Searching for Artemisinin Production Improvement in Plants and Microorganisms

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Abstract: The endoperoxide sesquiterpene lactone artemisinin which is isolated from the plant *Artemisia annua*, and its semi-synthetic derivatives, are potent, novel, antimalarial drugs. They are effective against multidrug-resistant *Plasmodium* strains and have become essential components of the so-called Artemisinin-based Combination Therapy, that is recommended by the World Health Organization as the treatment of choice for malaria tropica. Moreover, artemisinin and its derivatives show additional anti-parasite, antitumor, and anti-viral properties. The plants, however, are very poor resources for the drug, as the content of artemisinin is low (from 0,1 to 1,5 % of dried leaves) and dependent on seasonal and somatic variations as well as the infestation of bacteria, fungi and insects. A chemical synthesis of the compound is complex and uneconomic. Therefore, artemisinin is in short supply and remains unaffordable for most people in malaria-endemic countries. Thus, many researchers have focused on enhancing the production of artemisinin, first, through traditional breeding and in *in vitro* plant tissue cultures and, then, by heterologous expression systems (a semi-synthetic approach) with the use of genetically-modified or transgenic microbes. In this review, we summarize the progress made in the production of artemisinin by the biotechnological approach.

Keywords: Artemisinin, ACTs, biosynthesis, metabolic engineering.

1. INTRODUCTION

Malaria is still the most destructive and dangerous parasitic infection in the developing world [1,2]. There are nearly 250 million cases of malaria annually, causing almost a million deaths, mostly of children under 5 years old. About half the world's population (3.3 billion people) lives in areas that have a substantial risk of malaria transmission. It is widespread in tropical and subtropical regions, including parts of the Americas, Asia and Africa. Malaria is a disease that overwhelmingly affects areas of poverty, but an estimated 30,000 cases among international travelers is also recorded annually [3].

This vector-borne infectious disease is caused by protozoan parasites of the genus *Plasmodium* (phylum *Apicomplexa*). Although human malaria transmitted by female *Anopheles* mosquitoes has four *Plasmodium* species as its etiological agents, *P. faciparum, P. vivax, P.ovale and P.malariae,* the most widespread and severe disease is caused by *P. falciparum,* which transiently infects the liver before invading the red blood cells of the mammalian host [4,5].

Currently, there is only a limited number of drugs, which can be used to treat or prevent malaria. Quinine, an aminoquinoline alkaloid that is isolated from the bark of the *Cinchona* species, is one of the oldest and most important antimalarial drugs. Subsequently developed synthetic derivatives, particularly chloroquine, were used the most in the treatment of malaria.

Because of a widespread and unsupervised use of malaria drugs, chloroquine-resistant *P. falciparum* emerged in the early 1960s and rapidly spread around the world. Today there are reported cases of *Plasmodium* parasite resistance to most of the currently available antimalarial treatments [4].

A new class of antimalarials - artemisinin derivatives - was first isolated and developed in China in the 1980s. Artemisinin, which is extracted from the plant *Artemisia annua (Asteraceae),* has been used for centuries in Chinese folk medicine to treat fever. This sesquiterpenoid endoperoxide can be chemically converted into several active derivatives.

Artemisinin and its derivatives such as artesunate, artemether, and dihydroartemisinin are extremely potent antimalarials, that act rapidly against both the parasite's asexual and sexual stages, which could potentially help to reduce the rate of malaria transmission [6].

Besides acting more rapidly and effectively, artemisinin derivatives are less toxic than other antimalarials. The only potentially serious adverse effects are rare hypersensitivity reactions [7]. Moreover, artemisinins exhibit a clinically important activity against other parasites. They kill the younger stages of the trematodes and are effective in the treatment of schistosomiasis and fascioliasis [8,9]. Besides, artemisinin derivatives have anti-inflammatory properties and also inhibit angiogenesis and cell growth in several neoplastic cell lines, so they may have a potential role to play in cancer chemotherapy [10,11].

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The exact mechanism of artemisinin action has still not been fully explained, but it is known that 1,2,4-trioxane structure, particularly its peroxide bond, is necessary for its activity. It has been proposed that the endoperoxide bridge is activated by iron, resulting in the production of oxygen-centered free radicals, which are then converted into carbon-centred free radicals by an intramolecular hydrogen abstraction from CH2 groups on the periphery of the artemisinin [12]. The mechanism(s) by which the radicals kill the parasite may involve either the alkylation of specific parasite proteins or nonspecific damage to various intracellular targets, including proteins and membranes. A recently proposed target of artemisinin is the *P. falciparum* sarco/endoplasmic reticulum Ca^{2+}-ATPase (SERCA) homolog, PfAT. Uhlemann *et al* [13] suggested that a single amino acid (Leu263) in transmembrane segment 3 of SERCAs determines the susceptibility to artemisinin.

