Enzymes and methods for fermentative production of monoterpene esters

The present ~~invention~~disclosure relates to an alcohol acyl transferase which is capable of esterifying a tertiary monoterpene alcohol such that at least 30% by mass of said tertiary monoterpene alcohol is esterified, preferably within 36 h, 24 h, 18 h, 12 h, 6 h, 3 h, 2 h, 1h, 45 min or 30 min, more preferably in a microbial cell. The ~~invention~~disclosure further relates to a nucleic acid comprising a nucleic acid sequence encoding the alcohol acyl transferase of the ~~invention~~disclosure, or a complementary sequence thereof, and a vector or gene construct comprising the nucleic acid of the ~~invention~~disclosure. Further provided by the present ~~invention~~disclosure is a host cell comprising the vector or gene construct of the ~~invention~~disclosure, and a transgenic non-human organism comprising the nucleic acid of the ~~invention~~disclosure, the vector or gene construct of the ~~invention~~disclosure, or the host cell of the ~~invention~~disclosure. The ~~invention~~disclosure also concerns a method for preparing a monoterpene ester, comprising esterifying a monoterpene alcohol to a monoterpene ester, in the presence of an alcohol acyl transferase of the ~~invention~~disclosure. Specifically, it provides a method for preparing linalyl acetate, comprising esterifying linalool to linalyl acetate, in the presence of an alcohol acyl transferase of the ~~invention~~disclosure. The ~~invention~~disclosure further pertains to the use of the alcohol acyl transferase of the ~~invention~~disclosure, the nucleic acid of the ~~invention~~disclosure, the vector or gene construct of the ~~invention~~disclosure, the host cell of the ~~invention~~disclosure, or the transgenic non-human organism of the ~~invention~~disclosure (i) for heterologous reconstitution of a terpene biosynthetic pathway; (ii) for producing an industrial product, preferably a flavour or fragrance, a biofuel, a fuel composition, a fuel compound, e.g., a blowing agent for diesel fuel compositions, a pesticide, an insect repellent or an antimicrobial; (iii) for producing an aliphatic and/or aromatic monoterpene ester from a monoterpene alcohol, preferably from a tertiary monoterpene alcohol; (iv) for detoxifying a monoterpene alcohol in a microorganism, thereby increasing monoterpene production in said microorganism; (v) in combination with a GPP synthase and/or S- or R- linalool synthase; (vi) for increasing the beneficial effects of acetylation in that the hydrophobic acetate partitions more readily go into an organic phase, as compared to the monoterpene alcohol; (vii) for expressing the alcohol acyl transferase of the ~~invention~~disclosure such that the ratio of monoterpene acetate to monoterpene alcohol is greater than 5:1 or 10:1 or (viii) in a microbial production system for monoterpene esters. The ~~invention~~disclosure also provides a kit comprising the alcohol acyl transferase of the ~~invention~~disclosure, the nucleic acid of the ~~invention~~disclosure, the vector or gene construct of the ~~invention~~disclosure, the host cell of the ~~invention~~disclosure, or the transgenic non-human organism of the ~~invention~~disclosure, and optionally at least one monoterpene alcohol, preferably a tertiary monoterpene alcohol. Finally, the ~~invention~~disclosure relates to a method for the production of a fuel and/or biolubricant compound, wherein the method comprises the steps of: a) Producing one or more monoterpene esters by any one of the methods of the ~~invention~~disclosure; b) optionally, purifying the one or more monoterpene esters produced in step a); and c) converting part or all of the one or more monoterpene esters of step a), or the optionally purified one or more monoterpene esters of step b), to one or more fuel and / or biolubricant compound , preferably selected from the group consisting of: tetrahydrolinalool; 2,6-dimethyloctane (DMO); saturated C20 hydrocarbon dimers; saturated C30 hydrocarbon trimers; hydrogenated methylcyclopentadiene dimers; saturated high density multi-cyclic hydrocarbon compounds suitable for missile propulsion; and hydrogenated C40+ oligomers suitable to produce biolubricant additives; d) optionally combining the one or more fuel or biolubricant

compound with additional compounds suitable for a fuel and / or biolubricant; wherein the fuel and / or biolubricant composition has in sum between and including 0.01 % (w/w) to 99.99 % (w/w), of the fuel or biolubricant compound produced from the one or more monoterpene esters obtainable by one of the methods of the ~~invention~~disclosure.

