THIOESTERASES AND THEIR USE

TECHNICAL FIELD

Disclosed are nucleotide sequences encoding thioesterase enzymes, methods of their production, and their use in methods of forming thioesters.

BACKGROUND

Thioesters are important flavouring compounds for, amongst other things, cheese, vegetable, meat and coffee products. Particularly useful thioesters include, but are not limited to, methyl butanethioate, (hereinafter “MTB”), methyl thioacetate (hereinafter “MTA”) and methyl thiopropionate (hereinafter “MTP”).

Thioesters can be made synthetically. However, because of food trends, and health and wellness concerns, there is particular demand for flavour compounds, such as thioesters, that are either obtained directly from natural products, or produced via biological processes. A particular advantage of such compounds is that they may be termed “natural” and potentially labeled as such on consumable products.

It is known that various bacteria produce thioesters during fermentation. However, the yields and product ratios of these existing processes are difficult to influence.

Accordingly, it would be beneficial to develop a more predictable and potentially more economically viable method of producing thioesters that could be used in consumable products and labeled as “natural”.

DETAILED DESCRIPTION

The applicant has now identified nucleotide sequences encoding thioesterase enyzmes.

This finding enables the nucleotide sequences to be used in methods enabling the efficient production of thioesters that can be added to consumable products and labeled as “natural”. Furthermore, these nucleotide sequences may be used in screening methods to identify other wild type bacteria capable of forming thioesters.

According to a first illustrative embodiment, there are provided nucleotide sequences, encoding enzymes having thioesterase activity comprising the nucleotide sequences, or functional equivalents thereof, disclosed in SEQ.ID. Nos.1, 3, 5, and 7.

Without limitation, and only by way of illustration, the nucleic acids may be isolated from bacteria belonging to the Brevibacterium family. According to certain embodiments, the nucleic acids may be isolated from bacteria belonging to the Brevibacterium Linens species, a non limiting examples is strain American type culture collection (herein after ATCC) 9174 (BL2).

Functional equivalents of the nucleotide sequences include those nucleotide sequences that by virtue of the degeneracy of the genetic code possess a different nucleotide sequence to those disclosed herein, but that encode the same amino acid sequence with the same activity.

Functional equivalents encompass naturally occurring variants of the sequences described herein as well as synthetic nucleotide sequences, e.g., those nucleotide sequences that are obtained by chemical synthesis or recombination of naturally existing DNA. Functional equivalents may be the result of, natural or synthetic substitutions, additions, deletions, replacements, or insertions of one or more nucleotides.

Examples of functional equivalents include those nucleic acid sequences comprising a sense mutation resulting from the substitution of at least one conserved amino acid which does not lead to an alteration in the activity of the polypeptide, and thus can be considered functionally neutral.

Conservative substitution tables providing functionally similar amino acids are well known in the art. For example, one exemplary guideline to select conservative substitutions includes (original residue followed by exemplary substitution): ala/gly or ser; arg/lys; asn/gln or his; asp/glu; cys/ser; gln/asn; gly/asp; gly/ala or pro; his/asn or gln; ile/leu or val; leu/ile or val; lys/arg or gln or glu; met/leu or tyr or ile; phe/met or leu or tyr; ser/thr; thr/ser; trp/tyr; tyr/trp or phe; val/ile or leu. An alternative exemplary guideline uses the following six groups, each containing amino acids that are conservative substitutions for one another: 1) Alanine (A), Serine (S), Threonine (T); 2) Aspartic acid (D), Glutamic acid (E); 3) Asparagine (N), Glutamine (Q); 4) Arginine (R), Lysine (1); 5) Isoleucine (I), Leucine (L), Methionine (M), Valine (V); and 6) Phenylalanine (F), Tyrosine (Y), Tryptophan (W). Another alternative guideline is to allow for all charged amino acids as conservative substitutions for each other whether they are positive or negative.

Individual substitutions, deletions or additions that alter, add or delete a single amino acid or a small percentage (for example up to 26%, up to 20%, up to 10%, or up to 5%) of amino acids in an encoded sequence are also considered to be functional equivalents.

Other non-limiting examples of functional equivalents include fragments, orthologs, splice variants, single nucleotide polymorphisims, and allelic variants.

Such functional equivalents will have 60%, 75%, 80%, 90%, 95% or greater homology to the nucleotide sequences disclosed herein.

Nucleotide sequence homology may be determined by sequence identity or by hybridisation.

Sequence identity may be determined using basic local alignment search tool technology (hereinafter BLAST). BLAST technology is a heuristic search algorithm employed by the programs blastn which is available at http://www.ncbi.nlm.nih.gov.

If homology is determined by hybridisation, the nucleotide sequences should be considered substantially homologous provided that they are capable of selectively hybridizing to the nucleotide sequences disclosed herein.

Hybridisation should be carried out under stringent hybridisation conditions at a temperature of 42^{o}C in a solution consisting of 50% formamide, 5 x standard sodium citrate (hereinafter SSC), and 1% sodium dodecyl sulphate (hereinafter SDS). Washing may be carried out at 65^{o}C in a solution of 0.2xSSC and 0.1% SDS.

Background hybridization may occur because of other nucleotide sequences present, for example, in the cDNA. Any signal that is less than 10 fold as intense as the specific interaction observed with the target DNA should be considered background. The intensity of interaction may be measured, for example, by radiolabelling the probe, e.g. with 32P.

The nucleotide sequences may also comprise one or more of the following: a suitable 5' untranslated region, a promoter to enable expression in appropriate host cells, a suitable 3' untranslated region, a stop codon and tags.

Non limiting examples of tags include, but are not limited to, membrane export tags and tags used for detection of the thioesterases including, but not limited to, His tag, glutathione-S-transferase tag (GST).

The 5' untranslated region may comprise other operators or motifs that influence the efficiency of transcription or translation. The 3' untranslated region may comprise other signals such as a signal for transcriptional termination.

Non limiting examples of operators or motifs that influence transcription or translation include, but are not limited to, signals required for efficient polydenylation of the transcript, ribosome binding sites, recognition sites e.g. EcoR1.

It is well within the purview of the person skilled in the art to select suitable 5' and 3' untranslated regions, tags, stop codons, and operators or motifs that influence transcription or translation, depending on the host cells in question and the desired result. Non limiting examples are given in the examples included herein.

The nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof, may be used to produce thioesterase enzymes comprising the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalents thereof.

Expression of the enzymes with thioesterase activity comprising one or more of the amino acid sequences, or functional equivalents thereof, disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, may be effected by well established cloning techniques.

According to another illustrative embodiment, there is provided a host cell transfected with one or more of the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof. Such host cells are capable of expressing an enzyme with thioesterase activity comprising one or more of the amino acid sequences, or functional equivalents thereof, disclosed in SEQ.ID. Nos. 2, 4, 6, and 8.

Suitable host cells include prokaryote and eukaryotic cells of bacterial, fungal, plant or animal origin.

According to an illustrative embodiment the cells are bacterial or fungal cells.

In another illustrative embodiment the cells are selected from one or more of Escherichia coli, Brevibacterium, Cornynebacterium, Arthrobacter, Pseudomonas, Nocardia, Methylobacteri, Lactobacillus, Lactococcus, Streptococcus, Pediococcus, Oenococcus, Leuconostoc, Weisella, Carnobacterium, and Tetragenococcus. Proprionibacterium sp., Bifidobacterium spp., Enterococcus spp., Corynebacterium glutamicum, Arthrobacter *sp.,* Micrococcus luteus *and* Staphylococcus equorum. Geotrichum candidum, Yarrowia lipolytica, Kluyveromyces lactis, Debaryomyces hansenii, Saccharomyces cerevisiae.

Host cells may be transfected with the nucleotide sequences, or functional equivalent thereof, transiently or stably, as is well known in the art.

Any known method for introducing nucleotide sequences into host cells may be used. It is only necessary that the particular genetic engineering procedure used be capable of successfully introducing the desired nucleotide sequences, or functional equivalents thereof, into the host cell capable of expressing the desired amino acid sequences, or functional equivalents thereof. These methods may involve introducing cloned genomic DNA, cDNA, synthetic DNA or other foreign genetic

material into a host cell and include the use of calcium phosphate transfection, polybrene, protoplast fusion, electroporation, liposomes, microinjection, expression vectors, and the like.

Expression vectors, both as individual expression vectors or as libraries of expression vectors, comprising at least one nucleic acid sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalent thereof, may be introduced and expressed in a cell's genome, a cell's cytoplasm, or a cell's nucleus by a variety of conventional techniques.

It is well within the purview of the person skilled in the art to select a suitable technique.

