Whitepaper

Producing terpenes naturally and sustainably using microorganisms

EV BIOTECH

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Microbially produced terpenes

We can use microorganisms to produce terpenes.

These terpenes are natural and have a much higher terpene yield than naturally extracted terpenes, making fermentation a more sustainable and economically viable production method.

Over the past five years, demand has grown tremendously for more sustainably sourced ingredients in different industries such as flavours and fragrances, food and feed, and cosmetics[1,2]. This growth is due to an increase in consumer awareness of the impact that their behaviour has on the environment. At the same time, these industries are asked to produce their products naturally, as consumers are concerned that synthetic products may be related to health issues. In response to these changes in demand, many companies are setting up new natural product lines and adopting new company goals, such as natural sourcing and sustainable supply chains.

Terpenes are a large group of chemicals that have multiple functionalities - making them interesting for various products in different industries, such as flavouring agents in the food industry or active ingredients in the cosmetics industry. About 55,000 different terpenes have been identified, with a broad spectrum of application and functionality. Currently, these molecules are mainly produced synthetically at a low cost, but change in consumer awareness is driving a growth in demand for naturally sourced terpenes[2]. In the flavours and fragrances industry, for example, the global market size for naturally sourced terpenes is expected to continue to grow with a CARG of 5.2% from 3,880 million dollar in 2019 to 4,990 million dollar in 2024[2].

Terpenes can be extracted naturally from plant material, but the yield of terpenes per kg of material is very low. Hence, terpene plant extraction is not the most economically attractive of producing natural flavour or fragrance ingredients. Next to natural extraction and chemical synthesis, a third technology to produce terpenes is microbial fermentation.

These microbially produced terpenes are natural and have a much higher terpene yield than naturally extracted terpenes, making fermentation a more sustainable and economically viable production method.

EV Biotech has developed a platform microorganism for the production of terpenes, which produces the basic substrates for terpenes. Upon a client's request, production can be tuned towards a specific terpene by making only a few genetic alterations to this organism. This results in a broad range of different terpenes produced by EV Biotech's platform microorganism.

In a similar way as we utilise microorganisms to brew beer, we can use microorganisms to produce terpenes.

Terpenes and their functionalities

The diversity in functionality within the group of terpenes makes them interesting to several industries.

Terpenes are highly volatile, meaning they can easily evaporate into the surroundings which makes them interesting as fragrance ingredients or flavour enhancers.

Terpenes are a large group of secondary metabolites mostly originating from plants[4,5]. They have been used for many years for their medical properties, including anticancer, anti-inflammatory and antioxidative, as well as for pleasant scents caused by their volatile nature.

Terpenes are divided into different groups depending on the amount of carbon units (e.g., the length of the molecule).

The established groups are the monoterpenes, sesquiterpenes, diterpenes, sesterpenes, and triterpenes. Monoterpenes dominate the global terpene market for the flavours and fragrances industry.
The expected market of monoterpenes will grow from 2,790 million dollar in 2019 to 3,450 million dollar in 2024, with a CAGR of 4.3%[2]. Main drivers of this growth are geraniol, linalool, menthol and citral.

Flavours and Fragrances

Plants can produce pleasantly scented terpenes to attract pollinators and seed dispersers[8]. Linalool for example is a terpene that is used in the fragrance industry for its lavender or bergamot odour[2]. Another example is L-carvone, a terpene with a spearmint character that is used in the flavour industry in chewing gum, mouth wash and beverages[2].

