



(51) International Patent Classification:

C12N 9/10 (2006.01) *C12P 1/00* (2006.01)
C07K 14/33 (2006.01) *C12P 7/16* (2006.01)

(21) International Application Number:

PCT/EP2013/064302

(22) International Filing Date:

5 July 2013 (05.07.2013)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

12175385.9 6 July 2012 (06.07.2012) EP

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(81) Designated States (*unless otherwise indicated, for every kind of national protection available*): AE, AG, AL, AM,

AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (*unless otherwise indicated, for every kind of regional protection available*): ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))
- with sequence listing part of description (Rule 5.2(a))

(54) Title: ENGINEERED CLOSTRIDIUM STRAIN FOR BUTANOL PRODUCTION

(57) Abstract: The present invention relates to improved thiolase variants, as well as to microorganisms expressing the same and to processes of using the same. Such processes can be used for improved production of butanol. Preferably the improved thiolase variants have a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of wildtype thiolase thIA from *C. acetobutylicum*.



TITLE

ENGINEERED CLOSTRIDIUM STRAIN FOR BUTANOL PRODUCTION

DESCRIPTIONFIELD OF INVENTION

The present invention is related to the field of industrial microbiology and genetic engineering of microorganisms. More specifically, the invention relates to a process for producing a chemical compound using an acetoacetyl coenzyme A-dependent metabolic pathway in a microbial host, such as the genus *Clostridium*, the chemical being in particular *n*-butanol.

BACKGROUND OF THE INVENTION

1-Butanol (*n*-butanol or just butanol) is an important commodity chemical, produced today almost exclusively from petrochemical resources. The fermentative production of butanol is – in present understanding - restricted to the genus *Clostridium*, a group of Gram-positive, spore forming anaerobes comprising mainly species from terrestrial habitats (Dürre 1998, Dürre 2011). The biosynthesis of butanol requires a C₄-specific biosynthetic pathway deviating from a central metabolic intermediate such as the activated C₂ compound acetyl-CoA. Such a carbon-carbon bond formation constitutes an important metabolic step and the thiolase is a typical biocatalyst mediating this reaction.

Among solventogenic Clostridia, *Clostridium acetobutylicum* has been first discovered and to date is the best characterized solventogenic *Clostridium* species. *Clostridium* strain improvements for biotechnological applications have been obtained with random mutagenesis and plate screening approaches (US 4,757,010; US 6,358,717). Considering the recent development of new metabolic engineering and analytical tools for members of the genus *Clostridium*, several rational approaches to improve butanol production were conducted (Lee, *et al.*, 2008, Papoutsakis, 2008, Lütke-Eversloh & Bahl, 2011) These are, however, limited to the manipulation of gene expression patterns such as gene knockout or downregulation, respectively, and/or gene overexpression to manipulate metabolic fluxes towards the desired product.

Thiolase (acetyl-coenzyme A [CoA] acetyltransferase, EC 2.3.1.19) plays a key role in the production of butanol and other metabolites in organisms including *C. acetobutylicum* and its

activity may determine the ratio of C3 & C4 products (e.g. butanol, acetone and butyrate) to (C2 products (e.g. ethanol and acetate); (Wiesenborn *et al.* (1988). Wiesenborn *et al.* (1988) further described that thiolase may be inhibited by micromolar levels of Coenzyme A [CoA].

In an attempt published by Stiller *et al.* (2008) that aimed to direct the flux from acetate towards butanol formation, the aldehyde/alcohol dehydrogenase gene *adhE1* (*adh*) and the thiolase gene *thlA* (*thl*) were homologously overexpressed in the solvent-negative degenerated strain *C. acetobutylicum* M5. Whereas *adhE1* overexpression resulted in increased titers of the sum of all alcohols (including ethanol and butanol) additional *thlA* overexpression could not alter the phenotype of acetate being the main fermentation product in the *C. acetobutylicum* M5 strain. Moreover, butanol production of a *C. acetobutylicum* ATCC 824 strain comprising a downregulated acetone pathway and *adhE1* overexpression could not be increased by simultaneous *thlA* overexpression (Sillers, *et al.*, 2008, Sillers, *et al.*, 2009). These findings clearly indicated that increased amounts of *thlA* transcripts do not directly lead to an increased flux into the C₄ pathway.

In conclusion, it is desired to obtain bacteria capable of producing more C3- and C4-products (such as butanol), but altering gene expression is apparently not sufficient to satisfy this desire.

PROBLEM TO BE SOLVED

It is one object of the present invention to provide an improved process for the microbial production of butanol, as well as an improved enzyme suitable to be used in the process. It is particularly desired that said improved enzyme is useful as biocatalyst for microbial butanol production, and shows improved performance compared with enzymes of the present state of the art, particularly under physiological conditions i.e. when expressed in a strain capable of producing butanol and/or other C3-/ C4-products. Further goals of the invention that have been solved will become apparent from the following description of the invention.

BRIEF DESCRIPTION OF THE INVENTION

The invention provides an improved process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway. Said process is improved because it has a thiolase activity with higher specific activity and/or with reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of a wildtype enzyme, such as the polypeptide having SEQ ID NO: 1. In a preferred embodiment, the thiolase variants of the invention show reduced sensitivity towards its physiological inhibitor coenzyme A (CoA-SH). The process of the invention can be performed by expressing the enzyme of the invention

recombinantly, leading to a recombinant (micro)organism, such as for example a recombinant *Clostridium acetobutylicum* expressing the thiolase of the invention. Said enzyme surprisingly shows improved productivity of butanol.

The inventors achieved the invention by analyzing the polypeptide sequence of a wildtype thiolase, generating a library of mutant thiolase genes, followed by a high-throughput screening assay, employing *Escherichia coli* as host cells for expression of the mutant thiolase gene library. Screening of this library resulted in the identification of various thiolase enzyme variants, including a thiolase enzyme variant with significantly increased activity in the presence of free CoA-SH. The most preferred (optimized) thiolase variant has three amino acid substitutions (R133G, H156N, G222V) and its gene was expressed in *C. acetobutylicum* ATCC 824 to assess the effect of reduced feedback-inhibition on solvent production.

In addition to a clearly delayed ethanol and acetone formation, the ethanol titer was increased by 46%. Butanol titer was increased by 18%, while the final acetone concentrations were similar to the vector control strain.

The invention also provides host cells expressing the enzyme of the invention and/or suitable in the process of the invention, as well as nucleic acids encoding the enzyme of the invention.

ITEMS

The following items are comprised in the present invention

- [1] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1.
- [2] A process of item 1, wherein said thiolase has 5% or higher, preferably 10% or higher, more preferably 20% or higher, even more preferably 30% or higher activity, as compared to the parental wildtype enzyme under physiological conditions.
- [3] A process of any one of item 1 or 2, wherein activity of said thiolase is not inhibited by 10 μ M CoA-SH.
- [4] A process of any one of item 1-3, wherein said chemical is a C4 alcohol.
- [5] A process of item 4, wherein said chemical is butanol, preferably n-butanol.
- [6] A process of any one of items 1-5, wherein the production is done in a host cell belonging to the genus *Clostridium*.