According to an illustrative embodiment, expression vectors may be used to transfect host cells with the nucleic acid sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof.

In another aspect of the present invention there is provided a vector comprising at least one nucleotide sequence disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalent thereof.

Any suitable expression vector may be used. Non limiting examples of types of vectors include bacteriophage, plasmid, or cosmid DNA expression vectors; viral expression vectors, or bacterial expression vectors.

It is well within the purview of the person skilled in the art to select a suitable expression vector depending on the host cells in question and the desired effect.

In an illustrative embodiment the vector is selected from pBL, pET-22b(+), Pgem-5z, pGIV1.

After transfection, the transfected cells may be cultured using standard culturing conditions well known in the art.

It will be apparent to the skilled person that different cells require different culture conditions including appropriate temperature and cell culture media. It is well within the purview of the person skilled in the art to decide upon culture conditions depending on the cells in question and the desired end result.

In another aspect, there is provided a method of producing an enzyme with thioesterase activity comprising one or more of the amino acid sequences, or functional equivalents thereof, disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, comprising inserting a nucleotide sequence comprising at least one nucleotide sequence disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalent thereof, into a vector; transforming a host cell with the vector and growing the transformed host cell in a suitable culture medium.

As stated above, it will be apparent to the skilled person that different cells require different culture conditions including appropriate temperature and cell culture media. It is well within the purview of the person skilled in the art to decide upon culture conditions depending on the cells in question and the desired end result.

Information on appropriate culturing media and conditions with respect to certain cells may be found on the American type culture collection (ATCC) Website: http://www.lgcstandards-atcc.org/Home/tabid/477/Default.aspx

In a particular illustrative embodiment the cells used were Brevibacterium, Cornynebacterium or EColi cells and the culture medium was dairy or whey based.

In another particular illustrative embodiment the cells used were Brevibacterium, Cornynebacterium or EColi cells and the culture medium was selected from tripticase-soy, de Man-Rogosa-Sharpe (MRS), Elliker's, M17, nutrient broth or LB medium. Cells were incubated overnight at 37^{o}C.

In order to increase the yield of the thioesterase enzymes, the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof, may be over expressed by placing them under the control of a strong constitutive promoter.

It is well within the purview of the person skilled in the art to select a suitable constitutive promoter depending on the host cells and vector in question.

If desired the thioesterase enzymes comprising the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalents thereof, may be isolated from the culture medium using methods well known in the art.

The isolated thioesterase enzymes comprising at least one of the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalent thereof, or the transfected host cells comprising at least one of the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5 and 7, or functional equivalent thereof, may be used to produce thioesters.

In another aspect there is provided a method of producing thioesters comprising contacting at least one thioesterase enzyme comprising at least one amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalent thereof, and/or at least one host cells comprising at least one of the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5 and 7, or functional equivalent thereof, with at least one suitable substrate, incubating the mixture, isolating the crude product containing the thioester and, purifying the crude product to obtain only the thioester.

Incubation may be carried out at a temperature of 20^0C to 40^0C, at a pH of 4 to 9, for a time period ranging from 1 to 100 hours.

In an illustrative embodiment incubation is carried out at a temperature of 25^0C to 38^0C, at a pH of 6 to 8, for a time period ranging from 1 to 72 hours.
Short chain fatty acid coenzyme A derivatives have been found by the applicant to be suitable substrates for the thioesterase enzymes comprising at least one of the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalent thereof.

In an illustrative embodiment the suitable substrate comprises a short chain fatty acid coenzyme A (SCFA-CoA).

In another illustrative embodiment a suitable substrate comprises a C1-C8 SCFA-CoA.

The transfected host cells and/or enzymes of the present invention may be used in mobilised or immobilised form.

In an illustrative embodiment the enzymes and/or host cells are used in immobilised form.

Any purification technique may used to purify the crude product and it is well within the purview of the person skilled in the art to decide on such a technique. Non limiting examples of purification techniques include: affinity purification, centrifugation, chromatography.

In another aspect of present invention there is provided a thioester obtainable or produced by a method as described herein.

In another aspect there is provided a kit for producing thioesters comprising at least one thioesterase enzyme comprising at least one amino acid sequence disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalent thereof, and/or at least one host cells comprising at least one of the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5 and 7, or functional equivalent thereof, and at least one suitable substrate.

The kit may be used to carry out the method, as herein disclosed, for producing thioesters.

In an illustrative embodiment the suitable substrate comprises a short chain fatty acid coenzyme A (SCFA-CoA).

In another illustrative embodiment the suitable substrate comprises a C1-C8 SCFA-CoA.

The C1-C8 SCFA-CoA substrate may be provided in a concentration from 0.01μM - 500μM, 0.01μM - 200μM, 0.01μM -50μM.

It is known that the quantity and ratio of thioesters present in cheese affect its flavour. The thioesters are produced by microbes present in the cheese during its manufacturing and ripening. These microbes are often added to the cheese as starter cultures.

In another aspect there is provided a method of flavouring cheese comprising adding to a cheese during its manufacture process at least one thioesterase enzyme comprising at least one amino acid sequences disclosed in sequence ID numbers 2, 4, 6, and 8, or functional equivalent thereof, and/or at least one host cells comprising at least one of the nucleotide sequences disclosed in sequence ID numbers 1, 3, 5 and 7, or functional equivalent thereof.

In an illustrative embodiment the thioesterase enzyme(s) and/or host cell(s) are added as part of a starter culture.

In another illustrative embodiment the thioesterase enzyme(s) and/or host cell(s) are added as part of a starter culture added to milk.

In another aspect there is provided a cheese product obtainable by or produced according to the method herein above defined.

The nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof, may be used to screen and identify wild type organisms containing genes encoding the thioesterase enzymes encoded by the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalents thereof.

In another aspect there is provided the use of the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof, as markers in screening methods to screen for wild type organisms capable of producing the thioesterase enzymes encoded by the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalents thereof.

In another aspect there is provided a method of identifying organisms capable of producing the thioesterase enzymes encoded by the amino acid sequences disclosed in SEQ.ID. Nos. 2, 4, 6, and 8, or functional equivalents thereof, comprising using the nucleotide sequences disclosed in SEQ.ID. Nos. 1, 3, 5, and 7, or functional equivalents thereof, as markers in screening methods.

The organisms identified in the method may be any prokaryotic and eukaryotic organisms.

In an illustrative embodiment the organisms are wild type organisms.

Any known screening method may be used. Examples of well-known screening methods include, but are not limited to, computer driven nucleotide or protein homology searches of public sequence databases, nucleotide screening via high-throughput sequencing, and polymerase chain reaction (PCR) screening.

The thioesters formed by the methods disclosed herein may be isolated and purified for use as flavouring compounds in many compositions and consumable products.

In another aspect there is provided compositions comprising at least one thioester obtained or produced by the methods herein described.

In another aspect there is provided a method of creating, or modifying the flavour of a composition comprising adding to said composition, at least one thioester formed as disclosed herein.

The thioesters may be present or added into a composition in neat form or in a solvent, or they may first be modified, for example by entrapment with an entrapment material such as for example polymers, capsules, microcapsules, nanocapsules, liposomes, precursors, film formers, absorbents such as for example by using carbon zeolites, cyclic oligosaccharides and mixtures thereof, or they may be chemically bound to a substrates which are adapted to release the thioesters upon application of an exogenous stimulus such as light, enzyme, or the like.

One type of thioester may be the sole flavouring component of a composition. Alternatively, multiple types of thioesters may be used in combination.

The composition may additionally comprise other flavourant ingredients and excipients, for example carrier materials, conventionally used in flavour compositions.

Said other flavourant ingredients include, but are not limited to, natural flavours, artificial flavours, spices, seasonings, and the like. Exemplary flavouring ingredients include synthetic flavour oils and flavouring aromatics and/or oils, oleoresins, essences, distillates, and extracts derived from plants, leaves, flowers, fruits, and so forth, and combinations comprising at least one of the foregoing.

Further examples of other flavourant ingredients can be found in “Chemicals Used in Food Processing”, publication 1274, pages 63-258, by the National Academy of Sciences.

Said excipients conventionally used in flavour compositions include, but are not limited to, solvents (including water, alcohol, ethanol, oils, fats, vegetable oil, and miglyol), binders, diluents, disintegranting agents, lubricants, flavouring agents, coloring agents, preservatives, antioxidants, emulsifiers, stabilisers, flavour-enhancers, anti-caking agents, and the like.