Pharmaceutical Ingredients

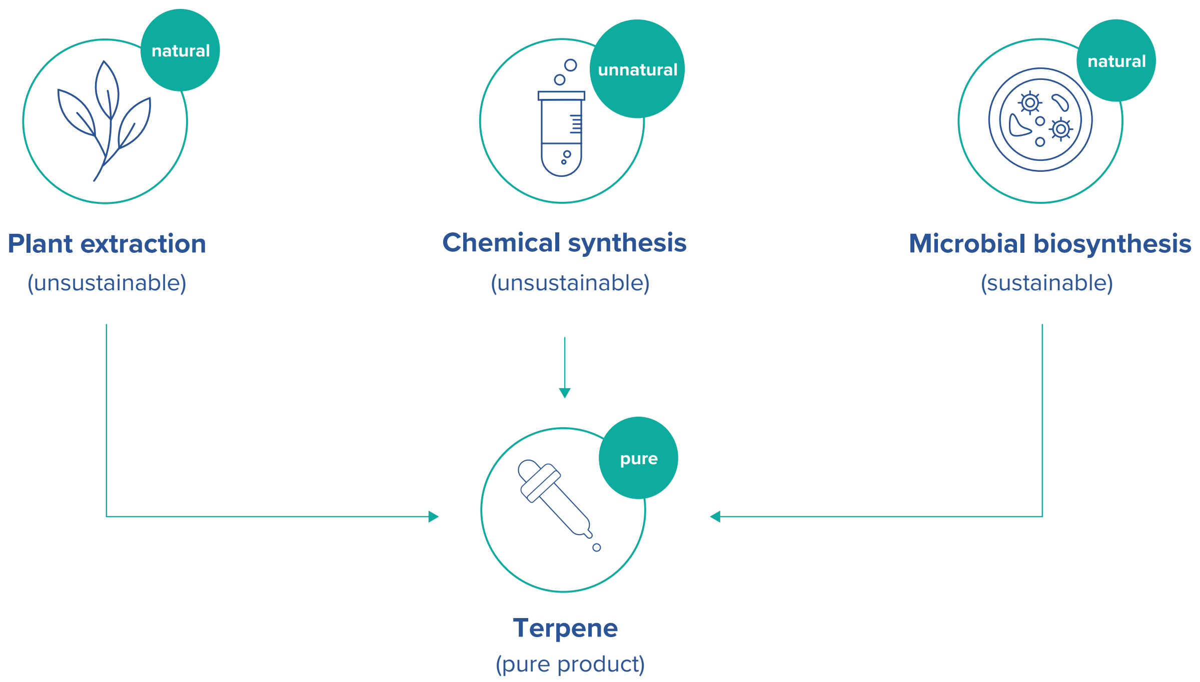
Some terpenes have antibacterial and antifungal properties, which can protect the plant against intruders. These properties make terpenes useful as active ingredient in medicines, cosmetics, and animal feed. Due to the broad range of terpenes, there is also a broad range of antibacterial mechanisms of action[6]. The best known terpene with a medical function is artemisinin, an antimalarial drug that inhibits the growth of the parasite that causes malaria[4]. Another terpene with pharmaceutical properties is nerol, often used in skin care products for its regenerative qualities and its antiseptic, antibacterial, anti-inflammatory nature[7]. This makes nerol an interesting active ingredient for the treatment of acne and scar tissue.

Biofuel

A few terpenes have been studied as potential alternative biofuel, as they have properties similar to diesel fuel. Bisabolane is a terpene that has comparable fuel properties as D2 diesel[9]. Bisabolane even has a higher energy density per volume than D2 diesel, making it an interesting potential biofuel9. Other examples of terpenes for fuel purposes are farnesene[9] and sabinene[10].

Terpene production

Terpenes can be produced in three different ways: via plant extraction, through chemical synthesis, or via microbial biosynthesis[8].



Plant extraction

Terpenes can be distilled from oils obtained by plant extraction, which are called essential oils[4]. Extraction of terpenes from plant material has a very low yield and low purity, making the production unsustainable and uneconomical[11]. For example, the yield of terpenes from lavender such as linalool is 0.3-0.8%, meaning that for 1 kg of terpenes from lavender approximately 120-300 kg of lavender is required[3]. The yield of terpenes from Rose geranium such as geraniol is even less, only 0.1%. This means that the production of 1 kg of terpenes from Rose geranium requires 1000 kg leaves of the plant[3]. In addition, terpene extraction from plants results in a mixture of different terpenes that requires purification. Another downside of using botanical sources for the extraction of terpenes is the dependence on harvest, growth season and distribution from across the world[12].