During the past few decades, intense scientific research focused on the most abundant secondary metabolites in all living organisms, the terpenes. More than 55,000 terpenoid substances are widely distributed among different families of natural products found in all biological kingdoms.

Many terpenoids are secondary metabolites as they are commonly, not primarily, essential for growth, development, or reproduction of any organism. However, this classification does not expand on the broad additional effects of these secondary metabolites that keep an ecosystem functioning. These substances play important roles and may provide plants with evolutionary advantages in relation to their distinct chemosensory properties such as smell. Amongst others, they may exert insecticidal effects thus protecting plants and crops against pests and pathogens, or may act as pollinator attractants in reproductive processes.

Many terpenoids are renowned for their economic importance being widely used as base structural moiety in the production of drugs, flavours, fragrances, pigments, and disinfectants. For example, the monoterpene alcohol linalool which is the main essential oil constituent of rosewood, Aniba rosaeodora, is among the most frequently used ingredients in perfume production. In addition, the sesquiterpene lactone, artemisinin, extracted from the shrub Artemisia annua, is used in the first-line treatment of malaria. The tricyclic diterpene taxol, isolated from the bark of the Pacific yew tree, Taxus brevifolia, and its structural analogs, are used as anticancer agents.

Terpenes are primarily synthesized in plants via common biosynthetic routes. In spite of their diverse structures and functions, all terpenes are built up of isoprene units (five- carbon atoms) following the isoprene rule. According to the number of isoprene units in their structure which are connected through head-to-tail addition, terpenes are classified according to their number of carbon atoms or sesquiterpenoid moieties, respectively: monoterpenes (C10), sesquiterpenes (C15), diterpenes (C20), triterpenes (C30), or polyterpenes having up to 30,000 connected isoprene units. Just like terpenes, terpenoids are likewise classified according to the number of isoprene units they are constituted of and are further named with the suffix -oids, as in monoterpenoids (C10), or in sesquiterpenoids (C15).

Isopentyl diphosphate (IPP) and its electrophilic isomer, dimethylallyl diphosphate (DMAPP), are the universal precursors in the biosynthesis of terpenes. Starting from these two building blocks, linear prenyl diphosphates are synthesized by a group of enzymes belonging to the prenyltransferases. IPP and DMAPP are condensed by the catalytic effect of the

difficult to access by enzyme active site pockets, possibly because of the steric accessibility of the alcohol group.

Linalyl acetate (CAS number 115-95-7) is an acetyl ester typically formed from linalool. So far, linalyl acetate is produced chemically, and no fermentative large-scale processes for producing linalyl acetate have been reported yet; see, e.g., https://de.wikipedia.org/wiki/Linalylacetat. Linalyl acetate is an important flavour and fragrance molecule. However, an alcohol acyl transferase which is capable of esterifying linalool or other tertiary monoterpene alcohols in economically significant amounts has not yet been identified. In a recent study, trace activity on linalool has been reported for a BAHD acyl transferase from common thyme (Thymus vulgaris), when transiently expressed in Nicotiana benthamiana (doctoral thesis “Die Biosynthese von acetylierten Monoterpenen in Thymian (Thymus vulgaris)” by Natalie Wiese, June 2020; https://opendata.uni-halle.de/handle/1981185920/34248). However, such a trace activity with small side production of linalyl acetate in very specific experimental settings can disappear under conditions common in biocatalytic or biotechnological production on an industrial scale, or simply be unsuited for large-scale production or uneconomical for such use.

Consequently, producing linalyl acetate by biocatalysis or by biotechnological approaches is hindered by the lack of an alcohol acyl transferase which is capable of esterifying tertiary monoterpene alcohols, e.g. linalool, in economically significant quantities.

Thus, the technical problem underlying the present ~~invention~~disclosure may be seen as the provision of means and methods complying with the aforementioned needs. The technical problem is solved by the ~~embodiments~~ examples characterized in the claims, herein below and the Examples.

In an aspect, there is provided an alcohol acyl transferase, comprising an amino acid sequence selected from the group consisting of:

a) an amino acid sequence as shown in any one of the sequences of SEQ ID NO: 2, SEQ ID NO: 15 or SEQ ID NO: 16;

b) an amino acid sequence having alcohol acyl transferase activity with i) at least 89% sequence identity at the amino acid level with SEQ ID NO: 2, or ii) having at least 60% sequence identity with SEQ ID NO: 15 or SEQ ID NO: 16, having alcohol acyl transferase activity; and

c) a fragment of the amino acid sequence of a) or b),

wherein the alcohol acyl transferase is capable of esterifying a tertiary monoterpene alcohol such that at least 30 % by mass of said tertiary monoterpene alcohol is esterified.