Further examples of flavourant ingredients and excipients conventionally used in flavour compositions may be found in ”Perfume and Flavour Materials of Natural Origin”, S. Arctander, Ed., Elizabeth, N.J., 1960; in "Perfume and Flavour Chemicals", S. Arctander, Ed., Vol. I & II, Allured Publishing Corporation, Carol Stream, USA, 1994; in “Flavourings”, E. Ziegler and H. Ziegler (ed.), Wiley-VCH Weinheim, 1998, and “CTFA Cosmetic Ingredient Handbook”, J.M. Nikitakis (ed.), 1st ed., The Cosmetic, Toiletry and Fragrance Association, Inc., Washington, 1988.

Other suitable and desirable ingredients of flavour compositions are described in standard texts, such as “Handbook of Industrial Chemical Additives”, ed. M. and I. Ash, 2nd Ed., (Synapse 2000).

The thioesters formed as disclosed herein may be used in compositions at a concentration of up to 100%.According to certain embodiments, the thioesters may be included in the compositions at a concentration in the range of about 0.01% to about 99%. According to other embodiments, the thioesters may be included in the compositions at a concentration in the range of about 1% to about 99%.

In another aspect there is provided a method of creating, enhancing or modifying the flavour of a consumable product comprising adding to said consumable product at least one thioester formed as disclosed herein.

The thioesters formed as disclosed herein, or compositions containing at least one thioester as disclosed herein, can be added to consumable products by using conventional techniques to directly admix said thioesters or composition into the consumable product.

The quantities in which the thioesters formed as disclosed herein may be added to consumable products may vary within wide limits and depend, inter alia, on the nature of the consumable product, on the effect desired, the purpose of adding the thioesters formed as disclosed herein, to a consumable product, for example enhancing or creating a taste, and on the nature and quantity of any other components comprised in the consumable product, for example other flavour ingredients. It is well within the purview of the person skilled in the art to decide on suitable quantities of the thioesters formed as disclosed herein to incorporate into a consumable product depending on the end use and effect required.

Typical non-limiting concentrations, of the thioesters formed as disclosed herein, in ppm by weight based on the weight of the consumable product, are about 500 ppm to about 0.01 ppm, more particularly about 250 ppm to about 0.01 ppm, still more particularly about 100 ppm to about 1 ppm.

In another aspect there is provided a consumer product comprising at least one thioester obtained or produced by the methods herein described.

The term consumable product(s) as used herein means any product that is intended to be placed in the oral cavity and ingested, or to be used in the mouth and then discarded. Suitable consumable products include, but are not limited to, sauces, condiments, foodstuffs of all kinds, confectionery products, baked products, sweet products, savoury products including meat flavoured and meat products and vegetable flavoured and vegetable products, dairy products, beverages, oral care products and combinations thereof.

Exemplary foodstuffs include, but are not limited to, chilled snacks, sweet and savoury snacks, fruit snacks, chips/crisps, extruded snacks, tortilla/corn chips, popcorn, pretzels, nuts, other sweet and savoury snacks, snack bars, granola bars, breakfast bars, energy bars, fruit bars, other snack bars, meal replacement products, slimming products, convalescence drinks, ready meals, canned ready meals, frozen ready meals, dried ready meals, chilled ready meals, dinner mixes, frozen pizza, chilled pizza, soup, canned soup, dehydrated soup, instant soup, chilled soup, UHT soup, frozen soup, pasta, canned pasta, dried pasta, chilled/fresh pasta, noodles, plain noodles, instant noodles, cups/bowl instant noodles, pouch instant noodles, chilled noodles, snack noodles, dried food, dessert mixes, sauces, dressings and condiments, herbs and spices, spreads, jams and preserves, honey, chocolate spreads, nut-based spreads, and yeast-based spreads.

Exemplary confectionery products include, but are not limited to, chewing gum (which includes sugarised gum, sugar-free gum, functional gum and bubble gum), centerfill confections, chocolate and other chocolate confectionery, medicated confectionery , lozenges, tablets, pastilles, mints, standard mints, power mints, chewy candies, hard candies, boiled candies, breath and other oral care films or strips, candy canes, lollipops, gummies, jellies, fudge, caramel, hard and soft panned goods, toffee, taffy, liquorice, gelatin candies, gum drops, jelly beans, nougats, fondants, combinations of one or more of the above, and edible compositions incorporating one or more of the above.

Exemplary baked products include, but are not limited to, alfajores, bread, packaged/industrial bread, unpackaged/artisanal bread, pastries, cakes, packaged/industrial cakes, unpackaged/artisanal cakes, cookies, chocolate coated biscuits, sandwich biscuits, filled biscuits, savoury biscuits and crackers, bread substitutes,

Exemplary sweet products include, but are not limited to, breakfast cereals, ready-to-eat (“rte”) cereals, family breakfast cereals, flakes, muesli, other rte cereals, children's breakfast cereals, hot cereals,

Exemplary savoury products include, but are not limited to, salty snacks (potato chips, crisps, nuts, tortilla-tostada, pretzels, cheese snacks, corn snacks, potato-snacks, ready-to-eat popcorn, microwaveable popcorn, pork rinds, nuts, crackers, cracker snacks, breakfast cereals, meats, aspic,

cured meats (ham, bacon), luncheon/breakfast meats (hotdogs, cold cuts, sausage), tomato products, margarine, peanut butter, soup (clear, canned, cream, instant, UHT),canned vegetables, pasta sauces.

Exemplary dairy products include, but are not limited to, ice cream, impulse ice cream, single portion dairy ice cream, single portion water ice cream, multi-pack dairy ice cream, multi-pack water ice cream, take-home ice cream, take-home dairy ice cream, ice cream desserts, bulk ice cream, take-home water ice cream, frozen yoghurt, artisanal ice cream, dairy products, milk, fresh/pasteurized milk, full fat fresh/pasteurized milk, semi skimmed fresh/pasteurized milk, long-life/UHT milk, full fat long life/UHT milk, semi skimmed long life/UHT milk, fat-free long life/UHT milk, goat milk, condensed/evaporated milk, plain condensed/evaporated milk, flavoured, functional and other condensed milk, flavoured milk drinks, dairy only flavoured milk drinks, flavoured milk drinks with fruit juice, soy milk, sour milk drinks, fermented dairy drinks, coffee whiteners, powder milk, flavoured powder milk drinks, cream, yoghurt, plain/natural yoghurt, flavoured yoghurt, fruited yoghurt, probiotic yoghurt, drinking yoghurt, regular drinking yoghurt, probiotic drinking yoghurt, chilled and shelf-stable desserts, dairy-based desserts, soy-based desserts,

Exemplary beverages include, but are not limited to, flavoured water, soft drinks, fruit drinks, coffee-based drinks, tea-based drinks, juice-based drinks (includes fruit and vegetable), milk-based drinks, gel drinks, carbonated or non-carbonated drinks, powdered drinks, alcoholic or non-alcoholic drinks.

Thioesters are particularly important flavouring ingredients for dairy products, particularly cheese flavoured products. In particular, the thioester MTB, preferably used in combination with MTA and/or MTP, is desired for providing an authentic cheese flavor with good flavor impact.

In an illustrative embodiment the consumable product, comprising at least one thioester formed as disclosed herein, is a cheese flavoured product.

In another illustrative embodiment the consumable product is a cheese flavoured product comprising MTA, and/or MTB, and/or MTP formed as disclosed herein.

SEQUENCE IDENTIFICATION

The invention has been described with reference to the following sequence ID numbers:

SEQ ID No. 1 - Depicts a nucleotide sequence encoding the thioesterase enzyme 425.
SEQ ID No. 2 - Depicts an amino acid sequence of the thioesterase enzyme 425.
SEQ ID No. 3 - Depicts a nucleotide sequence encoding one of the thioesterase 3320.
SEQ ID No. 4 - Depicts an amino acid sequence of the thioesterase enzyme 3320.
SEQ ID No. 5 - Depicts a nucleotide sequence encoding the thioesterase enzyme 1875.
SEQ ID No. 6 - Depicts an amino acid sequence of the thioesterase enzyme 1875.

SEQ ID No. 7 - Depicts a nucleotide sequence encoding the thioesterase enzyme 1874.
SEQ ID No. 8 - Depicts an amino acid sequence of the thioesterase enzyme 1874.

The following examples are set forth to describe the invention further detail and to illustrate the disclosed methods and products. However, the examples should not be construed as limiting in any manner.

EXAMPLES

Example 1 – Thioesterase gene cloning for protein expression.

DNA sequences of thioesterase enzymes (hereinafter TE) TE 0425, TE 1875, TE 3320, and TE 1874 were obtained from *B. linens* strain ATCC9174 by amplification using polymerase chain reaction (PCR) with gene-specific primers (Table 1).