As monotherapies, artemisinin derivatives are effective treatments for uncomplicated malaria, but because of their very rapid clearance in plasma, a complete cure requires a longer treatment (up to 7 days), which is often not completed and leads to developing resistant strains of *Plasmodium*. To avoid the development of drug resistance against artemisinin derivatives, a combination therapy is currently used. The ACTs (artemisinin-based combination therapies) are recommended by WHO as first-line treatment for malaria tropica. The drug-combinations include the following: artemether and lumefantrine, artesunate and amodiaquine, artesunate and mefloquine, artesunate and sulfadoxine and pyrimethamine combinations [5].

Unfortunately, because of their high cost, ACTs remain too expensive for the majority of people who need them. Because artemisinin is a complex molecule, its total chemical synthesis is not economic [14]. Nowadays, the only source for the drug is extraction from dried leaves and influorescences of *A. annua*. *A. annua* is a very labor-intensive crop with a lengthy growing cycle; the period from the time of planting to artemisinin extraction is 12-18 months [15]. Moreover, the content of artemisinin in the plant is very low (from 0,1 to 1,5 % of dried leaves) and is dependent on seasonal and somatic variations as well as an infestation of bacteria, fungi and insects [16]. Also, both the extraction and purification of artemisinin are difficult and costly. These processes currently rely on methods that make use of organic solvents such as hexane and petroleum ether, which are relatively inefficient, potentially unsafe, and environmentally damaging [17].

A growing demand for ACTs, leading to a shortage of supply, as well as the high cost of the therapies, making them unaffordable to most of the people in malaria endemic countries, generated a need for an additional source of artemisinin, that is consistent, reliable, pure, and inexpensive. Biotechnological alternatives to produce artemisinin more efficiently and economically became increasingly important. The major classes of potential artemisinin production are i/ metabolically engineered native host ii/fermentation of metabolically engineered cell culture, and iii/ fermentation of metabolically engineered bacteria or yeast. In this review, we summarize the progress made in the production of artemisinin with the use of biotechnological methods.

2. BIOSYNTHESIS OF ARTEMISININ

Artemisinin is a sesquiterpene lactone, that belongs to terpenoids. This class of compounds originates from the universal precursor isopentenyl diphosphate (IPP) and its allylic isomer, dimethylallyl diphosphate (DMAPP) (isoprene unit) [18]. Terpenoid building blocks are formed through the condensation of IPP moieties by prenyltransferases. Sesquiterpenoids are derived from farnesyl pyrophosphate [19]. There are two distinct pathways for the production of the terpenoid backbones, namely, the acetate mevalonate pathway (MVA) and the 1-deoxy-D-xylulose-5-phosphate (DXP) or 2C-methyl-D-erythritol-4-posphate pathway (MEP), as shown in Fig. (**1**). The MVA pathway begins with the precursor acetyl-CoA and proceeds through the intermediate mevalonic acid (MVA), while the MEP pathway leads from the condensation of pyruvate and glyceraldehyde-3-phosphate via 1-deoxyxylulose-5-phosphate (DXP) as the first intermediate. Higher plants exhibit both pathways, which are separated in different compartments, and the MVA pathway is localized in the cytosol, while the MEP pathway is in plastids [20,21]. The interaction between these two pathways is still unclear, but recent studies have revealed an exchange of intermediates between the cytosol and the plastids [22-24].

On the mevalonate pathway, three molecules of acetyl-coenzyme A undergo condensation to form 3-hydroxy-3-methylglutaryl CoA (HMG-CoA) in the reactions catalyzed by acetoacetyl-CoA thiolase and HMG-CoA synthase. HMG-CoA is subsequently reduced by the enzyme HMG-CoA reductase (HMGR) to mevalonic acid (MVA). 3-Hydroxy-3-methylglutaryl coenzyme A reductase catalyzes the irreversible reaction and its activity correlates with terpenoids synthesis and accumulation [25]. MVA is phosphorylated to mevalonate-5-diphosphate in an ATP-dependent reaction and decarboxylated to yield IPP.

The alternative pathway to producing IPP starts with the condensation of pyruvate and glyceraldehyde 3-phosphate, which is catalyzed by 1-deoxy-D-xylulose-5-phosphate synthase. This enzyme is a rate-limiting step on the MEP route [26]. Obtained in the condensation, 1-deoxy-D-xylulose-5-phosphate (DOXP) is subsequently converted by the IspC enzyme to 2C-methyl-D-erythriol-4-phosphate (MEP). In the next step, MEP reacts with CTP to yield 4-diphosphocytidil-2C-methyl-D-erythriol (CDP-ME), which is further converted to 4-diphosphocytidyl-2C-methyl-D-erythriol-2-phosphate (CDP-ME2P) and 2C-methyl-D-erythriol-2,4-cyclodiphosphate (MEPC). The next two steps, catalyzed by 1-hydroxy-2-methyl-2-(E)-butenyl-4-diphosphate synthase (IspG) and 4-hydroxy-3-methyl-2-(E)-butenyl-4-diphosphate reductase (IspH), are not fully understood. IspG converts MEPC to 2-methyl-2-(E)-butenyl diphosphate, which is then transformed to IPP by IspH [27].