- [7] A process of item 6, wherein such host cell belongs to one of the following species: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. butyricum*, *C. pasteurianum*, *C. saccharobutylicum*, *C. ljungdahlii*, *C. thermocellum*, *C. thermobutyricum*, *C. cellulolyticum*.
- [8] A process of any one of items 1-7, wherein butanol is produced by non-natural butanol producing strains.
- [9] A process of any one of items 1-8, wherein butanol is produced by a strain belonging to the genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Ralstonia*, *Saccharomyces*.
- [10] A process of any one of items 1-9, wherein butanol is produced by a strain belonging to the species *E. coli*, *B. subtilis*, *L. brevis*, *L. plantarum*, *P. putida*, *P. fluorescens*, *P. aeruginosa*, *R. eutropha*, *S. cerevisiae*.
- [11] A polypeptide having thiolase activity characterized in that it has 70 % or more, preferably 75 % or more, more preferably 80 % or more, more preferably 85 % or more, more preferably 90 % or more, more preferably 95 % or more, such as 96 % or more, 97 % or more, 98 % or more, 99 % or more sequence identity with SEQ ID NO: 1, provided that at least one, such as any combination of two, or all three of the residues 133, 156, 222 display a mutation (selected from substitution, deletion or insertion) with respect to SEQ ID NO:1.
- [12] The polypeptide of item 11 wherein said mutation(s) selected from
- any one,
 - any combination of two, and
 - preferably all three (SEQ ID NO: 2)
- of the following substitutions: R133G, H156N, G222V.
- [13] The process of any one of item 1 to 10, characterized in that the thiolase activity is provided by the enzyme of any of claims 11 to 12.
- [14] A nucleic acid encoding a polypeptide selected from the group consisting of (a) the polypeptide of any one of items 11 or 12 and (b) the enzyme having the thiolase activity defined in any one of claims 1 to 10.
- [15] A vector comprising the nucleic acid of item 14.

[16] A host cell carrying the nucleic acid of item 14 or the vector of claim 15.

Moreover, the present invention provides the following items:

[1] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1, and wherein the activity of said thiolase is not inhibited by 10 μ M CoA-SH.

[2] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1, and wherein the thiolase is a mutant protein having at least one mutation selected from

- any one,
- any combination of two, and
- preferably all three (SEQ ID NO: 2)

of the following substitutions: R133G, H156N, G222V.

[3] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1, and wherein the thiolase is a mutant protein having at least one mutation into the CoA-SH binding loop domain with respect to the parental wildtype enzyme.

[4] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1, wherein the thiolase is a mutant protein having at least one mutation into the CoA-SH binding loop domain with respect to the parental wildtype enzyme, and wherein the determination of thiolase activity is performed by a 3-hydroxybutyryl-CoA dehydrogenase-coupled spectrophotometric assay.

DESCRIPTION OF FIGURES

Figure 1. Thiolase assay development in 96-well microtiter plates.

(a), Cultures of recombinant *E. coli* strains expressing wildtype ThIA CAC2873 were used to prepare cell crude extracts which were subjected to spectrophotometric thiolase activity measurements according to NADPH decrease at 340 nm. Time course of absorbance at 340 nm ($A_{340\text{ nm}}$) of the coupled ThIA/PhaB assay in crude extracts of *E. coli* BL21 (DE3) pASK-IBA3::thIA^{WT}.

(b), Reproducibility of the coupled ThIA/PhaB assay in crude extracts of *E. coli* BL21 (DE3) pASK-IBA3::thIA^{WT}. Distribution of 144 independent thiolase activity measurements; the dashed line indicates the mean value. The average thiolase activity of *E. coli* pASK-IBA3::thIA^{WT} was 71 ± 4 U/mg, exhibiting a standard deviation of only 5.2 % ($n = 144$). In order to screen for ThIA mutants with reduced sensitivity towards free CoA-SH (the physiological inhibitor), the assay was repeated under the same conditions with the addition of 10 μ M CoA-SH each, revealing 27 ± 1 U/mg of the wildtype ThIA activity. Hence, *C. acetobutylicum* wildtype thiolase activities in crude extracts of recombinant *E. coli* constituted the reference values for the subsequent library screening.

Figure 2. Amino acid sequence alignment of thiolases from *C. acetobutylicum* (wildtype) and *Z. ramigera*.

Sequence alignment was performed using ClustalW 2.1; "" represents identical, ":" highly similar and "." similar amino acid residues. CAC2873, thiolase of *C. acetobutylicum*; Z.ram., thiolase of *Z. ramigera*. The catalytic histidine and cysteine residues are underlined, the residues of the loop domain involved in the CoA binding are shaded.

Figure 3. Screening of the *E. coli* pASK-IBA3::thIA^{MUT} library.

Thiolase activities were measured in cell crude extracts without (a) and in the presence of 10 μ M free CoA-SH (b). The relative distribution as percent of wildtype thiolase activity without (c) and in the presence of 10 μ M free CoA-SH (d) are shown. A total of 1620 clones of the *E. coli* BL21 (DE3) pASK-IBA3::thIA^{MUT} library were analyzed for thiolase activities and sensitivity towards free CoA-SH. As illustrated in the Figure, mutant thiolase activities exhibited a wide range of both increased and reduced values as compared to the wildtype ThIA activity (Figure 3a, c), and only 56 clones (< 4 %) revealed no thiolase activity. Interestingly, such wide-spread values were not observed when the same clones were subjected to thiolase activity measurements in the presence of 10 μ M CoA-SH, i. e. 80 % of the mutants showed activities within ± 20 % of the wildtype ThIA activity (Figure 3b, d). Finally, 14 ThI^{MUT} variants were identified by significantly increased activity, i. e. > 150 % of the ThI^{WT} activity in the presence of free CoA-SH. It is noteworthy that increased activity

values of both assays detected during the screening did not correlate to each other. The arrow in (d) indicates the 14 positive clones (ThIA^{MUT} variants with significantly increased activity) selected for further characterization.

Figure 4. Thiolase inhibition by free CoA-SH.

Open squares and dashed line, ThIA^{WT} ; closed circles and solid line, ThIA^{OPT} . Mean values of six independent measurements are shown. The clearly reduced sensitivity of ThIA^{OPT} towards free CoA-SH in comparison to ThIA^{WT} is illustrated. Remarkably, at a concentration of 50 μM CoA-SH, ThIA^{OPT} showed a more than 10-fold higher thiolase activity than ThIA^{WT} .

Figure 5. Fermentation profiles of recombinant *C. acetobutylicum* strains.

Closed circles, *C. acetobutylicum* pT:: thIA^{OPT} ; open diamonds, *C. acetobutylicum* pT (vector control). Average data of three independent replicates for each strain are shown. *C. acetobutylicum* pT:: thIA^{OPT} produced similar amounts of acetone but higher levels of ethanol and butanol as compared to the vector control strain. Acetone and ethanol formation was clearly delayed whereas butanol synthesis began at the same time as in the control culture. Expression of thIA^{OPT} resulted in a significantly increased butanol production during the stationary growth phase, elevating the final butanol titer from 142 mM (10.5 g/l) to 168 mM (12.4 g/l).

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides an improved process for the production of a chemical using an acetoacetyl-CoA-dependent metabolic pathway. The present invention thus relates to a process for the production of a chemical involving a higher flux into the acetoacetyl coenzyme A-dependent metabolic pathway.

The process of the invention is improved because it has a thiolase activity with higher specific activity and/or with reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of a wildtype enzyme, such as the polypeptide having SEQ ID NO: 1. Said improved variants of the enzyme thiolase thIA that have higher activity and/or reduced feedback inhibition are also comprised in the invention. Surprisingly microbial strains expressing such an enzyme variant show higher productivity and/or selectivity towards butanol production although prior art approaches employing microbial strains overexpressing such wildtype enzyme had not shown the respective effect Stiller et al. (2008).

In particular, high-throughput applications were developed to identify the thiolase variants of the invention.