The primers include restriction sites to allow directional cloning of the gene in the pET-22b(+) vector (Novagen), while avoiding sites that occur within the genes to be cloned. The 5' primers include an XbaI site followed by the sequence AGGAGGATTAACATATG, which provides a strong *E. coli* ribosome binding site and ATG start codon. After the ATG codon each 5' primer includes an in-frame gene-specific sequence of 14-20 bases starting with the first base of the second codon of the open reading frame encoding the specific protein.

The 3' primers includes a SalI or XhoI site and a gene-specific sequence corresponding to the 17-23 base 3' sequence of the specific open reading frame except for the native stop codon.

The pET-22b(+) vector is designed by Novagen to provide both inducible expression in *E. coli* and protein purification by the binding to nickel columns of a polyhistidine domain incorporated into the expressed protein. Transcription of genes cloned into this vector is designed to require the addition of the inducer IPTG, and use of the pET-22b(+) vector system for the purposes outlined in this invention was performed in full accordance to the manufacturer's recommendations.

For gene expression, *E. coli* BL21(DE3) host cells, which express the T7 RNA polymerase required for the employed pET-22b(+) vector, were used.

Cloning of the resulting gene-specific XbaI to SalI or XhoI fragments into the pET-22b(+) vector generated open reading frames with an in-frame sequence encoding a C-terminal His tag domain and a stop codon from the vector backbone.

Table 1. Gene-specific primers used to clone TE genes into the pET-22b(+) vector

Gene	5' Site	3' Site	Primers Used[1]
TE 0425	*Xba*I		GAATTCTAGAAGGAGGATTAACATATGTCCGTGAAGAC ATTCGAG
		*Sal*I	AGATCTGTCGACGCCGGCCTCGGCGCCGTATTC
TE 1875	*Xba*I		CAATTGTCTAGAAGGAGGATTAACATATGTGTTCACGA TCCTATCGCGG
		*Xho*I	AGATCTCTCGAGTCGGTTGCGACCCCGCATCGGT
TE 3320	*Xba*I		GAATTCTCTAGAAGGAGGATTAACATATGAATCCGCAG TCGGACGCA
		*Xho*I	AGATCTCTCGAGGTTGGCCTCCTTCGGCATGGGGA
TE 1874	*Xba*I		GAATTCTAGAAGGAGGATTAACATATGAGCGATGCAGA ACCCACAG
		*Xho*I	AGATCTCTCGAGCTGCGGAGCCTTCACACGGAC

Following polymerase chain reaction (PCR) amplification of the thioesterase genes with a mixture of the proofreading Pwo and Taq polymerases (Expand 20 kb^{plus} PCR System, Roche Diagnostics GmbH), the PCR products were cloned without restriction digestion into the pGEM-5Z vector, using a pGEMT Vector System I cloning kit (Promega Corporation).

After ligation to the vector DNA and electroporation, *E. coli* DH5α transformant clones with the putative gene inserts were selected on LB agar plates containing 50μg/mL ampicillin. The pGEM-5Z cloning allowed an initial screening of clones for inserts using the blue/white β-galactosidase gene inactivation screen. Transformants were then screened for appropriately-sized plasmids by electrophoresis on 0.8 % agaraose gels of plasmid DNA recovered from the ampicillin resistant colonies.

In an alternative method to the above, the PCR products were cloned directly into pET-22b(+) after restriction digestion at the 5' and 3' restriction sites included in the PCR primer sequences.

Plasmid DNAs from selected colonies were then digested with restriction enzymes to confirm the presence of insert DNA with the predicted restriction sites flanking the inserts and sizes using agarose gel electrophoresis of the digestion products. Inserts passing these screens were sequenced using primers binding to vector sites flanking the inserts. These were the SP6 and T7 promoter primers for pGEM-5Z clones and the T7 promoter and T7 terminator primers for the pET-22b(+) clones.

Sequencing was done using big dye terminator methodology. Clones with the expected sequences were identified in each case by perfect computer-based homology matches to their respective gene in the *B. linens* strain ATCC 9174 genome sequence.

For the sequences cloned initially in the pGEM-5Z vector, the inserts were recovered from appropriate restriction digestions by elution from preparative agarose gels and then transferred into pET-22b(+) and screened as described above. After obtaining the expected pET-22b(+) constructs in *E coli* DH5α cells, the plasmids were recovered and transformed by electroporation into *E. coli* BL21(DE3) cells for protein expression.

Example 2 – Purification of the cloned thioesterases

Protein expression was induced in the *E. coli* BL21 (DE3) cells prepared in example 1 through the addition of 0.5mM of IPTG to batch cultures (100 to 250mL) comprising the aforementioned *E. coli* cells.

The *E. coli* cells were then incubated for 2-4 h, after which time purification of the His-tagged proteins was performed under native conditions using the nickel-charged resin sold under the trade name Ni-NTA agarose (Invitrogen).

The incubated *E.coli* cells were then added to a solution comprising 50 mM of Na-phosphate (pH 8.0), 20 mM of imidazole, 0.5 M of NaCl buffer, and disrupted by vortexing with glass beads.

The solution comprising the disrupted cells was then added to a purification column comprising 1.5 ml of NI-NTA agrose resin, prepared as directed by the manufacturer.

The cloned thioesterase proteins were extracted from the purification column with a buffer containing 50 mM of imidazole. After extraction the proteins were further eluted with 250 mM of the same buffer.

The extracted proteins were then dialyzed against 50 mM TrisHCl buffer (pH 8.0) at 4˚C. The buffer was changed 4 times throughout the process.

Each thioesterase protein was then concentrated using 3K Amicon Ultra-4 cartridges (Millipore, Inc., Billerica, MA) employed as directed by the manufacturer.

The concentrations of each of the individual thioesterase proteins were determined using a micro-Lowry kit employed as directed by the kit supplier (Sigma).

Example 3 – SFCA's as substrate

The ability of purified TE 425, TE 3320, TE 1875, and TE 1874 to produce thioesters from fatty acids was determined. Unless stated otherwise the term solution should be interpreted as being a water based solution.

A short chain fatty acid (hereinafter SCFA) mixture containing 10 mM of each of formic acid (C_1), acetic acid (C_2), propionic acid (C_3), butyric acid and iso-butyric acid (C_4), valeric acid and iso-valeric acid (C_5) and hexanoic acid (C_6) was prepared. This mixture was then diluted to yield a solution with a final total C_1 to C_6 SCFA concentration of 80 µM.

The TE 425, TE 3320, TE 1875 and TE 1874 proteins purified as described in example 2 were each separately diluted to yield separate solutions with final enzyme concentrations of 0.35 µM.

Four sample solutions comprising 100 mM of phosphate buffer (pH 7.2), 100 µM of bovine serum albumin (hereinafter BSA), 25 µM of methanethiol (hereinafter MeSH), 25 µM of coenzyme A (hereinafter CoASH), 2.5 µM of a composition containing equal amounts of pyridoxal-phosphate, pyridoxamine and pyridoxal (hereinafter pyridoxal cocktail), 80 µM of the (C1-C6) SCFA mix prepared above, 10 µM of furfuryl alcohol, and 100 µl of one of the four 0.35 µM enzyme solutions, were prepared.

Control samples were also prepared.

Control 1 was a solution comprising 100 mM of phosphate buffer (pH 7.2), 100 µM of BSA, 25 µM of MeSH, 25 µM of CoASH, 2.5 µM of pyridoxal cocktail, 80 µM of the (C1-C6) SCFA mix, and 10 µM of furfuryl alcohol, but no enzyme.

Controls 2 -5 were solutions comprising 100 mM of phosphate buffer (pH 7.2), 100 µM of BSA, 25 µM of MeSH, 25 µM of CoASH, 2.5 µM of pyridoxal cocktail, 80 µM of the (C1-C6) SCFA mix, 10 µM of furfuryl alcohol, and 100 µl of one of the four 0.35 µM enzyme solutions, but no (C1-C6) SCFA mix.

The contents of the samples and controls are illustrated in table 2.

Table 2.

Samples (1-4)	**Control 1 (no enzyme)**	**Controls 2-5 (no substrate)**
100 mM of phosphate buffer	100 mM of phosphate buffer	100 mM of phosphate buffer
100 µM of BSA	100 µM of BSA	100 µM of BSA
25 µM of MeSH	25 µM of MeSH	25 uM of MeSH
25 µM of CoASH	25 µM of CoASH	25 µM of CoASH
2.5 µM of pyridoxal cocktail	2.5 µM of pyridoxal cocktail	2.5 µM of pyridoxal cocktail
80 µM (C1-C6) SCFA mix	80 µM (C1-C6) SCFA mix	
100 µl of one of the four 0.35 µM enzyme solutions		100 µl of one of the four 0.35 µM enzyme solutions
10 µM of furfuryl alcohol	10 µM of furfuryl alcohol	10 µM of furfuryl alcohol

After preparation all samples were then incubated at 37^{o}C for 35 min, with solid phase microextraction (SPME) carried out at the same time.