The two basic elements, IPP and DMAPP, are further used to build linear prenyl diphosphates. Enzymes called prenyltransferases catalyze the synthesis of geranyl diphosphate (geranyl diphosphate synthase), farnesyl diphosphate (farnesyl diphosphate synthase), geranylgeranyl diphosphate and farnesyl geranyl diphosphate [28].

The first committed step on the biosynthetic pathway of artemisinin is the cyclization of FDP to amorpha-4,11-diene

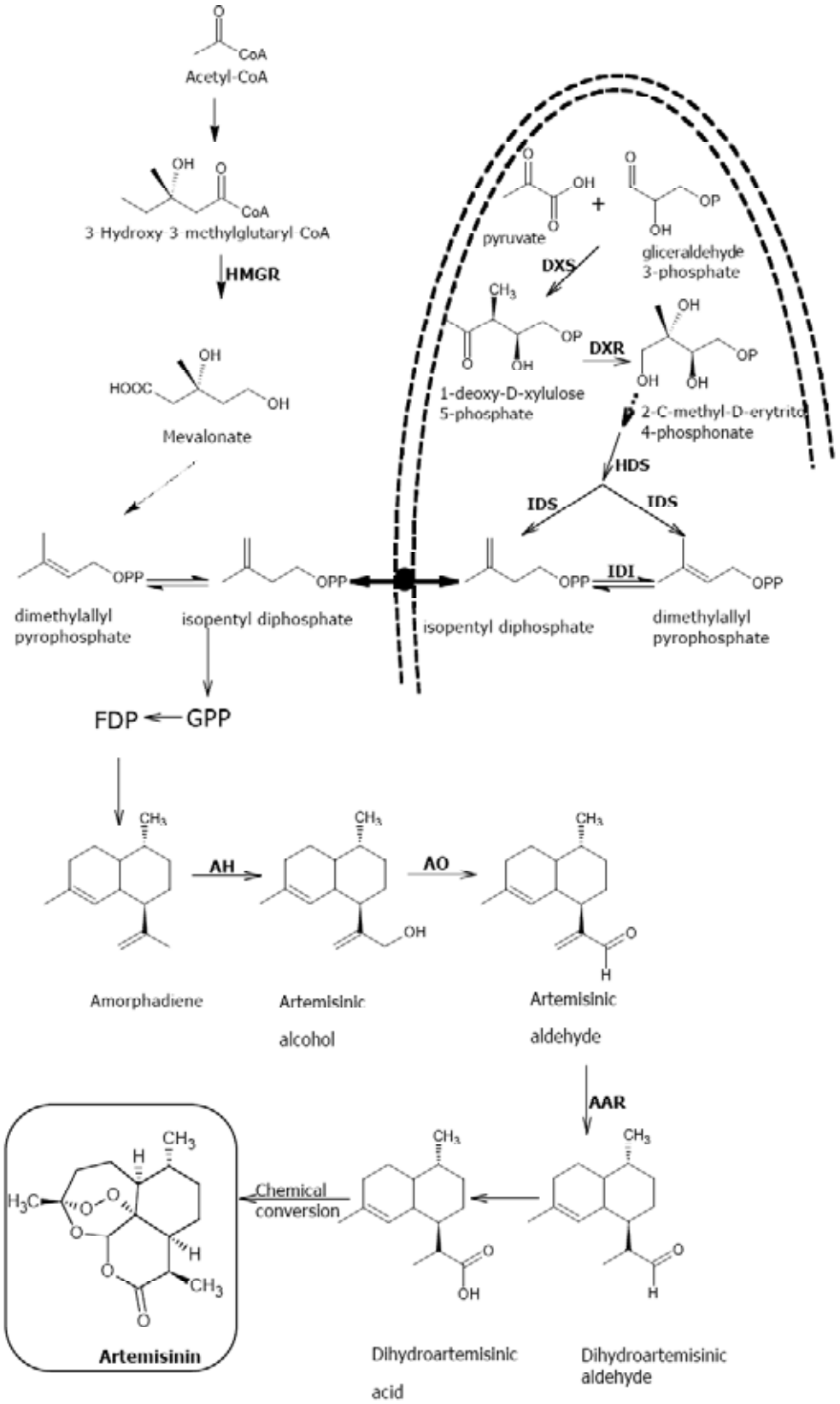

Fig. (1). Overview of artemisinin biosynthesis cytosolic and plastidic pathway. The key enzymes, which might be the subject of metabolic engineering are indicated. HMGR, 3-hydroxy-3-methylglutaryl coenzyme A reductase; DXS, 1-deoxy—D-xylulose-5-phosphate synthase; DXR, 1-deoxy-D-xylulose-5-phosphate reductoisomerase; HDS, hydroxyl-2-methyl-2(*E*)-butenyl 4-phosphate synthase; IDS, isopentenyl diphosphate: dimethylallyl diphosphate synthase; IDI, isopentenyl diphosphate isomerase; AH, amorpha-4,11-diene hydroxylase; amorphadiene oxidase (CYP71AV1); AAR, artemisinic aldehyde-Δ11(13)-reductase.

[29-31]. The enzyme catalyzing that reaction, amorpha-4,11-diene synthase (ADS) from *Artemisia annua*, has been isolated, cloned and biochemically characterized [32]. The further steps on the pathway are still being investigated. However, the metabolic reaction, leading from amorpha-4,11-diene to artemisinin, has been identified, this being based upon an analysis of both the intermediates and corresponding enzyme activities. Amorpha-4,11-diene is converted by a putative cytochrome P450 dependent hydroxylase to artemisinic alcohol. The subsequent biosynthesis steps include the oxidation of artemisinic alcohol to artemisinic aldehyde (by amorphadiene oxidase CYP71AV1), the reduction of the C11-C13 double bond in artemisinic aldehyde to dihydroartemisinic aldehyde (by artemisinic aldehyde-Δ11(13)-reductase), and the oxidation to dihydroartemisinic acid, possibly by a trichome-specific aldehyde reductase [33-35]. Artemisinin is thought to be formed from dihydroartemisinic acid in spontaneous rather than enzyme-catalyzed reactions [36]. The keto-enol can be obtained from dihydroartemisinic acid in the presence of singlet oxygen, that is created either by UV absorption or by visible light in the presence of photosensitizers, or from hydrogen peroxide. The tertiary allylic hydroperoxide intermediate is formed in a singlet oxygen reaction. After cleavage, a second hydroperoxide is formed in the presence of triplet (ground state) oxygen. The last step is the formation of the acetal peroxide and the acetal lactone groups.