It is considered in the art that random mutagenesis of enzymes often comes with the disadvantage that a large fraction if not all so-obtained mutants are not improved *vis-à-vis* the starting variant (which may be the wildtype variant). Reasons may include for example that mutagenesis of catalytically or structurally important residues can oftentimes not be tolerated. On the other hand, detailed information on the role of each residue in catalysis or structure of an enzyme are often unknown. Researchers therefore oftentimes feel discouraged to employ random mutagenesis approaches, particularly if no comprehensive structural and functional studies on the starting variant (which may be the wildtype) exist.

In an attempt to circumvent the disadvantages often going along with random mutagenesis, or more precisely, in order to minimize the chance of generating mainly inactive mutants and maximize the chances of obtaining improved enzyme variants, the inventors of the present invention applied a two-step process. In the first step, they aligned the amino acid sequence of CAC2873 with the *Z. ramigera* thiolase. This was done in view of the sequence homology of the *Z. ramigera* thiolase (for Alignment, see Figure 2), which had previously been subjected to detailed biochemical and crystallographic analyses. As a result from this alignment, the inventors expected that the amino acid residues 119-249 of CAC2873 form a large loop domain which interacts with the CoA moiety of the substrate (Meriläinen et al. 2009; Meriläinen et al. 2008).

In the second step, the inventors of the present invention designed a mutagenesis protocol aiming at introducing random mutations into the loop region, i.e. the region of residues 119-254, i.e. the catalytic residues Cys89, His348 and Cys378 were not involved. The correctness of the mutant library was validated by DNA sequencing of 48 randomly selected clones. The sequenced variants in the so-obtained mutant library of ThIA comprised an average number of 2.5 amino acid substitutions in the region of residues 119-254.

Thus, in one aspect, the present invention relates to a two-step process for making ThIA variants. Any ThIA enzyme from any organism (preferred are *Clostridium* species) can be used as starting sequence, which is aligned in the first step to the *Z. ramigera* thiolase shown in Figure 2. The alignment is done by ClustalW 2.1. A preferred region into which the mutations are to be introduced is the region which is homologous to the region corresponding to residues 120-249 of the *Z. ramigera* thiolase shown in Figure 2 ("loop region"). The term "loop region" is used without wishing to be bound to any theory simply to refer to a region that is homologous to the region corresponding to residues 120-249 of the *Z. ramigera* thiolase shown in Figure 2. In a preferred embodiment, CAC2873 (SEQ ID NO: 1; Figure 2) is the starting sequence for the mutagenesis approach, and more preferably, the region selected for mutagenesis is the loop region, i.e. the region corresponding to residues 119-249 of CAC2873. "Region selected for mutagenesis" can mean that mutations are

preferably introduced into this region, or – in a preferred embodiment – that mutations are introduced exclusively into said region. Thus, in a preferred embodiment, the improved thiolases of the present invention are variants of the ThIA enzyme (CAC2873) of *C. acetobutylicum* ATCC 824. In one embodiment, the thiolase gene *thIA* of *C. acetobutylicum* (CAC2873) is subjected to random mutagenesis. In a preferred embodiment, the CoA-SH binding loop domain is targeted for amino acid exchanges by random mutagenesis.

In a second step, mutations are introduced into the thiolase or into the loop region thereof. Although this is typically done by mutating the coding sequence, any desired mutant may optionally also be obtained by directly synthesizing the desired polypeptide. The coding sequence of the enzyme to be mutagenized can oftentimes be obtained from public databases. The corresponding nucleotide can then be obtained by designing PCR primers as known in the art and amplifying from a sample from the respective organism. Such a sample may for example be total genomic DNA or a colony of the respective microorganism (colony PCR). Alternatively, the coding sequence may be obtained by reverse-translating the amino acid sequence, and the respective oligonucleotide can be synthesized *in vitro*. Reverse translation may optionally be done by considering the codon usage of the organism in which expression is desired. Many methods for introducing mutations are known in the art. Random methods (methods for obtaining random mutants) include for example error-prone PCR. Alternatively, specific mutants can be introduced by site-directed mutagenesis. A variety of random and site-directed mutagenesis approaches is described by Sambrook and Russell 2001, and any such approach may be used. In cases in which introduction of mutations into one particular region of the starting sequence is desired (i.e. the loop region) is desired, the random mutagenesis may be done by any method suitable for such purpose known in the art, for example by introducing site-directed mutations into said particular region, or by excising said region from a nucleic acid construct comprising the entire coding sequence of the parental (e.g. wildtype) enzyme by means of unique restriction sites, followed by random mutagenesis of the excised region and by subsequent re-ligation into a nucleic acid construct corresponding to the one resulting from said excision by unique restriction sites. Preferably, the random mutagenesis is however done using synthetic oligonucleotides building the degenerated backbone, e.g. as described in Example 4 and Table 1.

Within this specification, mutation can mean any one or more selected from substitution, deletion and insertion, although substitution may be preferred. Within this specification, a mutant protein is any variant of a starting protein (parental protein) having at least one (preferred) mutation with respect to the starting protein (parental protein).

The obtained random mutants may be sequenced at this stage (for which the (plasmid) DNA encoding the variant may be isolated), although the sequencing at this stage is merely

optional.

In a third step, the obtained variants (mutants of the starting sequence) are screened for thiolase activity. To that end, the obtained variants are expressed in *E. coli*. Expression of the desired variant can be induced as described in the art (e.g. Sambrook and Russell 2001), depending on the promoter. For a tetracycline dependent system, expression can be induced by anhydrotetracycline as described in Example 2. After inducing expression, cells are further grown and subsequently harvested, e.g. by centrifugation. Crude extracts of the *E. coli* expressing the thiolase variants are prepared as described Example 3.

In one embodiment, the thiolase activity is tested in absence of the inhibitor. In another (preferred) embodiment, the thiolase activity is tested in presence of the inhibitor. Merely optionally, and to speed up the screening process, a high-throughput screening is done, employing multititer plates, such as the 96-well microtiter plate format.

The thiolase activity measurements serves to identify improved thiolase variants. In a preferred embodiment, recombinant *E. coli* strains are used as host cells for screening a plasmid-borne library of mutagenized *thlA* gene.

The determination of thiolase activities is performed by a 3-hydroxybutyryl-CoA dehydrogenase-coupled spectrophotometric assay as follows. The 3-hydroxybutyryl-CoA dehydrogenase activity is provided by a common 3-hydroxyacyl-CoA dehydrogenase or by the hbd protein of *C. acetobutylicum* or by the PhaB protein of *R. eutropha*. The 3-hydroxybutyryl-CoA dehydrogenase activity are provided either by purified enzymes or by crude extracts of native or recombinant host cells expressing the respective gene. The thiolase activities may be measured spectrophotometrically, such as for example in cuvettes or in (e.g. 96-well) microtiter plates. In particular, the assay for determination of the thiolase activity according to the present invention is as follows:

Assay for determination of the thiolase activity:

Cell crude extracts of *E. coli* are prepared by addition of 5 ml of BugBuster 10x Protein Extraction Reagent (Merck Bioscience, Darmstadt, Germany) which has previously been diluted 1:10 with 0.1 M Tris/HCl buffer, pH 6.0, per gram cell wet weight and the suspension is incubated at room temperature for 20 min. To remove the cell debris, the suspension is centrifuged at 4°C and 13,000 rpm for 30 min. The supernatant is transferred to a fresh vial and subsequently used for enzyme activity measurements, and the protein content is determined according to Bradford (Bradford 1976).