The 10 µM of furfuryl alcohol was added to the sample to correct for SPME variability.

For performing the SPME fibers coated with carboxen/polydimethylsiloxane were used. The coating thickness was 85mμm

The analytes absorbed or coated on the SPME fibres were then analysed by gas chromatography and mass spectrometry (GC-MS) at selected time intervals.

None of the enzymes demonstrated thioester synthesis activity under these assay conditions; i.e. no detectable production of methylthioacetate, methylthiopropionate, methylthiobutyrate, and methylthiovalerate was recorded using the SCFA mix as a substrate.

Follow up experiments with single SCFAs as the substrate gave a similar negative outcome.

Example 4 – Coenzyme A-SFCAs derivatives as substrates

Since thioester synthesis was not detected from SCFA substrates, the ability of purified TE 425, TE 3320, TE 1875, and TE 1874 to catalyze MTB production from butyryl-coenzyme A (hereinafter butyryl-CoA) instead of free butyrate was tested.

Four sample solutions were prepared by adding 5µM of one of the four thioesterase enzymes into a solution comprising 0.22 mM of BSA, 100 mM of phosphate buffer (pH 8.0), 0.03 mM butyryl-CoA, and 0.03 mM MeSH.

Six control samples were also prepared;
Control 7 was a solution comprising 0.22 mM of BSA, 100 mM of phosphate buffer (pH 8.0), 0.03 mM butyryl-CoA, and 0.03 mM MeSH, but no enzyme.

Controls 8 to 11 were solutions comprising 0.22 mM of BSA, 100 mM of phosphate buffer (pH 8.0), and 5 µM of the four thioesterase enzymes, but no butyryl-CoA.

Control 12 was a solution comprising 40µM of MTB only. This was used as a control for SPME detection of MTB.

The contents of the samples and controls are illustrated in table 3.

Table 3.

Samples 1-6	**Control 7**	**Controls 8-11**	**Control 12**
100 mM of phosphate buffer (pH 8.0)	100 mM of phosphate buffer (pH 8.0)	100 mM of phosphate buffer (pH 8.0)	
0.22 µM of BSA	0.22 µM of BSA	0.22 µM of BSA	
0.03 µM of MeSH	0.03 µM of MeSH	0.03 µM of MeSH	
0.03 µM of Butyryl-CoA	0.03 µM of Butyryl-CoA		
5 µm of one of the four enzymes (TE425, TE 3320, TE 1875, and TE1874)		5 µm of one of the four enzymes (TE425, TE 3320, TE 1875, and TE1874)	
			40µM MTB

After preparation all samples were incubated at 32°C. After 2 hrs of incubation SPME was carried out for 30mins with the temperature kept at 32°C throughout the procedure. This resulted in a total incubation time of 2.5 hrs.

10 µM of furfuryl alcohol was added to the sample to correct for SPME variability.

For performing the SPME fibers coated with carboxen/polydimethylsiloxane were used. The coating thickness was 85µm.

The analytes absorbed or coated on the SPME fibres were analysed using GC-MS at selected time intervals. Results from this analysis are shown in table 4.

Table 4. MTB production from butyryl-CoA and MeSH by TE enzymes

	m/z 118 peak area	Concentration of MTB (µM)
Control 7	0	0
Controls 8 – 11	0	0
Control 12	62.02	40
Sample 1 TE425	2.96	1.908
Sample 2 TE 1874	1.56	1.006
Sample 3 TE 3320	11.73	7.57
Sample 4 TE 1875	6.15	3.966

Control 12 only contained MTB. This control indicated the expected m/z ratio for MTB is 118.

Controls 7 to 12 did not show a peak at 118 and consequently it can be assumed that these samples do not contain any MTB (Table 3)

Samples 1, 2, 3, and 4 all show peaks at 118. This proves that in each case MTB has been formed and consequently that TE 425, TE 3320, TE 1875, and TE 1874 are thioesterase enzymes capable of producing thioesters from CoA-SCFA derivatives.

The amount of MTB produced by each enzyme can be estimated from the M/Z 118 corresponding GC peak area that should be roughly proportional to the amount of MTB in the sample. All peak areas and corresponding MTB concentrations are listed in table 4.

Example 5 – TE enzymes substrate preferences and product profiles

The ability of purified TE 425 and TE 3320 to catalyze thioester production from a mixture of SCFA CoA-derivatives was determined.

To investigate this activity, CoA-SFCA's were synthesized in a two step reaction.

A preparation of fatty acid anhydrides was made via the following method:
10 mmoles of N,N'-dicyclohexylcarbodiimide (DCC) was added to 50mL of dry carbon tetrachloride (CCl_4) (50 mL). This solution was then added to a second solution containing 20 mmoles of the SCFA mix in 150mL of dry CCl_4.

The reaction mixture was kept at room temperature for 5 hrs after which time the dicyclohexylurea (DCU) precipitate was removed by filtration. Any CCL_4 in the precipitate was removed by evaporation under reduced pressure. The solid residue was re-crystallised from acetone. The crystals were washed with CCL_4 to remove traces of chemicals. The products were then separated by chromatography to yield pure C_2 to C_8 SCFA anhydrides.

After the SCFA anhydrides had been prepared and separated 30 µm of each of the C_2 to C_8 SCFA anhydrides was added to a separate solution of 45 µmol of 2.5 µM of pyridoxal cocktail in 30 mL of ice cold water.

Sodium bicarbonate was added to all the samples to ensure that in each case the pH was 7-7.5. All the mixtures were kept in an ice-bath and shaken frequently for 60 min.

The C_2 to C_8 SCFA-CoA's were purified by precipitation with trichloroactetate (hereinafter TCA) and re-suspended in the water enzyme activity assay.

Once the SCFA CoA's had been formed, samples comprising different thioesterase and SFCA-CoA's were prepared by adding 35μM of one of the thioesterase enzymes, either TE 425 or TE3320, plus 120 μM of the C_2 to C_8 SCFA-CoA mixture into a solution containing 100 μM of BSA, 100 mM of phosphate buffer (pH 7.2), 25μM of MeSH, and 2.5μM of pyridoxal cocktail.

Control samples were also prepared;
Control 13 was a solution comprising 120 μM of the C_2 to C_8 SCFA-CoA mixture, 100 μM of BSA, 100 mM of phosphate buffer (pH 7.2), 25μM of methanethiol, and 2.5μM of pyridoxal cocktail, but no enzyme.

Controls 14 to 15 were solutions comprising 35μM of one of the thioesterase enzymes, either TE 425 or TE3320, 100 μM of BSA, 100 mM of phosphate buffer (pH 7.2), 25μM of methanethiol, and 2.5μM of pyridoxal cocktail, but no C_2 to C_8 SCFA-CoA mixture.

The contents of the samples and controls are illustrated in table 5.

Table 5.

Samples 1-2	**Controls 13**	**Controls 14-15**
120μM of the C_2 to C_8 SCFA-CoA	120μM of the C_2 to C_8 SCFA-CoA	
100 μM of BSA	100 μM of BSA	100 μM of BSA
25μM of MeSH	25μM of MeSH	25μM of MeSH
100 mM of phosphate buffer (pH 7.2),	100 mM of phosphate buffer (pH 7.2),	100 mM of phosphate buffer (pH 7.2),
2.5μM of pyridoxal cocktail	2.5μM of pyridoxal cocktail	2.5μM of pyridoxal cocktail
35μM of one of the thioesterase enzymes, either TE 425 or TE3320		35μM of one of the thioesterase enzymes, either TE 425 or TE3320

All samples were incubated at 37^{o}C for 35 min, then SPME was carried out for 30 mins with fibers coated (thickness = 85um) with carboxen/polydimethylsiloxane. This resulted in a total incubation time of 45 min.

10 μM of furfuryl alcohol was added to the sample to correct for SPME variability.

Analytes absorbed or coated on the SPME fibres were analysed by GC-MS at selected time intervals.

The results after incubation with equimolar mixture of SCFA-CoA derivatives, shown in table 6, confirm that TE 425 and 3320 have different substrate selectivity.