In a "dehydro" branch of the pathway, artemisinic acid is obtained from artemisinic aldehyde in a reaction catalyzed by amorphadiene oxidase. Artemisinic acid is thought to be nonenzymatically transformed into several compounds, including arteannuin B [14].

On both pathways, the majority of biosynthetic enzymes is known, making the introduction of the complete primary and secondary pathway in the heterologous hosts possible [37]. However, in the commonly used target hosts, the terpenoid and, thus, artemisinin biosynthesis pathways are regulated through complex networks, which are still rather unknown.

3. ARTEMISIN PRODUCTION IN PLANT CELLS AND TISSUE CULTURES

As mentioned above, currently the only possible source of artemisinin is a plant. There are three species, which are producing that compound: *Artemisia annua* (the most widely cultivated), *A. apiacea*, *A. caruifolia* and *A. lanceolata*. Artemisinin is localized exclusively in the glandular trichomes on the leaf surface. The content of artemisinin ranges from 0,1 to 1,5 % of dried leaves and is dependent on a seasonal and somatic variation as well as the infestation of bacteria, fungi and insects. Some attempts to increase the amount of artemisinin by conventional breeding have been conducted, but with no satisfying results so far [38].

As the yield of artemisinin obtained from plant extraction is low, its production from *in vitro* plant tissue cultures seems to be an attractive alternative. Plant cell culture could provide an environmentally friendly, renewable alternative for artemisinin supply. There were numerous attempts to obtain artemisinin from the calli, suspension cells, shoots, and hairy roots of *A. annua* during their cultivation *in vitro* [39-41]. As experiments with undifferentiated cultures of *A. annua* gave disappointing results, it has been postulated that a certain degree of differentiation in the tissue is necessary [42].

A successful cell suspension culture based approach was performed by A. Baldi and V.K. Dixit [43], who managed to obtain superior contents to previously reported contents of artemisinin by using mevalonic acid as a precursor and methyl jasmonate as an elicitor at optimal concentrations. Artemisinin was also detected in relatively high (0,16% dry weight) concentration in *A. annua* shoots cultured on 1/2 MS medium, that had been supplemented with 0.05 mg/l naphthaleneacetic acid, 0.2 mg/l benzyladenine (BA), and 1 % sucrose [44]. The presence of artemisinin in the root part of the *A. annua* plant has not been proved.

Some trials involved the induction of hairy roots with the use of *Agrobacterium rhizogenes* [45] and *Agrobacterium tumefaciens* [46]. Artemisinin content in the leaves of the regenerated plant was slightly higher than in the leaves of normally cultured plants.