For the NADPH-coupled thiolase assay, acetoacetyl-CoA reductase (PhaB) activity is provided by crude extracts of *E. coli* BL21 (DE3) pET30::*phaB1*. [Said crude extract is obtainable as follows: The *phaB* gene is amplified from chromosomal DNA of *R. eutropha*

H16 using primers phaB_fw and phaB_rv and cloned into the pET-30 Xa/LIC according the manufacturer's description (Merck Bioscience, Darmstadt, Germany, Cat. No. 69073-3). *E. coli* BL21 (DE3) pET30::phaB1 is cultivated in LB containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37°C to an OD₆₀₀ of 0.7, 24 mg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) is added and the culture is further incubated at 30°C for 4 h. Cells are harvested by centrifugation at 4°C and 5,000 × g for 10 min, and the cell pellets are stored at -20°C when necessary. Cell crude extracts are prepared as described above.] The PhaB activity is measured spectrophotometrically: 30 µl of crude extract are added to 0.9 ml of potassium phosphate buffer (0.05 mM, pH 6.0) containing 32 nM acetoacetyl-CoA and 100 nM NADPH, and the absorption is recorded at 340 nm (Haywood et al. 1988).

The condensation reaction of thiolase activity is determined in extracts of *E. coli* BL21 (DE3) pASK-IBA3::thlA in 50 mM potassium phosphate buffer, pH 6.0, comprising 1 mM dithiothreitol (DTT), 0.2 mM NADPH and 2 U PhaB (in crude extracts of *E. coli* BL21 (DE3) pET30::phaB1). The reaction is started by addition of 1 mM acetyl-CoA and monitored at 340 nm on an Ultrospec 3000 photometer (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany). The reverse thiolytic cleavage reaction is measured spectrophotometrically by acetoacetyl-CoA decrease at 303 nm as described by Hartmanis and Gatenbeck (1984).

Cell extract preparation of *C. acetobutylicum* strains and thiolase activity measurements are conducted as described in detail previously (Hartmanis and Gatenbeck 1984; Lehmann and Lütke-Eversloh 2011). The above assay for determination of the thiolase activity is done in order to identify *improved* thiolase variants (mutants) among all the obtained mutant thiolase variants. Mutant thiolase variants may also be referred to as *thlA*^{MUT}. Within the meaning of this specification, an improved thiolase mutant is a thiolase mutant (variant of the parent enzyme) having either (a) higher activity than the parent enzyme (the starting enzyme of the mutagenesis), (i.e. without addition of CoA-SH) or (b) higher activity than the parent enzyme in the presence of ≥10 µM CoA-SH, or (c), a combination of (a) and (b) (i.e. both (a) and (b)). Improved thiolase variants may also be referred to as *thlA*^{IMP}.

In a preferred embodiment of (a) improved thiolase variant of the invention shows 5% or higher, preferably 10% or higher, more preferably 20% or higher, even more preferably 30% or higher activity, as compared to the parental (e.g. wildtype) enzyme. When it is aimed at identifying enzymes of embodiment (a), then no CoASH is added to the assay for determining activity.

In preferred embodiment of (b), improved thiolase variant has 50% or higher, preferably 75% or higher, more preferably 100% or higher, even more preferably 200% or higher activity in the presence of 10 µM CoA-SH, as compared to the parental (e.g. wildtype) enzyme. This

includes improved thiolase mutants which are not (in a measurable way) inhibited by free CoA-SH. When it is aimed at identifying enzymes of embodiment (b), the 10 μ M CoA-SH is added prior to performing the assay.

In the two paragraphs above, parental (e.g. wildtype) enzyme refers to the polypeptide which served as basis for the mutagenesis. The parental (e.g. wildtype) enzyme can be any enzyme having thiolase activity, such as any variant of ThIA, particularly any (preferably wildtype) ThIA from a *Clostridium* species. In a preferred embodiment, the parental (e.g. wildtype) enzyme is the one shown as SEQ ID NO: 1.

The approach applied by the present inventors has also shown lead to the identification of particularly preferred sites for mutagenesis, i.e. 133, 156, 222 with respect to SEQ ID NO: 1. It is understood that mutation of at least one, such as any combination of two (i.e. 133 and 156; 133, and 222; or 156 and 222), or all three of the residues 133, 156, 222 can lead to an improved enzyme of the invention. Thus, the present invention comprises a polypeptide having thiolase activity characterized in that it has 70 % or more, preferably 75 % or more, more preferably 80 % or more, more preferably 85 % or more, more preferably 90 % or more, more preferably 95 % or more, such as 96 % or more, 97 % or more, 98 % or more, 99 % or more sequence identity with SEQ ID NO: 1, provided that at least one, such as any combination of two, or all three of the residues 133, 156, 222 display a mutation with respect to SEQ ID NO:1. Within the meaning of this specification, the term "mutation" refers to any mutation, selected from insertion, deletion and substitution, whereby substitution is preferred.

Preferred substitutions of position 133 are the ones wherein the R residue is exchanged by a small residue, such as G or A or S or C, or a non-polar residue, such as particularly a hydrophobic residue as G, A, L, I, V, W, Y, F, wherein G is most preferred. Preferred substitutions of position 156 are the ones wherein the H residue is exchanged by an amide residue, such as N or Q, whereby N is preferred. Preferred substitutions of position 222 are the ones wherein the G residue is exchanged by a hydrophobic residue, such as G, A, L, I, V, W, Y, F, wherein V is most preferred. Any combination of these preferred substitutions is also comprised in the invention. Thus, in the most preferred embodiment, said polypeptide is characterized in that said mutation(s) are selected among any one, any combination of two, and preferably all three of the following substitutions: R133G, H156N, G222V. The embodiment wherein all these three substitutions are combined is shown in SEQ ID NO: 2, and also referred to herein as *thIA*^{OPT}.

The genes of improved thiolase variants may be expressed in an industrial production organism for increasing the flux into an acetoacetyl-CoA-dependent metabolic pathway. The DNA sequence of improved thiolase variants described in the present invention may be adapted to the specific host organism, e. g. by changing the codon usage for enhanced

recombinant gene expression.

The improved thiolase enzymes may be employed in a process for producing technically important compounds deriving from an acetoacetyl-CoA-related biosynthetic pathway. More specifically, the improved thiolase enzymes may be involved in a process for producing an isoprenoid compound involving the mevalonate pathway. In a preferred embodiment, the improved thiolase enzymes are employed in polyhydroxyalkanoate formation by innate or recombinant hosts for the production of thermoplastic polyesters from renewable resources.

In another embodiment, improved thiolase enzymes are employed in biofuel production. In a preferred embodiment, a biofuel production process is based on the conversion of carbohydrates such as mono-, di-, or polysaccharides, optionally including lignocellulosic hydrolysates, into biofuels. The improved thiolase enzymes of the present invention are used in such a process.

The improved enzymes of the invention can be used in a process for producing a chemical along the acetoacetyl-CoA-dependent metabolic pathway; this pathway requires a thiolase activity. Any process employing an enzyme of the invention is therefore also comprised in the invention.

Preferably, the thiolase of the invention (to be used in the process of the invention) has 5% or higher, preferably 10% or higher, more preferably 20% or higher, even more preferably 30% or higher activity, as compared to the parental wildtype enzyme under physiological conditions. Physiological conditions are the conditions typically found in the organism in which the enzyme is (optionally recombinantly) expressed. It can be expected that a higher activity of the enzyme will lead to higher yield of the product of the respective pathway (e.g. butanol). The product yield may thus serve to indirectly measure the activity. For direct measurement of the activity, the enzyme is expressed as described above and the crude extract is tested for thiolase activity as described above.

Preferably, the thiolase of the invention (to be used in the process of the invention) has an activity which is (essentially) not inhibited by 10 μ M CoA-SH. This means that the activity in presence of this concentration CoA-SH is at least 90 % of the activity in the absence of CoASH.