Chemical synthesis

Terpenes can also be built from small and simple chemicals from a petrochemical feedstock using chemical synthesis[13]. This technique is complicated and time consuming due to the complex structure of terpenes[11]. Often dirty reagents such as organic solvents are required in the process. Additionally, the products created via chemical synthesis are not seen as natural products, and therefore not suitable to meet the growing demand for natural and sustainable ingredients.

Microbial biosynthesis

Using microbial biosynthesis for the production of terpenes would make this process more environmentally friendly as it uses renewable resources instead of petrochemical feedstock[11].

In addition, a recent carbon footprint analysis* showed that EV Biotech's fermentation-based production method is more sustainable than botanical extraction of terpenes[14].

This study showed that EV Biotech's terpene production is five to eleven times more sustainable than botanical extraction with low to high nitrogen fertilisation respectively, making microbial fermentation the most sustainable production method of producing terpenes.

The final product produced by microorganisms is considered "natural" according to the EC No 1334/2008 regulation of the European parliament for food additives and according to COSMOS standard for cosmetics[15–17]. Terpenes produced using fermentation would therefore meet the demand for natural products as well as the search for more sustainable products.

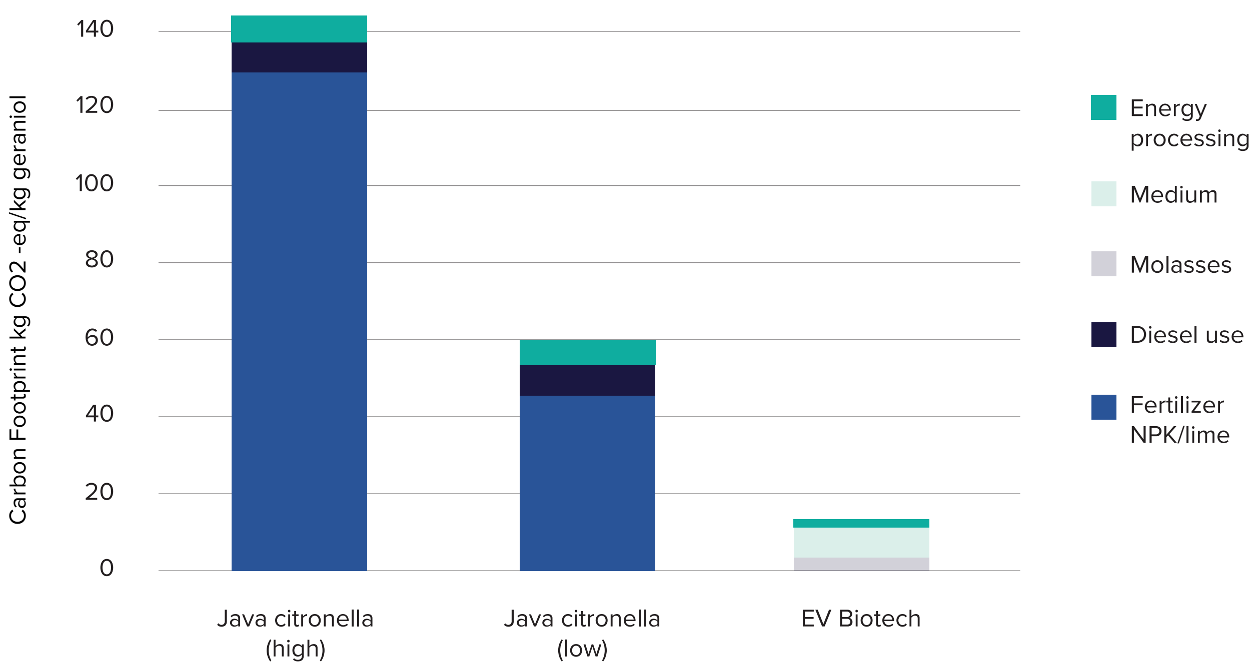


Figure 1: Carbon footprint of geraniol production

Terpenes at EV Biotech

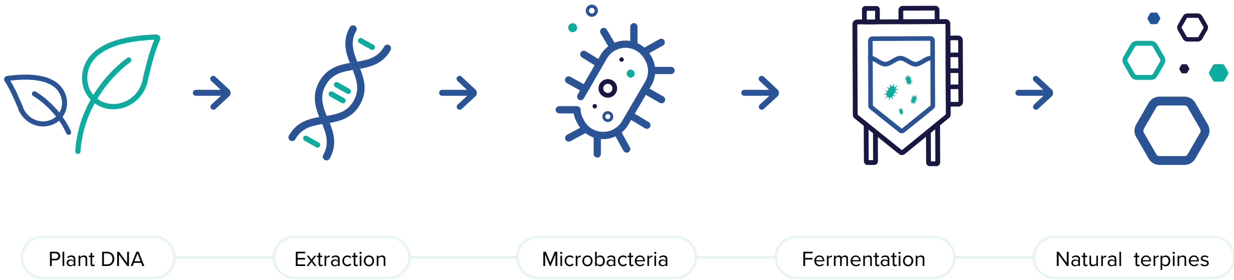
At EV Biotech we have developed a terpene plug-and-play system: a microorganism that produces the building blocks for terpenes and with a few alterations can produce specific, more complex terpenes.

At EV Biotech we have developed a terpene plug-and-play system: a microorganism that produces the building blocks for terpenes and with a few alterations can produce specific, more complex terpenes. The required components for producing terpenes are already present in the microorganism itself. For converting these starting components to terpenes, we utilise enzymes that originate from the most efficient terpene producers in nature: plants. These enzymes are built into the DNA of the microorganism, transforming it into a producer of the desired terpene.

