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One Potential Strategy for Biofuel Production: Isoprene Biosynthesis in Chlamydomonas Reinhardtii

Yiqun Zhou[1*], Kaiqi Liu[2]

[1]Faculty of Science, The University of Hong Kong, Hong Kong, China
[2]BDA School of The High School Affiliated to Rebmin University of China, Beijing, China
[*]Corresponding Author. Email: u3637553@connect.hku.hk

Abstract: World energy is facing multiple threats. On the one hand, non-renewable energy sources such as fossil energy, which account for a large proportion, are gradually depleting, but the demand is still high. On the other hand, most of the energy currently used, such as oil and coal, is directly related to climate change and air pollution. Renewable biosynthetic pathways currently account for a very small proportion of energy consumption and therefore have great potential for development. Isoprene has a wide range of uses and can be used as a target product for biosynthesis. The MEP pathway of *Chlamydomonas reinhardtii* UPN22 can be used to synthesize this terpene compound, and there is the possibility of further improving the synthesis efficiency through the combined effects of genetic engineering and the external environment. At this stage, the characteristics of the UPN22 strain may affect the collection of isoprene products. In addition, there is a lack of real data on the entire MEP pathway, and it is impossible to confirm how these strategies will affect the synthesis of isoprene.

Keywords: isoprene, MEP pathway, metabolic engineering, *Chlamydomonas Reinhardtii*.

1. Introduction

The development of technology drives the progress of productivity, causing the application of energy to expand to a wider extent than ever before, with the demand for fuels worldwide increasing a lot at the same time. According to Ritchie et al. [1], global consumption of oil and coal last year was 60 and 4 times what it was 100 years ago respectively, while those renewable energy types whose amount occupied only 14% of the total consumption. Among all the energy types, fossil fuel is a family of unrenewable primary energies that have been widely used for approximately [2] centuries, which is also an important factor that is threatening the environment all over the world. Studies have discovered that the public, especially the young, has been significantly affected by climate change and air pollution caused by fossil fuels, and many diseases like ADHD are highly related to exposure to polluted air [2]. In addition, the depletion of fossil fuels or other primary energy sources is predictable, forcing society to find a replacement for traditional energy resources [3].

Using organisms to produce energy is a development direction that many countries have tried for years, and the research results are called biofuels. Although biofuels have contributed less than 3% of total energy consumption in the last few years, they have the potential to improve the energy structure of the whole world [4]. The use of microbial metabolism to synthesize fuels belongs to the

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third and fourth generations of biofuels, which do not require the use of farmland and specific crops compared to the first generation of biofuel [5]. The second generation, which can consume waste to produce diesel and ethanol, does not need crops as materials but is unsuitable for large-scale production and has a high cost [6,7].

Isoprene is a semi-terpene hydrocarbon, and its C_5 skeleton can be used to synthesize a variety of bulk chemical products, such as rubber products. The demand for isoprene is huge, with an annual usage of over 850,000 tons. The huge consumption has led many countries to obtain isoprene through chemical synthesis or fractionation of petroleum products [8]. Currently, obtaining isoprene from organisms has the disadvantages of high cost and difficult operation. In nature, many plants release isoprene to resist high-temperature environments, and it is one of the main types of carbon emissions from plants. However, the relatively low boiling point (34°C) makes it difficult to collect isoprene [9,10]. Therefore, in an artificially controlled environment, the strategy of microbial biosynthesis is a possible development direction for obtaining isoprene.

Chlamydomonas reinhardtii is a photosynthetic autotrophic eukaryotic organism and a commonly engineered bacterium for biosynthesis. Like other microalgae, *C. reinhardtii* can both rapidly proliferate and absorb CO_2. It can also survive in sewage and utilize the nitrogen and phosphorus elements in it. Apart from that, in genetic engineering, the advantage of using *C. reinhardtii* is that its nuclear, chloroplast, and mitochondrial genes can be transformed and stably expressed [11,12].

This article will focus on the signaling pathway of isoprene metabolism in *C. reinhardtii* and explore strategies that may improve metabolic efficiency, including genetic engineering, environmental regulation, etc. At the same time, problems with the biosynthesis method of *C. reinhardtii* will be discovered, and the future possibilities of this process will be prospected.

2. The isoprene and its production

Isoprene is also called 2-methyl-1,3-butadiene with the molecular formula $CH_2=C(CH_3)-CH=CH_2$ [13]. Isoprene is a colorless organic substance with an aromatic odor that exists naturally in the form of gas or liquid. Isoprene belongs to the terpenoid family and is the most basic structure in this family of compounds, which can form more complex terpenoid compounds through multiple combinations [14].