As described herein, said thiolase is obtainable by the above-described screening methods for selecting improved thiolase variants among the mutant thiolase variants. Said screening may involve expression of the thiolase recombinantly in bacteria, such as *E. coli*. The screening may be done by spectrophotometric measurements of thiolase activities in bacterial cell crude extracts. Alternatively, the screening can include butyric acid utilization for phenotype selection. Said screening may include monitoring of polyhydroxyalkanoate accumulation and viable colony staining for phenotype selection.

Preferably, the chemical produced in the process of the invention is a C4 alcohol. C4-alcohol means any alcohol having four carbon atoms, wherein the number or type of non-H atoms is not particularly limited. Preferably, a C4 alcohol comprises one O atom as the only non-H atom, i.e. is butanol (including n-butanol, 2-butanol and tert-butanol), preferably n-butanol.

The present invention also provides a nucleic acid encoding the improved enzyme of the invention. This includes the enzyme variant defined by the specific mutations as defined above, as well as any enzyme having a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A, as defined above. In particular included is the nucleic acid encoding the polypeptide defined by SEQ ID NO: 2. The skilled person will understand that due to the degeneration of the genetic code, various nucleic acids may encode said polypeptide, and all of them are included in the invention. Said nucleic acids may be conveniently obtained by *in silico* reverse translation.

Said nucleic acid may have a sequence having 80 % or more, preferably 90 % or more, more preferably 95 % or more, more preferably 96 % or more (97 % or more, 98% or more) and most preferably 99 % or more sequence identity with SEQ ID NO: 13. In a particular embodiment of the said degree of sequence comprises or consists of the polynucleotide shown as SEQ ID NO: 13. SEQ ID NO: 13 encodes the polypeptide of SEQ ID NO: 2. In one embodiment the sequence of SEQ ID NO: 12 is excluded from the clouds of particular percentage identities mentioned in this paragraph; i.e. the invention also relates to a nucleic acid having 80 % or more (including all the more and most preferred embodiments listed above) identity to SEQ ID NO:13, provided that SEQ ID NO: 12 is excluded.

Said nucleic acid may be in any form, although DNA (single-stranded or double-stranded, i.e. with the respective complementary strand) is preferred.

The invention further relates to a vector comprising said nucleic acid. Any suitable vector is comprised in the invention, although circular nucleic acid vectors, particularly plasmids are preferred. The vector will typically, although not necessarily contain a promoter for expression of the above-defined nucleic acid and/or an origin of replication allowing for replication in the (or each of the) host cell in which the vector is to be propagated and/or the above-defined nucleic acid is to be expressed.

The invention further relates to a host cell comprising the above-defined nucleic acid or the above-defined vector. The host cell is not particularly limited, although the criteria for its selection are based on the following considerations. It is preferred that the enzymes of the present invention are employed in microbial butanol production. Organisms with a pronounced C₄ metabolism (preferably organisms capable of producing butanol) are a

preferred host for expressing optimized thiolase genes according to the present invention. Thus, the host cell may be selected from organisms comprising a relatively high metabolic flux into a C₄-specific pathway; this includes in particular bacteria belonging to the genus *Clostridium*. In the process of the invention, the host cell may belong to the genus *Clostridium*. More preferably, the host cell belongs to one of the following species: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. butyricum*, *C. pasteurianum*, *C. saccharobutylicum*, *C. ljungdahlii*, *C. thermocellum*, *C. thermobutyricum*, *C. cellulolyticum*.

In other embodiments, the thiolase of the present invention may be expressed in any other host organism, preferably unicellular organism such as bacteria, archaea and unicellular fungi such as yeasts. In this case, further genes encoding enzymes of the butanol biosynthetic pathway (such as any combination of enzymes selected from the following list, and preferably all of the following list: hydroxybutyryl CoA dehydrogenase, crotonase, butyryl CoA dehydrogenase, alcohol dehydrogenase) are optionally expressed (optionally recombinantly) in the host organism. Thus, microorganisms suitable for butanol production include, but are not limited to, organisms allowing heterologous gene expression such as Gram-negative bacteria (like *Escherichia coli*) or Gram positive bacteria (like *Bacillus subtilis*), or even eukaryotic organisms (like *Saccharomyces cerevisiae*). Thus, in this embodiment butanol is produced by non-natural butanol producing strains, i.e. a strain which does not naturally produce butanol. This includes more preferred embodiments wherein the enzyme of the invention is expressed by a strain belonging to the genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Ralstonia*, *Saccharomyces*, provided that the further enzymes for butanol production are expressed in said strain, said strain can be used for butanol production according to the invention. Preferably, the butanol is produced by a strain belonging to the species *E. coli*, *B. subtilis*, *L. brevis*, *L. plantarum*, *P. putida*, *P. fluorescens*, *P. aeruginosa*, *R. eutropha*, *S. cerevisiae*.

The host cell of the invention optionally also expresses a gene encoding for aldehyde/alcohol dehydrogenase, such as *adhE1* (*adh*) from a *Clostridium* species, such as *Clostridium acetobutylicum*. Said gene may be homologously or heterologously expressed. Overexpression is preferred.

In addition to the metabolic *in vivo* pathways, improved thiolase may be applied *in vitro*. For *in vitro* synthesis of butanol for example, the thiolase of the present invention, as well as further enzymes of the butanol biosynthetic pathway (such as hydroxybutyryl CoA dehydrogenase, crotonase, butyryl CoA dehydrogenase, alcohol dehydrogenase) are provided *in vitro*, together with the educts of the reaction and buffers.

Further, the enzymes of the invention may be used in a synthetic pathway. A synthetic

pathway may be designed *in silico* for developing a process for producing a desired compound which requires a carbon-carbon bond formation. In one embodiment, the carbon-carbon bond formation proceeds via thiolase-catalyzed acetoacetyl-CoA biosynthesis. This is particularly useful in embodiments wherein the thiolase-catalyzed reaction is the rate-limiting step of the synthetic pathway. Thus, thiolase enzymes as described in this invention may be used to achieve higher metabolic fluxes through the synthetic pathway by increased thiolase activity and/or alleviated feedback inhibition.

EXAMPLES

Example 1: Bacterial strains and general cultivation conditions

E. coli was cultivated in LB medium comprising per liter 5 g yeast extract, 10 g tryptone and 10 g NaCl, antibiotics for plasmid maintenance were added as required (Sambrook and Russell 2001). *C. acetobutylicum* was cultivated anaerobically at 37°C without shaking in Hungate tubes or serum bottles. Resazurin (7-hydroxy-10-oxidophenoxazin-10-ium-3-one) was added as a redox indicator for anaerobiosis at a concentration of 1 mg/l and residual oxygen was removed by addition of 50-100 µl titanium (III) nitrilotriacetic acid (NTA) solution (1.3 M NaOH, 0.16 M NTA, 0.27 M Na₂CO₃ and 1.3 % TiCl₃). Reinforced clostridial agar was used as solid medium, 40 µg/ml erythromycin was added for plasmid-containing strains of *C. acetobutylicum*. Procedures requiring strictly anaerobic conditions were conducted in an anaerobic chamber with 90 % N₂ and 10 % H₂. All strains used in this example are listed in Table I.

Table I. Bacterial strains, plasmids and oligonucleotides used in this study.