The plug-and-play system can be utilised to produce many different terpenes by utilising the genes of different organisms for different enzymes.

Pinene, for example, is a monoterpene that is produced by many pine trees and is responsible for the distinct pine scent. To produce pinene with the terpene plug-and-play system, the microorganism needs to produce an enzyme that can convert the building

Extraction of natural terpine



blocks into pinene. The responsible enzyme is pinene synthase, which can be found in the Sitka spruce, a large conifer. The genes correlating to this enzyme were built into the genome of the microorganism, resulting in pinene production by the microorganism. Following the same principle, EV Biotech has created microorganisms that produce different types of terpenes such as limonene or geraniol by using enzymes from plant sources. Limonene is produced by using the enzyme limonene synthase, which can be found in different citrus species. Geraniol is produced with a geraniol synthase enzyme found in sweet basil.

It all sounds relatively simple, while in fact the process is much more difficult. There are multiple enzymes for the conversion of a specific terpene, so how does one know which one will be the most successful? If the enzymes do not belong in the microorganism, will they influence the survival and what is the right balance to minimise this effect? Nutrients fed to the microorganism can have an effect on the yield, which nutrients will give the highest yields? Some enzymes produce by-products, will these by-products compromise the final product?

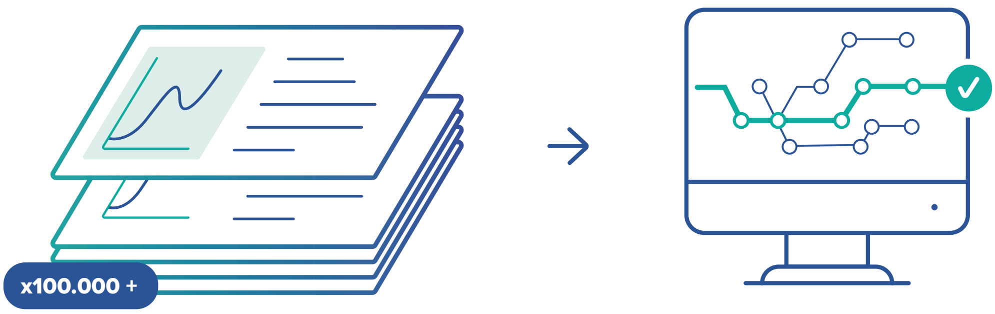
These are only a few examples of questions and hurdles, overcoming them could take a lot of time and resources. At EV Biotech, we solve them by using several proprietary computational tools that give insight in which strain engineering strategies have the highest chance of success. The tools reduce the time and effort needed in laboratory experiments by 90%. In addition, the modelling tools can foresee the different bottlenecks that could slow down the process, providing answers to the aforementioned questions.