Many different attempts were also made to increase artemisinin production by optimizing the chemical and physical environmental factors. For instance, Wang and Tan [47] reported that, with the ratio of NO_3/NH_4 at 5:1 (w/w), the optimum concentration of total nitrogen for artemisinin production was 20 mM and artemisinin production was 57% higher than in the standard MS medium.

A few studies showed that gibberellic acid (GA3), a plant inducing blooming hormone, can improve growth and artemisinin biosynthesis in the shoot cultures, root cultures and plantlets of *A. annua* [48,49]. The light and temperature influence on the artemisinin content was also investigated. Liu *et al.* [50] found an increase of artemisinin concentration when the hairy roots were cultured under the illumination of 3,000 lx for 16 h using several cool-white fluorescent lamps. The temperature can also affect the growth and artemisinin biosynthesis in the cultured *A. annua* hairy roots. The maximum hairy root growth was found at 25°C, but the highest artemisinin content was observed at 30°C [51].

The feeding of precursors and elicitation has proved to be an effective way to enhance artemisinin production in cell cultures. The addition of artemisinin precursors to the tissue culture medium increased the content of artemisinin in the tissue four times and eleven times in the spent medium [52]. The feeding of mevalonic acid alone, however, did not induce the enhancement of artemisinin production [53].

Other supplements, such as 5-azacytidine (that removes methyl groups from the DNA), colchicine (a mitosis inhibitor), miconazole (an inhibitor of sterol demethylase), and terbinaphine (the squalene epoxidase inhibitor) appeared to be too toxic for the cultures and did not increase the artemisinin production [44].

Unfortunately, given the cost of tissue culture, none of the reported yields appear to be economically feasible to serve as an alternative for the plant extraction at an industrial level [48].

4. PLANT GENETIC ENGINEERING

As the yield of artemisinin obtained from cell cultures was not satisfying, some attempts were made to genetically modify *A. annua* in order to increase the artemisinin content. The intention of this approach was either to overexpress the enzymes on the terpene biosynthetic pathway or to inhibit the enzymes of the pathways, that were competing for artemisinin precursors.

Chen *et al* [54] developed an *Agrobacterium tumefaciens*- mediated transformation system for *Artemisia annua* L. Using this system a cDNA encoding farnesyl diphospahte synthase was transferred into *A.annua* via *A.tumefaciens* strain L. In the transgenic plants thus obtained, the concentration of artemisinin was 2-3 fold higher than in a wild-type plant (8-10 mg/g DW). Transgenic *A. annua*, into which the 3-hydroxy-3-methylglutaryl CoA reductase (HMGR) gene from *Catharanthus roseus (L.) G. Don* was transferred, possessed a significantly higher HMGR activity when compared with wild-type controls, and the artemisinin content depicted an increase of 22.5 % when compared with wild-type control *A. annua* [55]. The inhibition of squalene synthase also increased the content of artemisinin about 3-fold. Squalene synthase was suppressed by means of a hairpin-RNA-mediated RNAi (RNA interference) technique and transgenic plants were obtained through *Agrobacterium tumefaciens*-mediated transformation. The concentration of artemisinin reached 31.4 mg/g dry weight [56], which was significantly higher than that obtained in the systems described above. Also, Yang *et al* reported that in transgenic *Artemisia annua* plants, that express the genomic integrated antisense squalene synthase gene (ASSS), the SS mRNA level in transgenic plants sharply decreased to 7.4% - 55.3%. The down-regulation of SS gene has also led to the coordinated overexpression of amorphadiene synthase (ADS), cytochrome P450 monooxygenase (CYP71AV1) and cytochrome P450 reductase genes together with the overproduction of artemisinin, although no complete correlation among the available experimental data has been shown [57].

Geng *et al* [58] investigated the effect of the expression of isopentenyl transferase, the enzyme representing the rate-limiting step in cytokinin biosynthesis in tumorous plant tissue on the artemisinin synthesis in *A. annua*. An isopentenyl transferase gene (*ipt*) from T-DNA was transferred into *Artemisia annua* leaf explants via *Agrobacterium tumefaciens.* As a result, cytokinins, chlorophyll and artemisinin contents increased at different degrees, while artemisinin increased 30-70% when compared with the control plants.

Moreover, some researchers assumed that other plants can also be hosts for possible artemisinin production. It was found that the wild type chicory extracts showed amorphadiene oxidase activity, suggesting that the expression of amorphadiene synthase in chicory could lead to obtaining artemisinic acid [59]. This concept could be the basis for commercial artemisinin production by using genetically-modified chicory. Furthermore, Wu and coworkers have engineered amorphadiene production in tobacco [60]. They found that the expression of FDP synthase and amorphadiene synthase targeted to plastids gave much better yields than cytosolic enzymes. The tobacco platform offers the basis for additional engineering.