Strain, plasmid or oligonucleotide	Relevant characteristics or sequence	Reference
Strains:		
<i>C. acetobutylicum</i> ATCC 824	Wildtype	American Type Culture Collection
<i>E. coli</i> (DE3)	BL21 F ⁻ , <i>ompT</i> , <i>hsdS_B</i> (r _B ⁻ , m _B ⁻), <i>gal</i> , <i>dcm</i> (DE3)	Studier and Moffatt 1986
<i>E. coli</i> DH5α	F ⁻ , φ80/ <i>lacZ</i> ΔM15, Δ(<i>lacZYA</i>), <i>recA1</i> , <i>endA1</i> , <i>hsdR17</i> (r _k ⁻ , m _k ⁺), <i>phoA</i> , <i>supE44</i>	Grant et al. 1990

thi1, *gyrA96*, *relA1*, λ

<i>E. coli</i> ER2275	<i>mcrA</i> , $\Delta mcrBC$, <i>hsdR</i> , <i>recA1</i>	Mermelstein and Papoutsakis 1993
<i>E. coli</i> BL21 (DE3) pET30:: <i>phaB</i>	Expression and purification of PhaB from <i>Ralstonia eutropha</i> with C-terminal His ₆ -tag, Amp ^R	this study

Plasmids:

pAN2	p15A, λ 3T1, Tet ^R	Heap et al. 2007
pET-30 Xa-LIC		Merck Bioscience, Darmstadt (Germany)
pASK-IBA3	F1 ori, P(<i>tetA</i>) ATG, <i>tetR</i> , <i>Strep</i> -tagII (C-terminal), Amp ^R	IBA GmbH, Göttingen (Germany)
pASK-IBA3:: <i>thiA</i> ^{WT}	F1 ori, P(<i>tetA</i>) ATG, <i>tetR</i> , <i>Strep</i> -tagII (C-terminal), Amp ^R , <i>thiA</i> wildtype gene of <i>C. acetobutylicum</i> (CAC2873)	this study
pASK-IBA3:: <i>thiA</i> ^{MUT}	F1 ori, P(<i>tetA</i>) ATG, <i>tetR</i> , <i>Strep</i> -tagII (C-terminal), Amp ^R , <i>thiA</i> mutant library	this study
pASK-IBA3:: <i>thiA</i> ^{OPT}	F1 ori, P(<i>tetA</i>) ATG, <i>tetR</i> , <i>Strep</i> -tagII (C-terminal), Amp ^R , <i>thiA</i> mutant (R133G, H156N, G222V)	this study
pT	ColE1, repL, Ery ^R , Amp ^R , P(<i>thiA</i>)	Mann et al. 2012
pT:: <i>thiA</i> ^{OPT}	ColE1, repL, Ery ^R , Amp ^R , P(<i>thiA</i>), <i>thiA</i> ^{opt} (R133G, H156N, G222V)	this study

Oligonucleotides:

thIA_fw_EcoRI	5'-AAAAAGAATTCATGAAAGAAGTTG-3'	(SEQ ID NO: 3)
thIA_rv_KpnI	5'- AAAAAGGTACCCTAGCACTTTTCTAGC- 3'	(SEQ ID NO: 4)
phaB_fw	5'- GGTATTGAGGGTCGCATGACTCAGCGC ATTG-3'	(SEQ ID NO: 5)
phaB_rv	5'- AGAGGAGAGTTAGAGCCCAGCCCATAT GCAGGC-3'	(SEQ ID NO: 6)
T7_fw	5'-TAATACGACTCACTATAGGG-3'	(SEQ ID NO: 7)
M13_rv	5'-CAAGGAAACAGCTATGAC-3'	(SEQ ID NO: 8)
pASK_fw	5'-GTTATTTTACCACTCCCTATC-3'	(SEQ ID NO: 9)
pASK_rv	5'-GTGCGCCATTTTTCCTTTCAC-3'	(SEQ ID NO: 10)
1107435.S1_rv	5'-GTTGCATAAAAAGGTCCATATC-3'	(SEQ ID NO: 11)

Example 2: Thiolase gene cloning and expression

General recombinant DNA techniques were conducted according to standard protocols (Sambrock and Russell 2001). All cloning procedures were validated by DNA sequencing (LGC Genomics GmbH, Berlin, Germany). The *thIA* gene (CAC2873) was amplified by PCR from chromosomal DNA of *C. acetobutylicum* ATCC 824 (Fischer et al. 2006) using the primers ThIA_EcoRI_fw and ThIA_KpnI_rv and ligated via *EcoRI* and *KpnI* restriction sites into the vector pASK-IBA3 resulting in plasmid pASK-IBA3::*thIA*^{WT}, which was transformnd

into *E. coli* BL21 (DE3). For protein expression, *E. coli* BL21 (DE3) pASK-IBA3::*thlA*^{WT} and *E. coli* BL21 (DE3) pASK-IBA3::*thlA*^{MUT} strains were cultivated in LB medium with 100 µg/ml ampicillin at 37°C until an OD₆₀₀ of 0.6-0.8. After addition of 20 µg/ml anhydrotetracycline (AHT), the cultures were incubated over night at 20°C and 180 rpm prior to crude extract preparation for enzyme activity assays.

The optimized *thlA* mutant gene identified in the library screening, SEQ ID NO:2; *thlA*^{OPT}, was subcloned via *EcoRI/KpnI* restriction of pASK-IBA3:: *thlA*^{OPT}, agarose gel purification, T4 DNA polymerase treatment and ligation into the pT vector, yielding plasmid pT::*thlA*^{OPT}. After *in vivo* methylation in *E. coli* (Heap et al. 2007; Mermelstein and Papoutsakis 1993), pT::*thlA*^{OPT} was electroporated into *C. acetobutylicum* ATCC 824 as described previously (Riebe et al. 2009). All plasmids and oligonucleotides used in this example are listed in Table I.

Example3: Enzyme activity measurements

Cell crude extracts of *E. coli* were prepared by addition of 5 ml of BugBuster 10x Protein Extraction Reagent (Merck Bioscience, Darmstadt, Germany) which was previously diluted 1:10 with 0.1 M Tris/HCl buffer, pH 6.0, per gram cell wet weight and the suspension was incubated at room temperature for 20 min. To remove the cell debris, the suspension was centrifuged at 4°C and 13,000 rpm for 30 min. The supernatant was used for enzyme activity measurements and the protein content was determined according to Bradford (Bradford 1976).

For the NADPH-coupled thiolase assay, acetoacetyl-CoA reductase (PhaB) activity was provided by crude extracts of *E. coli* BL21 (DE3) pET30::*phaB1*. For this, the *phaB* gene was amplified from chromosomal DNA of *R. eutropha* H16 using primers *phaB_fw* and *phaB_rv* and cloned into the pET-30 Xa/LIC according the manufacturer's description (Merck Bioscience, Darmstadt, Germany, Cat. No. 69073-3). *E. coli* BL21 (DE3) pET30::*phaB1* was cultivated in LB containing 100 µg/ml ampicillin and 25 µg/ml chloramphenicol at 37°C to an OD₆₀₀ of 0.6-0.8, 24 mg/ml isopropyl-β-D-thiogalactopyranoside (IPTG) was added and the culture was further incubated at 30°C for 4 h. Cells were harvested by centrifugation at 4°C and 5,000 × g for 10 min, and the cell pellets were stored at -20°C when necessary. Cell crude extracts were prepared as described above. The PhaB activity was measured spectrophotometrically: 10-50 µl of crude extract were added to 0.9 ml of potassium phosphate buffer (0.05 mM, pH 6.0) containing 32 nM acetoacetyl-CoA and 100 nM NADPH, and the absorption was recorded at 340 nm (Haywood et al. 1988).