TE 3320 showed a peak of activity for MTB and MTV formation (C4, C5); suggesting butyryl- and valeryl-CoA are preferred substrates. In contrast, TE 425 gave a slight peak at MTH formation, suggesting hexanoyl-CoA (C6) may be its primary substrate.

Table 6. Enzymatic activity of TE 3320 and TE 425 with C_2 to C_8 SCFA-CoA mixture as substrates

	Enzyme activity (by initial velocity method)											
Thioester Product*	MTA			MTP			MTB			MTV		
Control 13 (no enzyme)	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0	0.0	±	0.0
Controls 14-15 (no substrates)	0.2	±	0.11	0.3	±	0.15	0.2	±	0.12	0.2	±	0.12
TE 3320	0.2	±	0.11	0.1	±	0.22	0.05	±	0.05	0.1	±	0.04
TE 425	0.2	±	0.04	0.1	±	0.06	0.12	±	0.04	0.08	±	0.05

Thioester Product*	MTH			MTO		
Control 13 (no enzyme)	0.0	±	0.0	0.0	±	0.0
Controls 14-15 (no substrates)	0.1	±	0.06	0.1	±	0.03
TE 3320	0.1	±	0.05	0.1	±	0.05
TE 425	0.1	±	0.06	0.06	±	0.02

*MTA, methylthioacetate; MTP, methylthiopropionate; MTB, methylthiobutyrate; MTV, methylthiovalerate; MTH, methylthiohexanoate; MTO, methylthiooctanoate

Example 6 - Production of various thioesters by TE3320

A test sample was prepared by adding 100 µL of a solution comprising 0.35 µM of TE 3320 to a solution comprising 100 mM of phosphate buffer (pH 7.2), 100 µM of BSA, 25 µM of methanethiol, 2.5 µM pyridoxal cocktail, and 4.16 µM of each of Acetyl-CoA (C2), Propionyl-CoA (C3), butyryl-CoA (C4), valeryl-CoA (C5), Hexanoyl-CoA (C6), and Octanoyl-CoA (C8).

Two control samples were also prepared.

Control 16 was a solution comprising 100 mM of phosphate buffer (pH 7.2), 100 µM of BSA, 25 µM of methanethiol, 2.5 uM of pyridoxal cocktail, and 4.16 uM of each of Acetyl-CoA C2, Propionyl-CoA C3, butyryl-CoA C4, valeryl-CoA C5, Hexanoyl-CoA C6, and Octanoyl-CoA C8; but no enzyme.

Control 17 was a solution comprising 100 µL of a solution comprising 0.35 µM of TE 3320, 100 mM of phosphate buffer (pH 7.2), 100 µM of BSA, 25 µM of methanethiol, and 2.5 µM of pyridoxal cocktail; but no CoA- SFCA derivatives.

The contents of the samples and controls are illustrated in table 7.

Table 7.

Sample	**Control 16**	**Control 17**
100 mM of phosphate buffer (pH 7.2)	100 mM of phosphate buffer (pH 7.2)	100 mM of phosphate buffer (pH 7.2)
100 µM of BSA	100 µM of BSA	100 µM of BSA
25µM of methanethiol	25µM of methanethiol	25µM of methanethiol
2.5µM of pyridoxal cocktail	2.5µM of pyridoxal cocktail	2.5µM of pyridoxal cocktail
4.16 uM of each of Acetyl-CoA C2, Propionyl-CoA C3, butyryl-CoA C4, valeryl-CoA C5, Hexanoyl-CoA C6, and Octanoyl-CoA C8	4.16 uM of each of Acetyl-CoA C2, Propionyl-CoA C3, butyryl-CoA C4, valeryl-CoA C5, Hexanoyl-CoA C6, and Octanoyl-CoA C8	
0.35 µM of TE 3320		0.35 µM of TE 3320

The samples were then incubated at 32^{o}C. After 2hrs of incubation SPME was carried out. The SPME took 30mins and the temperature was kept at 32^{o}C throughout the procedure. This resulted in a total incubation time of 2.5hrs.

10 µM of furfuryl alcohol was added to the sample to correct for SPME variability.

For performing the SPME fibers coated with carboxen/polydimethylsiloxane were used. The coating thickness was 85µm.

The analytes absorbed or coated on the SPME fibres were then analysed using GC-MS.

The results from the GC-MS analysis are shown in table 8.

Table 8. Retention times, m/z fragment selection and TE 3320 enzymatic activity of thioester formation.

Thioester products	Sample TE 3320 Peak Area	Control 16 (No enzyme)	Control 17 (No substrate)
MTA (C2) 90m/z /7.9 min	1.3	0	0
MTP (C3)	3.5	0	0

104m/z /8.6 min			
MTB (C4) 118m/z /118; 9.3 min	4.7	0	0
Methylthiovalerate (C5) 132m/z /10.2 min	5.1	0	0
Methythiohexanoate (C6) 146m/z /11.12 min	3.2	0	0
Methylthiooctanoate (C8) 174m/z /13.32 min	3.4	0	0

Controls 16 and 17 both did not show peaks corresponding to any thioester products (Table 8). The sample containing the TE3320 enzyme showed peaks at 7.9, 8.6, 9.3, 10.2, 11.12, and 13.32 mins with m/z values of 90, 104, 118, 132, 146, and 174, respectively.

This result proves that the thioesters were formed and that TE3320 is a thioesterases capable of forming various thioesters from appropriate substrates.

The amount of each thioester produced by TE3320 can be estimated from the corresponding GC peak areas. Each peak corresponds to a particular thioester and the area of each peak should be roughly proportional to the amount of each corresponding thioester in the sample. All peak areas are listed in table 8.

The larger peak area sizes at 9.3 and 10.2 min with m/z values of 118 and 132, respectively, may indicate that TE3320 has a preference for C4 and C5 substrates.

Example 7 – Over expression of *B.linens* thioesterases

Over-expression of TE 425, TE 3320, TE 1875 and TE1874 in *B. linens* or other species of *corynebacteria* may be achieved via increased gene copy number by inserting a nucleotide sequence of the present invention into a suitable plasmid vector and transforming a host cell with said vector.

Suitable vectors include pGIV1, which is derived from the native plasmid pBLIN1 found in strain *B. linens* ATCC 9174. Features of pGIV1 include the pBLIN1 replication origin and adjoining *rep* genes, the pUC origin for replication in *E. coli*, a replaceable ampicillin resistance gene flanked by multiple cloning sites where promoter-gene constructs can be inserted, and a gene for kanamycin resistance to facilitate selection in *B. linens* host cells.

Promoter-gene constructs for TE 425, 3320, 1875 and 1874 are comprised of a suitable 5' untranslated region, with operators or promoter motifs that influence the efficiency of transcription or translation, plus the TE coding sequence.

Examples of suitable 5' untranslated ("promoter") regions include the 200 bp region immediately upstream of REBL2645, a gene encoding acetolactate synthase large subunit, which may be strongly upregulated by Met addition, as well as the 200 bp region immediately upstream of RBLE02060, a gene encoding methionyl aminopeptidase that may be constitutively expressed (Cholet et al. 2007, Appl. Microbiol. Biotechnol. 74:1320–1332).

Promoter-gene constructs were assembled following the general PCR strategy described in example 1. The promoter fragments are 200 base pairs in length and flanked on their 5' end by *Not*I sites and on their 3' end by *Eco*31I sites. In addition, the 3' promoter primers were constructed to include an ATG start codon, a strong ribosome binding site(AGGAGG) starting ten nucleotides upstream of the start codon, and the 'consensus' sequence CCAC between the ribosome binding site and the start codon. The AGGAGG hexamer is complementary to a region near the 3' end of the *B. linens* 16 S ribosomal RNA sequence.

Thioesterase gene fragments are 603 base pairs for the TE 1875 and 435 base pairs for TE 3320. Both are flanked on their 5' ends by *Eco*31I sites and on their 3' ends by *Hind*III sites. The 3' end of the thioesterase gene fragment includes the TGA stop codon immediately 5' to the HindIII site.

These fragments were digested with the appropriate pairs of restriction enzymes and then ligated in triple ligations into pBluescript (Stratagene, Agilent Technologies, Inc., Santa Clara, CA) that had been digested with *Not*I and *Hind*III. The RBLE02060 and REBL2645 promoter fragments were ligated with the TE 1875 and TE 3320 gene insert fragments in the four pair wise promoter-gene combinations and transformed into *E. coli* DH5 alpha cells.

The presence of correctly assembled promoter-gene combinations in plasmid DNA from prospective clones was confirmed by DNA sequence analysis (Table 9).