Our technology

At the core of EV Biotech's technology is the search for a global optimum of the strain, rather than identifying local optima for each different parameter separately.

The synergy between the computational (dry) lab and the laboratory (wet) lab allows to use output of experimental data as input for the computational tools directly, improving the system continuously to make better predictions about the most optimal strain. In this way, omics data creates a multidimensional model. The feedback loop of data between the dry lab and the wet lab is the magical recipe that will optimise the microorganisms to produce the purest product, have the highest yields and the most optimal titres, all tailored to the needs of the clients.

The first terpenes developed with the plug-and-play system are monoterpenes, as market research showed that monoterpenes dominate the terpene market. The first terpenes produced with the plug-and-play system were **limonene and pinene**.
Limonene is a monoterpene that is used in pharmaceuticals, as flavouring agent in food and beverages, and as fragrance in household products, cosmetics, and personal hygiene products. Pinene is another monoterpene, used as the main ingredient of turpentine and in many perfumes and personal



All omics data generated by our laboratory experiments are looped back into our models to improve them in finding the most optimal organism and pathway towards the desired end product.

care products. Currently, we are working on the production of **geraniol**, a monoterpene that smells like roses and can be extracted from rose oil, palmarosa oil, and citronella oil. Geraniol is used in cosmetics as fragrance or tonic, and can be used as insect repellent.

The variety of functionality of terpenes is what makes EV Biotech's terpenes plug-and-play system interesting for different industries and clients.

Currently, EV Biotech is working together with Delft Advanced Biorenewables (DAB bio) to develop both a process and a strain which can produce Geraniol at (pilot) scale.

As some terpenes have antimicrobial effects in a higher concentration, DAB bio's advanced bioreactor technology (FAST) reduces terpene inhibition on the microbe thereby increasing the productivity of the process. Together with DAB, we aim to facilitate a more efficient R&D process to ensure a higher success rate.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Unit 1 - Summary
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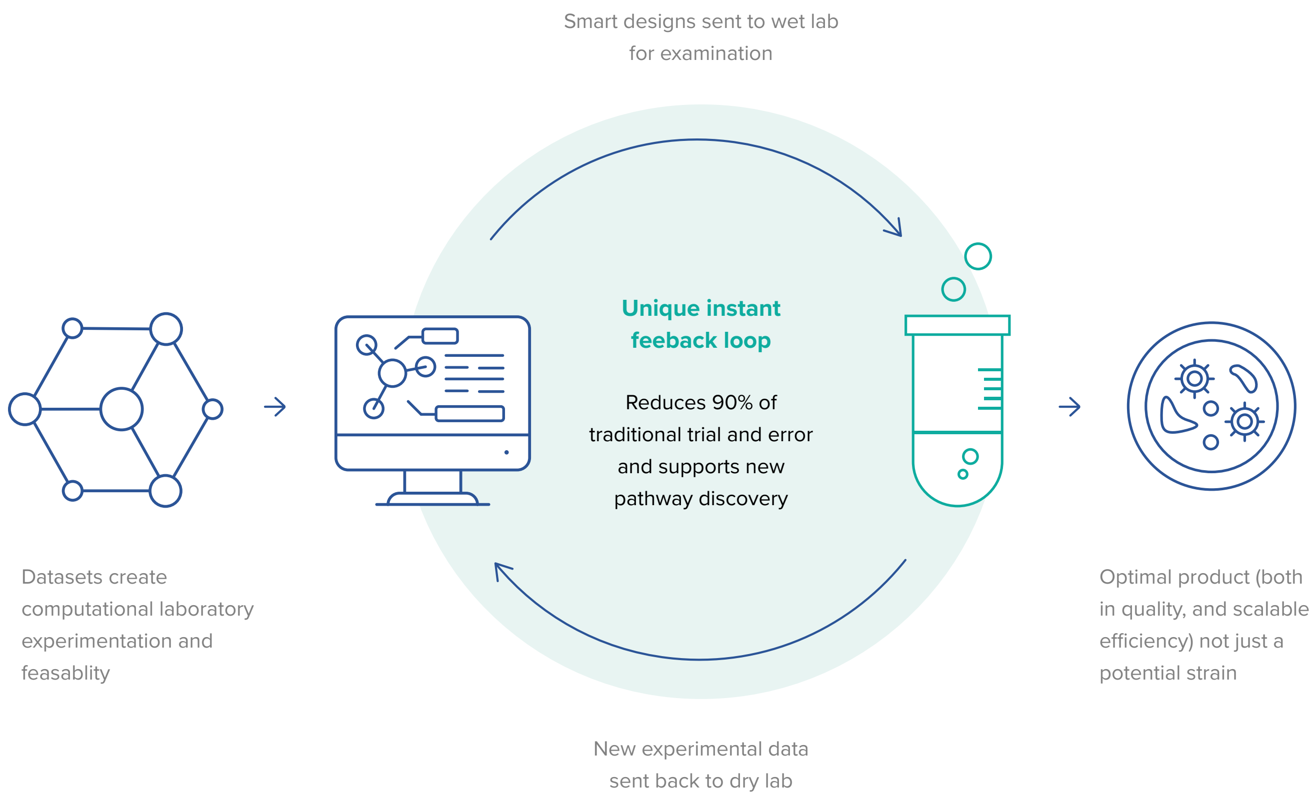
Customer journey