5. BIOSYNTHESIS USING ENGINEERED MICRO-ORGANISMS

Metabolic engineering in microbial cells for the sustainable production of artemisinin offers several advantages such as i/ environmentally friendly chemistry ii/ the use of inexpensive carbon sources iii/ the capability of genetic manipulation to increase the production yield iv/compatibility with large scale fermentation processes due to microorganims' relative insensitivity to shear stress, which is induced via impeller mixing in bioreactors [27]. Well characterized and genetically easy to manipulate hosts like *E. coli* and *S. cerevisiae* are most often utilized. The ultimate goal is to develop a manufacturing process, combining synthetic biology with synthetic chemistry. Microbiological "factories" are supposed to produce artemisinic acid, that will be chemically converted to artemisinin. In this way, a seasonal, independent supply of artemisinin of a consistent quality and cost could be provided [61].

The first attempts involved engineering *E. coli* for isoprene production. Martin *et al* optimized the production of the FDP precursor and expressed the enzyme, so catalyzing the first committed step on the artemisinin pathway, amorphadiene synthase, in *E. coli* [62]. As the approach based on the manipulation of the *E. coli* DXP/MEP pathway is limited, due to the control mechanisms present in the host, they bypass the endogenous DXP/MEP pathway by the introduction of an entire suite of exogenous genes on the MVA pathway from *Saccharomyces cerevisiae.* The expression of this heterologous pathway in *E. coli* led to a growth inhibition, owing to an accumulation of isoprenoid precursors. The simultaneous expression of a synthetic ADS gene reduced that effect and resulted in higher amorphadiene accumulation.

Pitera *et al* [63] discovered that *E. coli* cells growth inhibition was connected with the accumulation of the pathway intermediate 3-hydroxy-3-methyl-glutaryl-coenzyme A (HMG-CoA), which implies that the activity of HMG-CoA reductase was insufficient to balance the flux on the engineered pathway. The co-expression of HMG-CoA reductase, which converts HMG-CoA to non-toxic mevalonate, alleviated the toxicity, eliminated the pathway bottleneck and elevated mevalonate production.

A complimentary method for pathway optimization, that focuses on balancing gene expression, identifying rate-limiting enzymes, and decreasing the metabolic burden of the pathway on the host, has been described by Anthony *et al* [64]. The *E. coli* strain they engineered, containing stronger lacUV5 promoters, codon-optimized MevT and ADS operons, and additional copies of mevalonate kinase and amorphadiene synthase, was able to produce the highest amorphadiene titers in shake flasks observed so far (300mg/l/ OD600).

The subsequent step on the artemisinin biosynthetic pathway involves the oxidation of amorphadiene to artemisinic alcohol and, furthermore, to artemisinic acid. The first example of a high-level production of artemisinic acid in *E. coli* by using plant-derived P450s, amorphadiene oxydase (CYP71AV1) from *A. annua,* was demonstrated by Chang *et al* [65]. Although the expression of the native CYP71AV1 gene led to undetectable *in vivo* or *in vitro* CYP450 activity,

codon optimization coupled with N-terminal transmembrane domain modification enabled the first oxidation step *in vivo*, resulting in the generation of artemisinic alcohol at low concentrations (0.18-0.45 mg artemisinic alcohol/l). The replacement of the *Candida tropicalis* CYP450 reductase gene with the *A. annua* CYP450 reductase gene increased artemisinic alcohol production 12-fold to 5.6 mg artemisinic alcohol/l. Furthermore, the change in the plasmid vectors and *E. coli* strains increased the oxidation of amorpha-4,11-diene by more than 1,000-fold to 553 mg amorpha-4,11-diene/l.

An alternative to the approach described above is the *de novo* pathway design, using well-characterized, substrate-promiscuous enzymes. P450 BM3 from *Bacillus megaterium* has been mutated, based on the transition state complex modeling with amorphadiene. The introduction of the mutation F87A opened up the active site and relieved the steric hindrance on the ring structure of amorphadiene. Mutation A328L decreased the size of the P450BM3 binding pocket, restricting the mobility of amorphadiene. Mutations R47L/ Y51F greatly reduced the fatty acid oxidation. The mutations enabled the selective oxidation of amorphadiene to artemisinic-11*S*,12-epoxide at titers of 250 mg/1 in *E. coli.* The microbial production of artemisinic epoxide can be followed by high-yielding, selective transformations to dihydroartemisinic acid and, further, to artemisinin [66].

E. coli is a convenient host for the production of secondary metabolites, due to its rapid growth rate and ease in genetic modification. However, the functional expression of plant CYP genes in *E. coli* is challenging, because of the difficulties in proper peptide folding, membrane association, cofactor integration, and post-translational modification as well as other factor interactions [67].