Isoprene gas is widely distributed in the atmosphere, including in human breath. Trees and shrubs are the main sources of isoprene in the atmosphere, accounting for about a third of total hydrocarbon emissions, with the rest probably coming from algae and single-celled organisms [15]. Isoprene is considered to be very important for plant survival. According to Sharkey *et al.* [9], emitting isoprene can confer heat tolerance and tolerance to reactive oxygen species in plants, since C-H can react with free radicals from atmospheric oxygen, and as a result, generate hydroperoxides that enhance ozone formation. Isoprene released by plants is primarily generated via the methylerythritol phosphate (MEP) pathway, which occurs in chloroplasts within cells [9,16].

2.1. How isoprene works as fuel

Isoprene is a high-quality aviation fuel and an important polymer building block in the synthetic chemical industry [17]. Isoprene from renewable materials is seen as an alternative to petroleum-based products due to high oil prices, ongoing supply, and environmental concerns [18]. And because the chemical structure of isoprene is very suitable for addition and substitution, it can easily form C_{10}-C_{13} olefins, a common fuel carbon chain structure. The olefin product is found to have a higher cetane number and lower consistency than general diesel and therefore has great

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development potential [19]. Another example is 1,6-dimethyl-1,5-cyclooctadiene (DMCOD), a jet fuel produced by reacting isoprene as a precursor [20].

2.2. The biosynthesis of isoprene in microorganisms

In addition to being used as a fuel, isoprene has been found to have a wide range of uses, making the quest to increase production an urgent requirement. The main method of obtaining isoprene is to fractionate C_5 carbon chains from petroleum, which has been proven to face multiple problems, such as reduced production and high pollution. And even though large plants such as angiosperms can produce large amounts of isoprene, since it is not easy to collect the product (isoprene in gaseous form), using engineered microbes may be a better choice [21].

Two important pathways in the production of isoprene inside microorganisms are the MEP pathway and the Mevalonate (MVA) pathway [22,23]. The MEP pathway exists independently in green algae, cyanobacteria, and other organisms, and their MVA pathway genes are believed to have been eliminated during evolution to maintain higher synthesis efficiency. The MVA pathway is widely present in a variety of macroscopic and microscopic organisms, such as plants, which have both MEP and MVA pathways [23]. Both of them are related to dimethylallyl diphosphate (DMADP), a key precursor for isoprene synthesis [18,24]. Then, under the action of isoprene synthase (IspS), DMADP will be converted into isoprene [10,25].

One of the main differences between the two pathways is where they occur. The MEP pathway occurs in plastids, a type of organelle found in plant cells. The MVA pathway, on the other hand, takes place in the cytosol and peroxisomes of eukaryotic cells. This situation, on the other hand, determines the efficiency of the two pathways. To complete the MEP pathway, one more NADPH is required when compared with the MVA pathway, but cells with the MEP pathway can usually carry out photosynthesis, during which the large amount of NADPH produced far exceeds the consumption of the MEP pathway, so the net output is much higher than the latter [23,24].

Also, the MEP pathway uses glyceraldehyde 3-phosphate (G3P) and pyruvate as precursors (Pyr), while the MVA pathway uses acetyl-CoA [24,26]. 1 unit of G3P and 1 unit of Pyr can form 1 unit of DMADP and IDP during the MEP pathway, and a total of 3 units of acetyl-CoA is required for the MVA pathway to form the same amount of DMADP and IDP [24].

Besides, depending on the species of organisms, the carbon sources utilized by the two pathways may be different. For example, fungi with the MVA pathway or bacteria with the MEP pathway need to break down glucose to produce raw materials, while autotrophic organisms such as microalgae with the MEP pathway can use CO_2 as a carbon source [10,24].

3. Strategies that may help increase the efficiency of isoprene production

3.1. Pathway selection

As shown in **2.2**, both the MVA pathways and the MEP pathways can participate in synthesizing isoprene [22], and the first target of improving the isoprene production should focus on the differences between the two pathways. It is predicted to include three main directions: efficiency of the pathway itself, the species that own this pathway, and the materials required to be supplied for running the pathway.

The two factors have the ability to affect the efficiency of pathways. One is the rate-limiting enzymes, for MEP it could be the 1-deoxy-D-xylulose-5-phosphate synthase and for MVA it should be the hydroxyl-methylglutaryl-CoA reductase [24]. These are not final considerations as they can be regulated through genetic engineering. The other one is the energy required during the process. The study found that the two pathways operate almost independently. Although their products are similar, they rarely intersect (when they exist at the same time) [23], which makes it feasible and

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necessary to consider the energy consumption of the two separately. From **2.2**, it is clear that the net consumption of NADPH by the MEP pathway involved in photosynthesis is much smaller than that by the MVA pathway, and the molar amounts of G3P and Pyr required for synthesis are also smaller than that of acetyl-CoA in MVA [24].