After sequence confirmation the insert fragments were recovered as *Not*I-*Hind*III restriction fragments for transfer into the *B. linens* shuttle vector pGIV1. The vector was digested in the polylinker region with the *Not*I and *Hind*III enzymes, then individual promoter-gene inserts were ligated into the vector and transformed into *E. coli* DH5 alpha cells.

The presence of cloned promoter-gene combinations in recombinant plasmid DNA (pGIV1: TE plasmids) from prospective transformants was confirmed by DNA sequence analysis. Plasmid DNA was then isolated and transformed into *B. linens* ATCC 19391 using the protocol described by Leret et al. 1998 (Microbiol. 144:2827-2836) which is incorporated herein by referece.

Representative isolates of *B. linens* ATCC 19391 transformed with pGIV1: TE plasmids were selected, and over-expression of TE 425, 3320, 1875 or 1874 may be confirmed by real-time quantitative PCR

(Q-PCR) of TE mRNA transcripts using primers based on the sequenes provided in Table 1. It is well within the purview of the person skilled in the art to design appropriate Q-PCR primers from such sequences and quantify cognate mRNA transcripts.

Table 9. Nucleotide sequences for combined promoters and TE gene constructs. Modified ribosome binding site and start codon regions are underlined and in bold font.

promoters and TE gene constructs	Nucleotide sequences
RBLE02060 promoter and TE 1875:	CAGGCTCACCACGTCGCTGAGTCCGGAGAATTCGGGACCGGTCAC ATCTGCCCGTTCGCTCATCACTCCCCTTTGCTCAACATTGTGGCCT TCGCTGGCCGTTCCTTTCACTCTATTATCCTCTCCCACGGCAGGCC CGCACCGACAGGCACCGCAATTGTGGAATATCTGGGTTCAACCGC TCTAGAAT**AGGAGGCACCATG**TGTTCACGATCCTATCGCGGCCCT CACTGCAGCGGTGAAGGTGGCATGATGGGACATATGCACAACCGC ACCACGAATCCCCATCTGAACGAATTCACACGAGTCCTGTTGGAAC TGAGATGGGGAGACATGGATGCCTATGGCCACGTCAACAATGTCA CCCAGCTGCGTCTGCTCGAAGAGGCACGCGTCCGCGCTCTGGGCT CACCGACGCACAGCACCGATGCACCCACAACTCCAGGTCAGCTGG GAATCTCAGGCACGGTATCGGGGATCACAATTCCGGCGATCTTCG CCGAGGCTTCGAACACCACCGAGCTGCTCGTCGCCTCCCACGCGA TCGAATATCGCCGTCCCATTCCCTACCGTGCAGGTCCCATTGCCAT CGATCTCGTCATCAGCGAGGTCAAACCGGCCTCTGTGACGATCGG TTACAGCATCGCCGAACCCGATGGTTCGGTCGGCTATACGCTGGC AGAGACGGTCATCGTCTTTGTAGACAGGACGACCTCCCGACCGCG TCGCCTGACCCAGGAGGAGACAGCAGCAATGGAAGACGTCATTCG ACCTGCCGTACCGATGCGGGGTCGCAACCGATGA
RBLE2645 promoter and TE 1875:	GTCTCCCGTCTGATCGCCGGCAGCAACGAGATCCAGGAGATCGAG ATCAACCCGGTGCGCGTGACCCCGGATGGGGCATTGGCCGTCGA CGCGCTCGTCGTCACGAACCGAGACGACAACGATGGCAGCACCGA CAACGACAGCGGCAGCGACAACCCCGACAACGACAGCAGCACCG ACAACCCCGACA**AGGAGGCACCATG**TGTTCACGATCCTATCGCGG CCCTCACTGCAGCGGTGAAGGTGGCATGATGGGACATATGCACAA CCGCACCACGAATCCCCATCTGAACGAATTCACACGAGTCCTGTTG GAACTGAGATGGGGAGACATGGATGCCTATGGCCACGTCAACAAT GTCACCCAGCTGCGTCTGCTCGAAGAGGCACGCGTCCGCGCTCTG GGCTCACCGACGCACAGCACCGATGCACCCACAACTCCAGGTCAG CTGGGAATCTCAGGCACGGTATCGGGGATCACAATTCCGGCGATC TTCGCCGAGGCTTCGAACACCACCGAGCTGCTCGTCGCCTCCCAC GCGATCGAATATCGCCGTCCCATTCCCTACCGTGCAGGTCCCATTG CCATCGATCTCGTCATCAGCGAGGTCAAACCGGCCTCTGTGACGAT

	CGGTTACAGCATCGCCGAACCCGATGGTTCGGTCGGCTATACGCT GGCAGAGACGGTCATCGTCTTTGTAGACAGGACGACCTCCCGACC GCGTCGCCTGACCCAGGAGGAGACAGCAGCAATGGAAGACGTCAT TCGACCTGCCGTACCGATGCGGGGTCGCAACCGATGA
RBLE2060 promoter and TE 3320:	CAGGCTCACCACGTCGCTGAGTCCGGAGAATTCGGGACCGGTCAC ATCTGCCCGTTCGCTCATCACTCCCCTTTGCTCAACATTGTGGCCT TCGCTGGCCGTTCCTTTCACTCTATTATCCTCTCCCACGGCAGGCC CGCACCGACAGGCACCGCAATTGTGGAATATCTGGGTTCAACCGC TCTAGAAT**AGGAGGCACCATG**AATCCGCAGTCGGACGCACTTCCA GATGTCTCACTTGCCTCAGCCAGCAACTTCGTCGCCGCCTCGGGG CTCGTCATCGACGAGGTCACGAACACAAGCGTCCGCGGCCATGCC GATCTGGGCAGCGACCACCACACGCCTTGGGGCGTCGTCCACGG CGGCGTGTACACAACGCTCGTGGAGAGCACGGGAAGCATTGGTGC CAGCCACGCTGTGGGCGAGCGCGGCGAGTTCGCCGTCGGCATCC ACAACGCCACCGACTTTCTGCGCCCGACCGCCGGCGCCCGCGTTG CAGTCGAGGGCACCGCCCTGCATCAGGGCCGGACCCAGCAGCTG TGGGAGGTCATCATCACCGACACCTCATCGGACAAGGTCCTGGCC CGCGGCCAGCTGCGCCTGCAGAACGTCCCCATGCCGAAGGAGGC CAACTGA
RBLE2645 promoter and TE3320:	GTCTCCCGTCTGATCGCCGGCAGCAACGAGATCCAGGAGATCGAG ATCAACCCGGTGCGCGTGACCCCGGATGGGGCATTGGCCGTCGA CGCGCTCGTCGTCACGAACCGAGACGACAACGATGGCAGCACCGA CAACGACAGCGGCAGCGACAACCCCGACAACGACAGCAGCACCG ACAACCCCGACA**AGGAGGCACCATG**AATCCGCAGTCGGACGCACT TCCAGATGTCTCACTTGCCTCAGCCAGCAACTTCGTCGCCGCCTCG GGGCTCGTCATCGACGAGGTCACGAACACAAGCGTCCGCGGCCAT GCCGATCTGGGCAGCGACCACCACACGCCTTGGGGCGTCGTCCA CGGCGGCGTGTACACAACGCTCGTGGAGAGCACGGGAAGCATTG GTGCCAGCCACGCTGTGGGCGAGCGCGGCGAGTTCGCCGTCGGC ATCCACAACGCCACCGACTTTCTGCGCCCGACCGCCGGCGCCCGC GTTGCAGTCGAGGGCACCGCCCTGCATCAGGGCCGGACCCAGCA GCTGTGGGAGGTCATCATCACCGACACCTCATCGGACAAGGTCCT GGCCCGCGGCCAGCTGCGCCTGCAGAACGTCCCCATGCCGAAGG AGGCCAACTGA

Example 8 – Heterologous expression of thioesterases

Heterologous expression of TE 425, TE 3320, TE 1875 and TE1874 in *Lactobacillus casei* other lactic acid bacteria may be achieved via increased gene copy number by inserting a nucleotide sequence of the present invention into a suitable plasmid vector and transforming a host cell with said vector.

Construction of *thioesterase* gene construct plasmids (hereinafter TE plasmids) for heterologous expression was performed by PCR following the method described in example 1 except that a suitable vector containing suitable 5' untranslated region for heterologous expression of TE genes must be used.

Suitable vectors are those derived from *Lactobacillus casei* or other lactic acid bacteria and may include, but are not limited to, pHADH, a plasmid containing the cloned *dhic* gene from *L. casei* ATCC 334. (Broadbent et al. 2004, Appl. Environ. Microbiol. 70:4814-4820).