As eukaryotic organisms, *S. cerevisiae* are more suitable for an heterologous expression of plant genes, that are difficult to express in bacterial systems. The mevalonate pathway that produces FDP is well characterized in *S. cerevisiae* and may, therefore, be engineered to produce an optimal amount of FDP, resulting in an improved yield of the sesquiterpenes. Similarly, as with *E. coli*, metabolic engineering approaches to yeast involve an increasing flux through the mevalonate pathway by an overexpression of the tHMG gene encoding HMG reductase, reducing the flux toward ergosterol and an overexpression of the farnesyl diphosphate synthase encoding gene [27].

The Brodelius group introduced the amorphadiene synthase gene to yeast in two different ways, by plasmid transformation and by homologous recombination. A higher activity was observed for yeast, expressing the enzyme from the plasmid. The plasmid and the genome-transformed yeasts produced 600 and 100 μg/l of amorpha-4,11-diene, respectively, during 16-day batch cultivation [68]. The researchers concluded that the yield of sesquiterpenes is correlated with the gene amount and insufficient FDP synthesis is a limiting factor.

Yeast strains producing a high amount (up to 100 mg/l) of artemisinic acid was engineered by Ro *et al* in three subsequent steps [69]. They engineered the farnesyl diphosphate biosynthetic pathway to increase FDP production and decrease its use for sterols, transferred the amorphadiene synthase gene from *A. annua* and expressed cytochrome P450 monooxygenase (CYP71AV1), that performs a three-step oxidation of amorphadiene to artemisinic acid. Some genes responsible for the FDP synthesis were upregulated. The overexpression of a truncated, soluble form of 3-hydroxy-3-methylglutaryl-coenzyme A reductase (tHMGR) improved amorphadiene production approximately 5-fold. The gene responsible for FDP conversion to sterols, ERG9 encoding squalene synthase, was downregulated, using a methionine-repressible promoter (PMET3)13, that increased amorphadiene production an additional 2-fold. A further increase in the amorphadiene amount (up to 105 mg/l) was achieved by the combination of downregulating ERG9 and overexpressing upc2-1, a semi-dominant mutant allele enhancing the activity of UPC2 (a global transcription factor, regulating the biosynthesis of sterols in *S. cerevisiae*). The integration of an additional copy of tHMGR in the chromosome further increased amorphadiene production by 50% to 149 mg/l. After overexpressing the gene encoding FDP synthase, the production of amorphadiene increased by about 10%, owing to a decrease in cell density. All these manipulations led to a total enhancement in amorpha-4,11-diene biosynthesis up to 153 mg amorpha-4,11-diene/l, an elevation of nearly 500-fold of what has been reported previously [70]. Afterwards, CYP71AV1 and CPR genes have been transferred into yeast cells to enable an oxidation of amorphadiene producing artemisinic acid. In a shake flask, approximately 32 mg artemisinic acid/l was produced, but the pathway intermediates, artemisinic alcohol and artemisinic aldehyde, were present at negligible levels in the culture medium and cell pellets.

In a related development, the co-expression of farnesyl diphosphate synthase (*Fps2*), amorpha-4,11-diene synthase, amorpha-4,11-diene monooxygenase (*Cyp71av1*), cytochrome P-450 reductase, and artemisinic aldehyde Δ11(13) reductase (*Dbr2*) in yeast has been performed. The strain expressing *Fps2* and *Cyp71av1* (but not *Dbr2*) accumulated artemisinic acid to a level of 29.4 g/l culture. In the strain additionally expressing *Dbr2*, artemisinic acid accumulated to 11.8 g/l, and, in addition, dihydroartemisinic acid was found at a level of 15.7 g/l culture [71].

Another way of increasing amorphadiene content in yeast cells is to enhance the supply of acetyl-CoA to the mevalonate pathway. This approach has been attempted by Shiba *et al*, who engineered the pyruvate dehydrogenase bypass in *S. cerevisiae*, namely pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase (ALD6), and acetyl-CoA synthetase (ACS), converting pyruvate to acetyl-CoA [72]. The overexpression of acetaldehyde dehydrogenase alone reduced amorphadiene production, probably due to the accumulation of a byproduct acetate, which led to decreased cell mass. An overexpression of acetyl-coA synthase caused an 8-23% increase in production, while an overexpression of mutated ACS from *Salmonella enterica* together with ALD6 resulted in high amorphadiene accumulation [73].

6. COMMERCIAL SCALE PRODUCTION OF ARTEMISININ

The last step towards the commercial production of artemisinin is the development of a bioreactor appropriate for large-scale cultures. Many factors are important during the scale-up of any kind of cell culture, including the oxygen

transfer rate, heat transfer, mixing and the associated shear stress, the superficial air velocity associated with impeller-mixed cultures, and culture age and stability [74, 75]. Several types of bioreactors have been designed both for plant cell cultures and microbial cultures.