Developing a strain for the production of specific terpene at EV Biotech is tailored to the needs of a client.

Different properties such as the preferred organism, fermentation capacity or toxicity of the terpene are taken into account from the start. The process of strain development at EV Biotech has three stages:

1. Feasibility
2. Development
3. Support



Feasibility Study

The project will initially start with the Feasibility Study. This results in a feasibility report outlining risk estimation and a mitigation plan per different phase, project timelines, a patent scan, a techno-economic analysis, and the modelling/strain engineering strategy. The latter is a result of modelling all possible strategies and comparing them on different key performing indicators to select the highest performing strategies. As our parameters are tailored to meet the client's request and capacity, we aim to provide a thorough risk assessment on whether it is worthwhile continuing to the next phase of EV Biotech development. Following the feasibility report, a go/no go decision is to be made before the next stage is started.

Development

The second stage is the strain Development. During this stage there is a constant feedback loop between the computational modellers and the strain engineers.
This interplay leads to the optimisation of the microorganism and the production of the ingredient. During the whole development stage there is close contact with the client, deciding after each iteration if the project continues to the next round.

Support

The third and the last stage is when the strain is handed over to the client: Support. During this stage the optimised microorganism is transferred to the client and EV Biotech organises comprehensive tech transfer and consultancy. Additionally, EV Biotech offers optional scale-up optimisation and support packages.

As a client, you are in control of the project and our services are fine-tuned to ensure your demands are met.

Curious to know how we can offer tailored strain engineering solutions for your next R&D project?

Contact our Business Developer, Ronja Wabeke at r.wabeke@evbio.tech

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Our focus at EV

Ability to develop strains for a boundless repertoire of biobased compounds.

EV Biotech's current portfolio contains several different chemical compounds. Our computational tools remove the restrictions to produce certain molecules naturally. We can find the most optimal pathway to produce any compound, even if these pathways are not naturally occurring in the host microorganism.

Time of strain development is cut in half, from 6 to 3 years.

90% of laboratory experiments are substituted by digital experiments which can run many experiments in parallel, speeding up the process. Additionally, our computational modelling tools can predict bottlenecks and find solutions before time is wasted on it in the real laboratory.

Cost of strain development is cut in half.

Less experiments translate to less resources, less working hours and therefore less costs.

Successful output for working strains is increased.

Multiple rounds of optimisation lead to the most optimal strains with the desired yield and biomass.

Minimal use of resources.

Having less experiments minimises the use of resources, contributing to a more sustainable use of resources.

Green chemical production method with natural and non-GMO products

Microbial biosynthesis is the most sustainable production method for terpene production. Terpenes produced in this way are both natural and non-GMO[16,17].

Appendix

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