It is relatively easy to screen species that carry both pathways because they can be identified by the presence or absence of terpenoids in their products. When considering sustainability, algae is the main choice [27].

As for the raw materials needed, in this process, the availability and cost of the raw materials need to be considered. If possible, sustainability issues need to be considered as well.

As a result, the MEP pathway should be the more acceptable one. One of the most significant features of the MEP pathway is its carbon source, the CO_2 for many of the microalgae species with this pathway. It is predicted to result in zero carbon emission during the production – use process of the isoprene, as the bioreactor would be able to absorb the CO_2 from the burning of isoprene fuels [10,21]. Apart from that, consideration of the relationship between host cell types and the pathways they own was also necessary for ensuring the final choice.

3.2. Specie selection

As mentioned in **3.1**, photoautotrophic organisms are the main selection direction. Secondly, the characteristics of genes and mutants need to be considered.

C. reinhardtii is the final choice of bioreactor that will be used during the process of running the MEP pathway. It is the microalgae that only have the MEP pathway [23]. *Escherichia coli* can also be used for biosynthesis using the MEP pathway but with higher sustainability and cost [28]. On the one hand, without chloroplasts, *E. coil* cannot use CO_2 as a carbon source and organic substances such as glucose are necessary [24]. On the other hand, to increase isoprene production, overexpression of isoprene synthase (IspS) is required (this will be explained in **3.3**), which means the introduction of the IspS gene, which is commonly found in terrestrial plants, such as poplar [29,30]. In that case, eukaryotes are more likely to express related genes than prokaryotes because prokaryotes are unable to process exons and introns, which may affect protein expression.

The detailed category of *C. reinhardtii* we decided to use is the UPN22. UPN22 is a derivative of strain UVM4 [10,31,32]. UVM4 has been an important bio-factory for recombinant protein production in the last 20 years [28]. UVM4 strains have a larger size without flagella when compared to the wild type. It is a cell wall-deficient strain, making it easier to manipulate the organism and introduce foreign DNA. The cell wall of microalgae affects the collection of terpenoid products [33]. After expression, the protein will be released to the cell membrane's outer side [11]. UPN22 strains are engineered to express the Pseudomonas stutzeri WM88 phosphite oxidoreductase (ptxD) from its plastid genome [10]. The nitrate metabolism of these strains was complemented by the transformation of native nuclear genes nit1/nit2 [10]. Therefore, UPN22 strains are able to grow with phosphite as the sole source of phosphorus and nitrate as a nitrogen source [10]. As a result, it enables the mutant *C. reinhardtii* to grow in unfavorable conditions and treat pollution, especially the nitrogen and phosphorus [34], which cause algal bloom.

Therefore, when screening UPN22 strains, the wild type after gene knockout can be cultured on a phosphorus-free medium without organic phosphorus, and an appropriate amount of phosphite can be added as the only nitrogen source. After overnight incubation, the UPN22 strain should be able to grow while the other species should not show obvious colonies. However, how to screen whether the genes related to the enhancement of the MEP pathway are correctly transferred requires further design.

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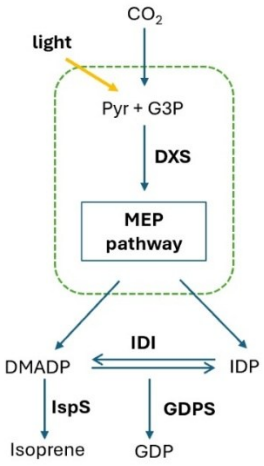
3.3. Enhancement of MEP pathway

The figure (**Figure 1**) below shows a simplified MEP pathway.



Green dashed line: chloroplast membrane; DXS: 1-deoxy-D-xylulose-5-phosphate synthase; IDI: Isopentenyl-diphosphate delta isomerase; IDP: isopentenyl diphosphate; GDP: geranyl diphosphate; GDPS: geranyl diphosphate synthase.

Figure 1: The MEP pathway inside *C. reinhardtii* from CO_2 to isoprene or GDP

According to **Figure 1**, three important points are significantly related to the improvement of isoprene production in *C. reinhardtii*: the DXS, the IDI with the balance, and the GDPS [10,24].

DXS is the first enzyme that is required during the MEP pathway. It can also be seen as the rate-limiting enzyme of the process [24]. DXS promotes the interaction between Pyr and G3P and can increase the number of raw materials entering the MEP pathway by overexpressing related genes, thereby improving the efficiency of the MEP pathway.

As shown in **Figure 1**, DMADP and IDP will convert to each other under the action of IDI, and only the DMADP is required for the synthesis of isoprene. As IspS can convert DMADP into isoprene, it is predicted that if DMADP is consumed excessively, the balance should be tilted towards DMADP, and more IDP will be converted into DMADP. Thus, the concentration of IspS in *C. reinhardtii* can be increased by adding the IspS gene, which is commonly found in land plants [10,29].