Bioreactors tested for hairy roots cultures included: liquid-phase reactors such as stirred tank, bubble column, and air-lift reactors; gas-phase reactors such as trickle bed, droplet phase and mist reactors; and, finally, hybrid reactors that are a combination of both [75-77]. The results of performed experiments showed that the hairy root cultures in the modified inner-loop airlift bioreactor grew more homogeneously than in the bubble column reactors [78]. Recently, a developed nutrient mist bioreactor, using ultrasonic transducers, produced nearly a three times higher level of artemisinin than the bubble column reactor [75,79]. To increase the efficiency of that bioreactor, the mist duty cycle, medium formulation, and gas composition were examined and optimized by Wyslouzil *et al* [80]. However, in a series of experiments performed by Kim *et al.*[81], roots grown in a mist reactor reached a lower dry mass than those in a bubble column reactor. To improve the growth rate of roots in a mist reactor, Towler *et al.* [82] investigated the effects of initial packing density and sugar availability on the growth rates of *A. annua* hairy roots. They reported that higher inoculum densities and higher sucrose concentrations in the medium increased the growth rate at a fixed mist duty cycle, while increasing the mist duty cycle at the constant sugar amount's decreased growth.

Despite recent advances, the use of bioreactors for large-scale plant cell cultivation is still very challenging and cannot be implemented without additional study and design changes [76,83].

The employment of bioreactors in the microbial fermentation seems to be more successful. Newman *et al* [84] used two-phase partitioning bioreactors (TPPB) with a hydrophobic n-dodecane overlay to trap amorphadiene, that is volatile and easily lost from a culture. The expression of a heterologous yeast mevalonate pathway and codon-optimized amorphadiene synthase in *E. coli* yields approximately 0.4 g/l of the amorphadiene product.

Lenihan *et al* [85] improved the production of artemisinic acid by *Saccharomyces cerevisiae* in bioreactors. A methionine repressible promoter (Pmet3) was used to down-regulate the squalene synthase gene, which increased FDP's availability for the synthesis of artemisinic acid. They designed a dissolved-oxygen-stat algorithm, simultaneously controlling the agitation and galactose feed. This algorithm enabled high density production in cultivations where glucose was used, first, for growth, followed by a galactose feed for growth and induction. Fermentation processes were developed, yielding cell densities of 120-200 OD600 and artemisinic acid titers as high as 2.5 g/l.

Further improvements in amorpha-4,11-diene production in *E. coli* by strain engineering and fermentation process development were performed by Tsuruta *et al* and led to a yield of 27.4 g/l. The yeast genes encoding HMG-CoA synthase and HMG-CoA reductase were replaced with more active equivalent genes from *Staphylococcus aureus*. The improved strain was cultivated under carbon and nitrogen restriction. Glucose restriction controls the production of acetate in high density fermentations by limiting the rate of cell growth, while the nitrogen restriction reduces amino acid and protein biosynthesis in the cells and increases carbon availability for amorpha-4,11-diene production. Although a full commercialization of that process requires some improvement, like the removal of antibiotic selection and expensive inducer IPTG, such a high an amount of amorphadiene is a significant step towards the development of a commercial process for the semi-synthetic production of artemisinin [86].

7. CONCLUSIONS

An access to effective and safe antimalarial drugs remains a key component of any malaria control program. Despite recent reports on resistance to certain artemisinin-derivatives [87], ATCs still remain the most valuable drugs for the treatment of this devastating disease. Among the new biotechnological approaches to increase the production and reduce the price, the engineered microorganisms seem to be most promising. Fermentation has been used for decades to produce important chemicals and medicine. Combining the scale-up process of fermentation with synthetic chemistry should result in a significant reduction in the ACTs price, making them more accessible to patients in poverty areas. Industrial fermentation processes also offer advantages in scalability, reliability, and flexibility, which allow a greater and faster ability in adjusting to market changes.

On the other hand, the advances which have been made in the characterization and redirection of metabolic pathways involved in terpenoids synthesis in plant make more realistic plant cell culture technology as a platform for the supply of terpenoid compounds, including ATCs. However, there are still many challenges that lie ahead in the commercial production of ACTs from either microorganisms or plants. In the latter, one of them is the use of genetic tools to modify the metabolism for higher yields of artemisinin, while in the microorganisms precursor supply is the problem. However, if successful, the metabolic engineering of ACTs could make an impact upon public health significantly.

ABBREVIATIONS

ACTs	=	Artemisinin-based combination therapies
ADS	=	Amorphadiene synthase
FDP	=	Farnesyl diphosphate
HMGR	=	3-hydroxy-3-methylglutaryl-coenzyme A reductase
IPP	=	Isopentenyl diphosphate
MEP	=	2C-methyl-D-erythritol-4-phosphate
MVA	=	Mevalonic acid

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