In another aspect, there is provided a nucleic acid comprising a nucleic acid sequence encoding the alcohol acyl transferase as defined above, or a complementary sequence thereof

In another aspect, there is provided a vector or gene construct comprising the nucleic acid as defined above.

In another aspect, there is provided a host cell comprising the vector or gene construct as defined above, wherein the host cell is preferably a bacterial cell, a yeast cell, a fungal cell, an algal cell or a cyanobacterial cell, a non-human animal cell or a non-human mammalian cell, or a plant cell, more preferably a bacterial cell, a yeast cell, or a fungal cell.

In another aspect, there is providd a transgenic non-human organism comprising the nucleic acid as defined above, the vector or gene construct as defined above, or the host cell as defined above.

In another aspect, there is provided a method for preparing a monoterpene ester, comprising esterifying a monoterpene alcohol to a monoterpene ester, in the presence of

(i) an alcohol acyl transferase

a) having an amino acid sequence as shown in any one of the sequences of SEQ ID NO: 2, SEQ ID NO: 15 or SEQ ID NO: 16; or

b) having an amino acid sequence with at least 60% sequence identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 15 or SEQ ID NO: 16, having alcohol acyl transferase activity; or

c) a fragment of the amino acid sequence of a) or b), having alcohol acyl transferase activity;

or

(ii) an alcohol acyl transferase comprising an amino acid sequence as shown in database accession number XP_006493396 (SEQ ID NO: 13) or UNIPROTKB-A0A2H5PUP1 (SEQ ID NO: 14), or an amino acid sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the amino acid sequence as shown in database accession number XP_006493396 (SEQ ID NO: 13) or UNIPROTKB-A0A2H5PUP1 (SEQ ID NO: 14), wherein the alcohol acyl transferase is capable of esterifying a monoterpene alcohol to a monoterpene ester, or

(iii) an alcohol acyl transferase of (i) or (ii), wherein the amino acid corresponding to the amino acid at position 371 and 372 of SEQ ID NO: 2 is not Tryptophan, or

(iv) an alcohol acyl transferase of (i) or (ii) further comprising at the positions corresponding to the positions of SEQ ID NO: 2 indicated in Table 1 any of those amino acids listed in Table 1 for those positions, or at the positions corresponding to the positions of SEQ ID NO: 2 indicated in Table 2 those amino acids listed in Table 2, wherein the alcohol acyl transferase is capable of esterifying a monoterpene alcohol to a monoterpene ester;

(v) any alcohol acyl transferase of i) to iv) that is capable of esterifying a monoterpene alcohol such that at least 30 % by mass of said monoterpene alcohol is esterified, preferably within 36 h, more preferably in a microbial cell; or

(vi) any alcohol acyl transferase of i) to v) that is capable of esterifying a monoterpene alcohol such that at least 50 μg of monoterpene ester per minute and per gram of alcohol acyl transferase is produced, at 30 °C and at a pH in the range of 6.0 to 8.5, and under conditions where the substrates are not limiting.

In another aspect, there is provided a method for preparing linalyl acetate, comprising esterifying linalool to linalyl acetate, in the presence of

(i) an alcohol acyl transferase

a) having an amino acid sequence as shown in any one of the sequences of SEQ ID NO: 2, SEQ ID NO: 15 or SEQ ID NO: 16; or

b) having an amino acid sequence with at least 60% sequence identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 15 or SEQ ID NO: 16, having linalool acyl transferase activity; or

c) a fragment of the amino acid sequence of a) or b), having linalool acyl transferase activity;

or

(ii) an alcohol acyl transferase comprising an amino acid sequence as shown in database accession number XP_006493396 (SEQ ID NO: 13) or UNIPROTKB-A0A2H5PUP1 (SEQ ID NO: 14), or an amino acid sequence with at least 60%, at least 65%, at least 70%, at least 75%, at least 80%, at least 85%, at least 90%, or at least 95% sequence identity to the amino acid sequence as shown in database accession number XP_006493396 (SEQ ID NO: 13) or UNIPROTKB-A0A2H5PUP1 (SEQ ID NO: 14), wherein the alcohol acyl transferase is capable of esterifying linalool to linalyl acetate, or