Also, the DMADP and IDP will be synthesized into GDP with the help of GDPS, and then become other terpenes and olefins. If the action of GDPS can be stopped, all the DMADP and IDP obtained from the MEP pathway will be used in the synthesis of isoprene. In that case, knocking out the synthetic GDPS gene is the most direct approach and has the potential to be inherited by daughter cells. Knocking out the synthetic GDPS gene is the most direct method, and it is possible to pass it on to daughter cells. Alternatively, GDPS can be inactivated by adding additional substances. For example, specific antibodies can be used to bind to GDPS, causing the enzyme protein to lose its action site.

3.4. Culture environment control

According to Abdallah *et al.* [11] and Perozeni et al. [35], algal cells like *C. reinhardtii* could be cultivated under mixotrophic conditions using Tris-acetate-phosphate (TAP) in shaker flasks at 25℃ and 100–150 μmol photons $m^{-2} \times s^{-1}$ of continuous white light unless otherwise stated [31,35]. In addition to these basic raw materials, metal ions can promote the growth of

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Chlamydomonas and maintain a high cell density in the culture medium [12]. The type of metal ion supplement is related to photosynthesis. And in the absence of N, Fe, and other elements, lipid bodies may accumulate [12].

However, different enzymes or genes have different requirements for the optimal environment. One of the external IspS gene sources of *C. reinhardtii*, *Populus grisea*, showed different activities of its IspS gene promoter in different parts, which was found to be the result of the combined effects of light and temperature [36]. For IspS to achieve the highest catalytic activity, the culture temperature may need to be close to 42°C. But this temperature is outside the optimal growth temperature range of Chlamydomonas reinhardtii and may not be suitable in the early stage of isoprene production (expansion of cell number) [10].

Also, the concentration of gas may affect the efficiency of *C. reinhardtii* simultaneously. CO_2 is the carbon source of the MEP pathway that is predicted to be used [24]. Research has discovered that a higher concentration of CO2 (5-20%) may increase the biomass in a short period [24].

4. The existing problems & prospects

4.1. The problems caused by strain

As a type of *C. reinhardtii* UVM4 strain, UPN22 cannot form a complete cell wall [28], making the outer gene easily get into the cells and the products can be released outside the cell membrane with no major hindrance. However, genes related to cell wall synthesis still exist, and their expression products are also released outside the cell membrane [37]. This leads to the need for suitable methods to separate isoprene from culture systems.

As mentioned in **3.2**, the UPN22 strain could be selected from the wild type of C. reinhardtii using a special nitrogen source instead of soybean meal. The current problem is that the success of other introduced genes (IspS and DXS synthase) cannot be determined through cultivation. This is because *C. reinhardtii* originally has the relevant genes. The new idea envisioned in this paper is to overexpress these genes, which is the most direct way to increase the proportion of relevant genes in the total genome.

As for how to improve it, it can be solved by constructing nutritionally deficient strains. The specific steps are as follows. First, we screened out genes related to metabolism in Chlamydomonas reinhardtii, such as genes for the synthesis of a key enzyme (avoiding genes related to photosynthesis, because using Chlamydomonas to absorb carbon dioxide and achieve carbon neutrality is the core idea of this design). After the cutting site is determined, the gene to be overexpressed is inserted into the cut position through the designed primers, so that the original gene sequence cannot function normally. In this case, the nutritional deficiency type discovered during the screening process is likely to be the desired special strain that has been introduced with the relevant gene sequence, which can increase the rate of the MEP pathway in the cell.

In addition, at present, the most serious problem comes from the unclear interaction between algal genomes, so more detailed research is needed to verify the above speculation, some of which have been found to increase isoprene production in *E. coil* [24,28].

4.2. The problems from the collection process

Given the boiling point of isoprene, once isoprene is produced, it can be collected from the headspace of the culture using a gas collection system. This typically involves trapping the isoprene in a solvent and then analyzing it using a gas chromatograph. The isoprene trapped in the solvent can then be extracted using a suitable method. For example, liquid-liquid extraction or solid-phase microextraction (SPME) can be used [38].

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5. Conclusion

Overall, the MEP pathway in microbial cells has been shown to be able to synthesize a variety of terpenoids, including isoprene. And *C. reinhardtii* UPN22 may be a more suitable host cell than *E. coil* for running the MEP pathway. The advantage of this strain is that it can potentially reduce carbon emissions from fuel production to combustion, which is a highly sustainable method. There are two strategies to improve the efficiency of the MEP pathway. One is to inhibit or overexpress some enzyme genes through genetic engineering, and the other is to create an external environment with high cell activity or high enzyme activity. However, there is a large gap in the research on microalgae biosynthesis, and the hypothesis lacks strong evidence. The extraction and separation of isoprene needs further study.

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