The condensation reaction of thiolase activities were determined in extracts of *E. coli* BL21 (DE3) pASK-IBA3::*thlA* in 50 mM potassium phosphate buffer, pH 6.0, comprising 1 mM dithiotreitol (DTT), 0.2 mM NADPH and 2 U PhaB (in crude extracts of *E. coli* BL21 (DE3) pET30::*phaB1*). The reaction was started by addition of 1 mM acetyl-CoA and monitored at 340 nm on an Ultrospec 3000 photometer (Amersham Buchler GmbH & Co. KG, Braunschweig, Germany). The reverse thiolytic cleavage reaction was measured spectrophotometrically by acetoacetyl-CoA decrease at 303 nm as described (Hartmanis and Gatenbeck 1984). Cell extract preparation of *C. acetobutylicum* strains and thiolase activity measurements were conducted as described in detail previously (Hartmanis and Gatenbeck 1984; Lehmann and Lütke-Eversloh 2011). Enzyme activity measurements in *E. coli* cell extracts are shown in table II.

Table II. Enzyme activities in cell extracts of *E. coli* hosts.

Strain	Acetoacetyl-CoA (PhaB)	reductase	Thiolase (ThlA)	
	NADH	NADPH	Thiolytic cleavage	Condensation
<i>E. coli</i> BL21 (DE3) pASK-IBA3 (control)	0.03 ± 0.02	0.02 ± 0.01	0.08 ± 0.04	0.2 ± 0.09
<i>E. coli</i> BL21 (DE3) pASK-IBA3:: <i>thlA</i> ^{WT}	0.05 ± 0.03	0.73 ± 0.09	65.94 ± 4.61	68.96 ± 1.54
<i>E. coli</i> Tuner (DE3) pET30:: <i>phaB1</i>	0.15 ± 0.02	34.27 ± 1.43	0.02 ± 0.003	0.3 ± 0.11

Example 4: Thiolase library construction

To construct the *thlA* mutant library (designated as pASK-IBA3::*thlA*^{MUT}), synthetic oligonucleotides building the degenerated backbone of the library were assembled without any amplification reaction involved (non-amplified library), and the diversity of the library was directly correlated to the amount of DNA molecules produced, i.e. 67 fmol/μl corresponded to 4 x 10¹⁰ molecules (Geneart AG, Regensburg, Germany). To generate the amplified library, 4 μl (268 fmol or 1.6 x 10¹¹ molecules) of the non-amplified library were amplified with the primers T7_fw and M13_rv, full length fragments were gel-purified and resuspended in 100 μl Tris/EDTA buffer. The concentration of the amplified library was determined by UV

spectroscopy as 400 ng/μl. The amplified library was digested with *EcoRI* and *KpnI* and ligated into the *EcoRI/KpnI*-restricted vector pASK-IBA3. Ligation reactions were transformed into *E. coli* BL21 (DE3) and the transformation rate was determined by plating of dilution series. The total number of transformants was 5.7×10^4 colony forming units (CFU). Total cells from the transformation plates were harvested for plasmid preparation. The concentration of the cloned library was determined by UV spectroscopy as 0.46 μg/μl. The plasmid preparations were sequenced with primers pASK_fw, pASK_rv and 1107435.S1_rv (Geneart AG, Regensburg, Germany). Total cells from the transformation plates were harvested, resuspended in 50 vol% glycerol and aliquots of the cell suspension comprising 3.9×10^{10} cells/ml were stored at -70°C. All strains, plasmids and oligonucleotides used in this example are listed in Table I.

Example 5: Screening procedure

The *E. coli* pASK-IBA3::*thlA*^{MUT} library was plated on LB agar plates plus 100 μg/ml ampicillin (LB + Ap) and incubated over night at 37°C. Colonies were transferred with sterile toothpicks into 96-well microtiter plates containing 200 μl LB + Ap and grown over night at 37°C. Each microtiter plate comprised two samples each of *E. coli* pASK-IBA3::*thlA*^{WT} and *E. coli* pASK-IBA3 (empty vector) as controls. 10 μl Aliquots of the cultures were used to inoculate fresh LB + Ap and the grown microtiter plates were stored at -70°C after addition of 10 vol% glycerol. The inoculated microtiter plates were incubated at 37°C until the cultures exhibited an OD₆₀₀ of 0.5-0.7. For induction of gene expression, 20 μg/ml AHT was added, and the cultures were incubated over night at 20°C. Subsequently, cell crude extracts were prepared using BugBuster 10x Protein Extraction Reagent (Merck Bioscience, Darmstadt, Germany) as described above to determine the thiolase activities of the *E. coli* pASK-IBA3::*thlA*^{MUT} library. For this, 150 μl potassium phosphate buffer (50 mM, pH 6.0) comprising 1 mM DTT and 0.2 mM NADPH were pipetted into the wells of fresh microtiter plates. When analyzing the CoA-SH sensitivity, the buffer contained 10 μM free coenzyme A (Sigma-Aldrich Chemie, Deishofen, Germany). After addition of 20 μl PhaB extract (2 U) and 10 μl cell crude extracts, the reaction was started by addition of 20 μl acetyl-CoA solution (20 mM). The absorption at 340 nm was recorded for 3 min in 3 s intervals on a SpectraMax M2e Multi-Mode Microplate Reader and the slope values were automatically calculated.

In order to validate the microtiter screening results, the 14 positive clones were cultivated at a larger scale and thiolase activities were spectrophotometrically determined in the cuvette format (Table III). None of the *ThlA*^{MUT} variants exhibited an increased overall thiolase activity, but differences to *ThlA*^{WT} were detected in the presence of 10-50 μM CoA-SH. Whereas 8 clones showed similar activities at various CoA-SH concentrations as the wildtype *ThlA*, the clones 9G4, 13D7, 19C10, 21B8, 25C8 and 28C9 revealed significantly

increased thiolase activities when CoA-SH was added to the assay (Table III). Subsequent DNA sequencing of the respective plasmids exhibited that all 6 positive clones had exactly the same genotype, indicating that in fact only one novel *ThIA*^{MUT} variant was isolated. Basically, the 6 independently identified hits confirmed the reliability of the screening procedure to find improved thiolase mutants. Thus, clone 9G4 was chosen as a representative for subsequent experiments and was referred to as *E. coli* BL21 (DE3) pASK-IBA3::*thIA*^{OPT}.

Table III. Thiolase activities of putative positive clones identified in the *E. coli* BL21 (DE3) pASK-IBA3::*thIA*^{MUT} library screening.

ThIA variant	Thiolase activity in the presence of free CoA-SH					
	0 μ M	10 μ M	20 μ M	30 μ M	40 μ M	50 μ M
wildtype	68.96 1.54	$\pm$ 25.60 0.84	$\pm$ 8.44 $\pm$ 0.67	1.30 $\pm$ 0.03	0.28 $\pm$ 0.03	0.11 $\pm$ 0.03
7D5	69.80 3.41	$\pm$ 26.23 1.58	$\pm$ 8.54 $\pm$ 0.13	1.33 $\pm$ 0.08	0.29 $\pm$ 0.04	0.13 $\pm$ 0.01
9G4	69.19 0.55	$\pm$ 34.60 1.67	$\pm$ 17.23 1.06	$\pm$ 9.83 $\pm$ 0.25	2.06 $\pm$ 0.10	1.18 $\pm$ 0.08
11B9	69.39 2.01	$\pm$ 24.81 0.58	$\pm$ 8.38 $\pm$ 0.06	1.22 $\pm$ 0.04	0.30 $\pm$ 0.05	0.10 $\pm$ 0.03
12B8	69.50 1.37	$\pm$ 25.23 0.23	$\pm$ 8.52 $\pm$ 0.09	1.30 $\pm$ 0.12	0.27 $\pm$ 0.03	0.11 $\pm$ 0.03
13D7	68.94 0.89	$\pm$ 36.45 0.68	$\pm$ 17.28 0.57	$\pm$ 11.87 0.08	$\pm$ 1.98 $\pm$ 0.17	1.17 $\pm$ 0.07
17C5	70.11 1.27	$\pm$ 24.58 0.77	$\pm$ 8.30 $\pm$ 0.41	1.06 $\pm$ 0.04	0.32 $\pm$ 0.02	0.13 $\pm$ 0.02
19C10	69.76 0.90	$\pm$ 37.89 0.53	$\pm$ 18.57 0.58	$\pm$ 10.52 0.28	$\pm$ 1.86 $\pm$ 0.76	1.43 $\pm$ 0.09
21B8	68.72 0.12	$\pm$ 34.13 1.06	$\pm$ 16.79 0.43	$\pm$ 11.32 0.20	$\pm$ 2.37 $\pm$ 0.09	1.38 $\pm$ 0.15