(iii) an alcohol acyl transferase of (i) or (ii), wherein the amino acid corresponding to the amino acid at position 371 and 372 of SEQ ID NO: 2 is not Tryptophan, or

(iv) an alcohol acyl transferase of (i) or (ii) further comprising at the positions corresponding to the positions of SEQ ID NO: 2 indicated in Table 1 any of those amino acids listed in Table 1 for those positions, or at the positions corresponding to the positions of SEQ ID NO: 2 indicated in Table 2 those amino acids listed in Table 2, wherein the alcohol acyl transferase is capable of esterifying linalool to linalyl acetate; or

(v) any alcohol acyl transferase of i) to iv) that is capable of esterifying linalool such that at least 30 % by mass of said linalool is esterified, preferably within 36 h, more preferably in a microbial cell; or

(vi) any alcohol acyl transferase of i) to v) that is capable of esterifying linalool such that at least 50 μg of monoterpene ester per minute and per gram of alcohol acyl transferase is produced, at 30 °C and at a pH in the range of 6.0 to 8.5, and under conditions where the substrates are not limiting.

In another aspect, there is provided the use of the alcohol acyl transferase as defined above the nucleic acid as defined above, the vector or gene construct as defined above, the host cell as defined above, or the transgenic non-human organism as defined above,

(i) for heterologous reconstitution of a terpene biosynthetic pathway;

(ii) for producing an industrial product, preferably a flavour or fragrance, a biofuel, a fuel composition, a fuel compound, a pesticide, an insect repellent or an antimicrobial;

(iii) for producing an aliphatic and/or aromatic monoterpene ester from a monoterpene alcohol, preferably from a tertiary monoterpene alcohol;

(iv) for detoxifying a monoterpene alcohol in a microorganism, thereby increasing monoterpene production in said microorganism, wherein the microorganism is preferably a bacteria or a fungus;

(v) in combination with a GPP synthase and/or a S- or R- linalool synthase;

(vi) for increasing the beneficial effects of acetylation in that the hydrophobic acetate partitions more readily go into an organic phase, in comparison to the monoterpene alcohol;
(vii) for expressing the alcohol acyl transferase as defined above such that the ratio of monoterpene acetate to monoterpene alcohol is greater than 5:1 or 10:1; or
(viii) in a microbial production system for producing monoterpene esters.

In another aspect, there is provided a kit comprising the alcohol acyl transferase as defined above, preferably the alcohol acyl transferase as defined above, the nucleic acid as defined above, the vector and/or gene construct as defined above, the host cell as defined above, and/or the transgenic non-human organism as defined above, and optionally at least one monoterpene alcohol, preferably a tertiary monoterpene alcohol.

In another aspect, there is provided a method for the production of a fuel and/or biolubricant compound, wherein the method comprises the steps of:
a) Producing one or more monoterpene esters by any one of the methods as defined above;
b) optionally, purifying the one or more monoterpene esters produced in step a); and using part or all of the one or more monoterpene esters of step a), or the optionally purified one or more monoterpene esters of step b), as fuel and / or biolubricant compound(s); and / or converting part or all of the one or more monoterpene esters of step a), or the optionally purified one or more monoterpene esters of step b), to one or more fuel and / or biolubricant compound(s).

The present invention relates to an alcohol acyl transferase, comprising an amino acid sequence selected from the group consisting of:
a) an amino acid sequence as shown in any one of the sequences of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 15 or SEQ ID NO: 16;
b) an amino acid sequence with at least 60% sequence identity at the amino acid level with SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 15 or SEQ ID NO: 16, having alcohol acyl transferase activity; and
c) a fragment of the amino acid sequence of a) or b), having alcohol acyl transferase activity.
The alcohol acyl transferase of the invention can be a naturally occurring enzyme. In another embodiment the alcohol acyl transferase of the invention is a synthetic (interchangeably used are: artificial or non-naturally occurring) enzyme.

In one embodiment the alcohol acyl transferase of the invention is capable of esterifying a monoterpene alcohol, preferably a tertiary monoterpene alcohol such that at least 30 % by mass of said alcohol is esterified, preferably within 36 h, more preferably in a microbial cell, as defined herein.