen from 2015 to 2016, in comparison to that of 2019 to 2020, reveals that all data are comparable. Specifically, numbers may have dropped in both cases but the percentage of listed publications in the year span of 2019–2020 is still greater than that of 2015–2016. Similarly, the percentage of patents filled during the peak of the pandemic (2020–2021) is still higher than that of patents filled during 2011–2013 and nearly all of those filled in 2014.

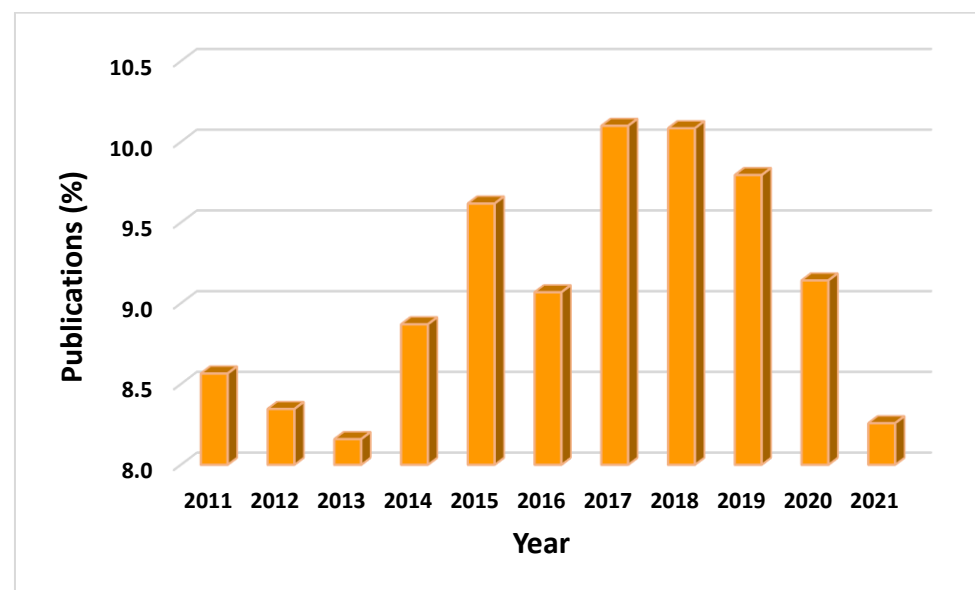


Figure 10. Publication of erythromycin patents per year during the years 2011–2021.

Statistically in the year span of 2011–2021, patents were published in many different languages, with the vast majority of them being published in the English language (68.22%),

followed by the Chinese (Mandarin) and the Japanese language (13.91% and 6.70%). It must be highlighted that the onset of erythromycin patent's publication was reported back in 1983, with only one document listed. Most patents were filled out between the years 2015 and 2018—numbers that later dropped but are expected to rise in the next decade [66]. A report on topical antibiotics market attributes claimed that the market size of antibiotics like erythromycin in 2023 reached USD 6.4 billion, which in the forecast period of 2024–2032 has a value projection of USD 10.4 billion [9,34,67].

Many applicants and of course inventors all over the globe, in 2011–2021, dealt with erythromycin patents and managed to fill out such documents. A huge number of applicants in this year span, including famous pharmaceutical companies, incorporations, universities, institutes and colleges, worked on erythromycin production-related publications. The University of California placed first in this “race” by filling 4.70% of the total patents, followed by Novartis AG, Genentech INC and Hoffmann la Roche. Moreover, we must refer to the applicant's and inventor's origin: reports in the time period of 2011–2021 showed that more than half of them—as expected—derived from the United States of America, while less than 5% were obtained from Germany and Japan, and less than 3% was shared between the rest of the listed countries [66]. Greece also marginally contributed with erythromycin patents in 2011–2021, with four published erythromycin patents. All of these patents were published in the Greek language, while 23 Greek applicants and 65 Greek inventors participated in those publications.

As of 2024, erythromycin thiocyanate is currently trending in the global market as recent insights claim its important role in eliminating cyanobacterial blooms [68]. This derivative is thought to be responsible for the hindrance of *M. aeruginosa*'s photosynthesis and possibly for the low promotion and high inhibition of soluble proteins' synthesis in synergy with enrofloxacin [69].

The development of semi-synthetic macrolides such as roxithromycin, dirithromycin, spiramycin, clarithromycin, josamycin, and especially azithromycin greatly altered the anti-infective drug picture and the medical practice procedures of the 20th century [9,70]. Yet, delving into the 21st century, telithromycin and solithromycin, as well as azithromycin and other macrolide erythromycin-derived antibiotics, reveal favorable activity [9,70], including an excellent antimicrobial potency towards some key erythromycin-resistant pathogens [9,71,72]. At this point, it is important to note that erythromycin, as well as clarithromycin and azithromycin, which are namely the main erythromycin-derived compounds, are substantially different in terms of properties and drug–drug interaction abilities [73]. Destined predominantly for human and veterinary medication, erythromycin is expected to thrive in the global market, since this molecule—either as a single drug or mainly in synergy with other drugs like levofloxacin [74] and retapamulin [75] or nanoparticles (NPs) such as Ag–ZnO NPs [76]—is able to effectively battle multi-drug resistant Gram-positive bacteria [10].

6. Conclusions

The current study demonstrated the importance of the antibiotic erythromycin A and illustrated in detail the catalytic production process. *Saccharopolyspora erythraea*—HOE107 strain—is used industrially to produce erythromycin A, by synthesizing the catalytic system of the metabolic path to the final production. There is certainly room for development, considering the TCA metabolic pathway, in which succinyl-CoA, the precursor to methylmalonyl-CoA, poses a limiting factor to the synthesis of DEB-6. Therefore, newer studies recommend the use of mutant strains, in which the gene cluster responsible for the activation of the TCA cycle is suppressed. A plethora of methods and patents has been proposed for the crystallization process, regarding the solubility of erythromycin and its derivatives to less-harmful, economical, and more environmentally friendly solutes. In this way, erythromycin A can be obtained in both higher yield and purity.

Despite the fact that erythromycin is currently placed in the shadow of its derivatives, namely azithromycin and clarithromycin, this molecule's excellent drug profile and an-

timicrobial, antibiotic and antifungal properties are believed to be the main reasons for its expected vast exploitation in the near future. Either in synergy with many other drugs, or as a drug-loading molecule in emulgels or nanoparticles, erythromycin is expected to play a significant role in drug delivery systems and thus aid in the amelioration of many pestering global diseases and conditions like COVID-19.

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