Suitable 5' untranslated region for heterologous expression in pHADH may be derived by PCR amplification of the putative ribosome binding site and promoter region for *dhic* in using primers P1 and P2 (Table 10) to obtain a 285 bp XbaI/BglII fragment. The coding regions for TE 3320 or TE 1875 were amplified from *B. linens* ATCC9174 genomic DNA using primers B71 and B72 or B73 and B74, respectively.

These reactions yielded the complete TE3320 coding region on a 456 bp BglII/BamHI fragment, or the complete TE1875 coding region on a 609 bp BglII/BamHI fragment. The start codons were one (TE3320) or two (TE1875) basepairs downstream of the BglII site. No recognition sites for these three restriction enzymes (or SacI, see below) are present in the TE genes or in the *dhic* promoter region.

Table 10. PCR primers used to clone TE genes into the pHADH-derived vector

	Primer			
Organism	**Anneal area**	**Name**	**Linker***	**Sequence**
L. casei ATCC 334	*dhic* promoter 5'	P1	XbaI	GTTATCTAGACGATT TCTTATGGAGAG
	dhic promoter 3'	P2	BglII	GTTTAGATCTCTTCC TTTCCAATTTGTCCA CTCACCAG
B. linens ATCC 9174	TE3320 5'	B71	BglII	GTTTAGATCTCATGAATCC GCAGTCGGACGCACTTC CAG

	TE3320 3'	B 72	Bam HI	GTTTGGATCCTC GAATCAGTGCCCAT CTCAGTTG
	TE1875 5'	B 73	BglII	GTTTAGATCTGCGTG TGTTCACGATCCTATCG CGG
	TE1875 3'	B 74	Bam HI	GTTTGGATCCAGTCA TCGGTTGCGACCC CGCATCGG

*Restriction endonuclease target site embedded in primer sequence to facilitate directional cloning.

The PCR fragments are digested with BglII and the promoter is separately ligated to each coding sequence. The ligated DNA samples are recovered and checked by PCR amplification with the primer combinations P1/B72 and P1/B74 (Table 10).

The promoter-TE coding region constructs were then digested with XbaI and BamHI and ligated into pBlueScript that was also digested with XbaI and BamHI and transformed into *E. coli* DH5 alpha. The presence of correctly assembled promoter-gene combinations in transformants was verified by PCR using primers P1xB72 (TE3320) and P1xB74 (TE1875) and DNA sequence analysis of insert regions from recombinant plasmids.

The recombinant plasmids are digested with SacI and BamHI and the promoter-coding region fragments were separated from the pBlueScript vector. The inserts were ligated into SacI/BamHI digested pHADH, which effectively replaces *dhic* with the promoter-TE coding region, and transformed into *E. coli* DH5 alpha.

The presence of correctly assembled promoter-gene combinations in transformants was verified by PCR using primers P1xB72 (TE3320) and P1xB74 (TE1875) and DNA sequence analysis of insert regions from recombinant plasmids.

The recombinant plasmids pTE 3320 and pTE 1875 were recovered from *E. coli* clones and transformed by electroporation into *L. casei* ATCC 334 or other lactic acid bacteria.

For L. casei, one of several methods to transform cells begins by inoculating stationary-phase cells at 2% (vol/vol) into 500 mL of MRS broth (Difco, Detroit, Mich., USA) and incubating at 37˚C until the suspension reached an absorbance at 600 nm (A600) of 0.8.

The cells were harvested by centrifugation at 5000xg, washed twice with sterile, distilled water, and suspended in 2.5 mL of ice-cold, sterile 30% polyethylene glycol 1450 (Sigma Chemical Co.). 3 µl of the TE plasmid constructs formed above, are mixed with 100 µl of cell suspension

in a 0.2 cm electroporation cuvette and placed on ice for 3 min. An electric pulse is delivered in a Bio-Rad Gene Pulser (Bio-Rad Laboratories, Richmond, Calif., USA) set to the following parameters: 2.5 kV, 25 μF, and 200Ω. After electroporation, 0.9 mL of warmed (37˚C) MRS broth was added, and the cells were incubated at 37˚C for 2h. Transformed cells were collected on MRS agar containing 5 μg of ERY (Sigma Chemical Co., St. Louis, Mo., USA) per mL.

The presence of the TE vector in lysates from putative transformants was confirmed by electrophoretic separation in 0.6% agarose gels with Tris acetate buffer (40 mM Tris, 20 mM acetic acid, and 2 mM Na_2EDTA, pH 8.1). It is well within the purview of the person skilled in the art to select an appropriate lysis procedure for isolation of plasmid DNA, depending on the host cells in question.

The fidelity of promoter-gene combinations in pTE 3320 and pTE 1875 transformants was verified by PCR using primers P1xB72 (TE3320) and P1xB74 (TE1875) and DNA sequence analysis of insert regions.

Overexpression of TE 425, 3320, 1875 or 1874 was confirmed by real-time quantitative PCR (Q-PCR) of TE mRNA transcripts using primers based on the cognate gene sequences.

Alternatively, TE activity in these hosts can be demonstrated by enzyme assay for cell lysates using the method described in example 4.

While the nucleotide and amino acid sequences encoding thioesterase enzymes, and various methods using and products incorporating the same, have been described in connection with various illustrative embodiments, it is to be understood that other similar embodiments may be used or modifications and additions may be made to the described embodiments for performing the same function disclosed herein without deviating there from. The embodiments described above are not necessarily in the alternative, as various embodiments may be combined to provide the desired characteristics. Therefore, the disclosure should not be limited to any single embodiment, but rather construed in breadth and scope in accordance with the recitation of the appended claims.

CLAIMS

1. A method of producing thioesters comprising contacting at least one thioesterase enzyme comprising the amino acid sequence disclosed in SEQ.ID. No. 6 or an amino acid sequence having at least 74%, 80%, 90% or 95% or greater sequence identity to the amino acid sequence of SEQ.ID. No. 6, and/or at least host cells comprising the nucleotide sequence of SEQ.ID. No. 5 or a nucleotide sequence having at least 75%, 80%, 90% or 95% or greater sequence identity to the nucleotide sequence of SEQ.ID. No. 5 with at least one suitable substrate comprising a C1-C8 SCFA-CoA, incubating the mixture, isolating the crude product containing the thioester and, purifying the crude product to obtain only the thioester.

2. Use of a kit for producing thioesters according to claim 1 comprising at least one thioesterase enzyme comprising the amino acid sequence of SEQ ID. No. 6 or an amino acid sequence having at least 74%, 80%, 90% or 95% or greater sequence identity to the amino acid sequence of SEQ.ID. No. 6 and/or at least host cells comprising at least the nucleotide sequence of SEQ.ID. No. 5 or a nucleotide sequence having at least 75%, 80%, 90% or 95% or greater sequence identity to the nucleotide sequence of SEQ.ID. No. 5 and at least one suitable substrate comprising a C1-C8 SCFA-CoA.

3. A method of flavouring cheese comprising adding to a cheese during its manufacture process at least one thioesterase enzyme comprising the amino acid sequence of SEQ.ID. No. 6 or an amino acid sequence having at least 74%, 80%, 90% or 95% or greater sequence identity to the amino acid sequence of SEQ.ID. No. 6, and/or at least host cells comprising the nucleotide sequence of SEQ.ID. No. 5 or a nucleotide sequence having at least 75%, 80%, 90% or 95% or greater sequence identity to the nucleotide sequence of SEQ.ID. No. 5 wherein the thioesterase enzyme is contacted with at least one suitable substrate comprising a C1-C8 SCFA-CoA.

4. The method of claim 3, wherein the thioesterase enzyme(s) and/or host cell(s) are added as part of a starter culture.

5. Use of a flavoured cheese product or a cheese flavoured product obtainable by or produced by the method of claims 3 or 4 in a consumable product.

6. Use of SEQ.ID. No. 5 or a nucleotide sequence having at least 75%, 80%, 90% or 95% or greater sequence identity to the nucleotide sequence of SEQ.ID. No.5 as marker in a method of screening for organisms capable of producing the thioesters encoded by said nucleotides sequence wherein the thioesterase enzyme is contacted with at least one suitable substrate comprising a C1-C8 SCFA-CoA.

7. Use of a composition comprising a thioester obtained by or produced by the method of claim 1 in a flavoured cheese product or a cheese flavoured product.

8. Use of a consumable product comprising a thioester obtained by or produced by the method of claim 1 which provides an authentic cheese flavor with good flavor impact.

9. Use of a consumable product according to claim 8, wherein the consumable product is selected from the group consisting of: a cheese flavoured product or flavoured cheese product.