22B7	69.85 1.19	± 24.85 0.98	± 8.47 ± 0.09	3.76 ± 4.83	0.32 ± 0.05	0.13 ± 0.02
25C8	70.33 0.53	± 35.92 0.47	± 17.33 0.53	± 9.95 ± 0.77	2.12 ± 0.18	1.34 ± 0.19
28B9	68.61 0.54	± 24.80 0.53	± 8.90 ± 0.73	1.02 ± 0.08	0.37 ± 0.02	0.15 ± 0.01
28C4	69.88 1.00	± 24.61 0.81	± 8.42 ± 0.30	1.12 ± 0.03	0.31 ± 0.05	0.14 ± 0.00
28C9	69.51 1.00	± 37.89 0.53	± 20.73 4.51	± 9.83 ± 0.33	2.00 ± 0.17	0.01 ± 1.23
30F3	71.03 0.79	± 24.56 0.65	± 8.41 ± 0.01	1.32 ± 0.20	0.29 ± 0.04	0.12 ± 0.01

Example 5: Fermentation experiments for solvent production

Cultivation experiments of recombinant *C. acetobutylicum* were performed in 200 ml MS-MES comprising 40 µg/ml erythromycin as described previously in detail (Lehmann et al. 2012a; Lehmann and Lütke-Eversloh 2011; Lehmann et al. 2012b). Samples were regularly drawn to determine the pH with a pH meter (Wissenschaftlich-Technische Werkstätten GmbH, Weilheim, Germany) and the Optical Density at 600 nm in a spectrophotometer (Zeiss Spekol 1100 photometer, Analytik Jena AG, Jena, Germany) using 1.5-ml plastic cuvettes with a light path of 1 cm. Cell-free supernatant samples were stored at -20°C until quantification of glucose (Bergmeyer 1983) and fermentation products. Acetate, butyrate, acetone, ethanol and butanol were determined according to Thormann et al. (Thormann et al. 2002) on an Agilent 7890A gas chromatograph (Agilent Technologies, Böblingen, Germany) equipped with a Chromosorb 101 (80/100 mesh, 2.0 m x 3.0 mm x 1.6 mm) glass column.

When the protein shown as SEQ ID NO: 2 was recombinantly expressed in the *C. acetobutylicum*, the following results were obtained: In addition to a clearly delayed ethanol and acetone formation, the ethanol titer was increased by 46%. Butanol titer was increased by 18%, while the final acetone concentrations were similar to the vector control strain.

Protein sequences shown in the sequence listing

SEQ ID NO: 1: Thl Wild type (*Clostridium acetobutylicum*)

SEQ ID NO: 2: Thl^{OPT}

SEQ ID NO: 12: Nucleic acid encoding polypeptide of SEQ ID NO: 1

SEQ ID NO: 13: Nucleic acid encoding polypeptide of SEQ ID NO: 2

Further oligonucleotide sequences shown in the sequence listing are also listed in Table I.

REFERENCES CITED IN THE DESCRIPTIONPATENT DOCUMENTS CITED IN THE DESCRIPTION

US 4,757,010 Production of clostridium acetobutylicum mutants of high butanol and acetone productivity, the resultant mutants and the use of these mutants in the joint production of butanol and acetone

US 6,358,717 Method of producing butanol using a mutant strain of *Clostridium beijerinckii*

US 2011/0027845 A1, Enhanced ethanol and butanol producing microorganisms and method for preparing ethanol and butanol using the same

US020110281313 A1, Improved production of acid and solvent in microorganisms

CN000101423813B Recombinant clostridium and construction method and use thereof

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acetone/butanol/isopropanol fermentation. Appl Microbiol Biotechnol 49:639-648.

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CLAIMS

- [1] A process for producing a chemical using an acetoacetyl-CoA-dependent metabolic pathway which has a thiolase activity with higher specific activity and/or reduced sensitivity towards the inhibitor coenzyme A compared to the activity and sensitivity of the polypeptide having SEQ ID NO: 1, wherein the thiolase is a mutant protein having at least one mutation into the CoA-SH binding loop domain with respect to the parental wildtype enzyme, and wherein the determination of thiolase activity is performed by a 3-hydroxybutyryl-CoA dehydrogenase-coupled spectrophotometric assay.
- [2] The process of claim 1, wherein the thiolase has 5% or higher, preferably 10% or higher, more preferably 20% or higher, even more preferably 30% or higher activity, as compared to the parental wildtype enzyme under physiological conditions.
- [3] The process of any one of claim 1 or 2, wherein activity of said thiolase is not inhibited by 10 μ M CoA-SH.
- [4] The process of any one of claims 1-3, wherein said chemical is a C4 alcohol.
- [5] The process of claim 4, wherein said chemical is butanol, preferably n-butanol.
- [6] The process of any one of claims 1-5, wherein the production is done in a host cell belonging to the genus *Clostridium*.
- [7] The process of claim 6, wherein such host cell belongs to one of the following species: *C. acetobutylicum*, *C. beijerinckii*, *C. saccharoperbutylacetonicum*, *C. butyricum*, *C. pasteurianum*, *C. saccharobutylicum*, *C. ljungdahlii*, *C. thermocellum*, *C. thermobutylicum*, *C. cellulolyticum*.
- [8] The process of any one of claims 5-7, wherein butanol is produced by non-natural butanol producing strains.
- [9] The process of any one of claims 5-8, wherein butanol is produced by a strain belonging to the genus *Escherichia*, *Bacillus*, *Lactobacillus*, *Pseudomonas*, *Ralstonia*, *Saccharomyces*.
- [10] The process of any one of claims 5-9, wherein butanol is produced by a strain belonging to the species *E. coli*, *B. subtilis*, *L. brevis*, *L. plantarum*, *P. putida*, *P. fluorescens*, *P. aeruginosa*, *R. eutropha*, *S. cerevisiae*.
- [11] A polypeptide having thiolase activity characterized in that it has 70 % or more, preferably 75 % or more, more preferably 80 % or more, more preferably 85 % or

more, more preferably 90 % or more, more preferably 95 % or more, such as 96 % or more, 97 % or more, 98 % or more, 99 % or more sequence identity with SEQ ID NO: 1, provided that at least one, such as any combination of two, or all three of the residues 133, 156, 222 display a mutation (selected from substitution, deletion or insertion) with respect to SEQ ID NO:1.

[12] The polypeptide of claim 11 wherein said mutation(s) selected from

- any one,
- any combination of two, and
- preferably all three (SEQ ID NO: 2)

of the following substitutions: R133G, H156N, G222V.

[13] The process of any one of claim 1 to 10, characterized in that the thiolase activity is provided by the enzyme of any of claims 11 to 12.

[14] A nucleic acid encoding a polypeptide selected from the group consisting of (a) the polypeptide of any one of claims 11 or 12 and (b) the enzyme having the thiolase activity defined in any one of claims 1 to 10.

[15] A vector comprising the nucleic acid of claim 14.

[16] A host cell carrying the nucleic acid of claim 14 or the vector of claim 15.

FIGURES

Figure 1.

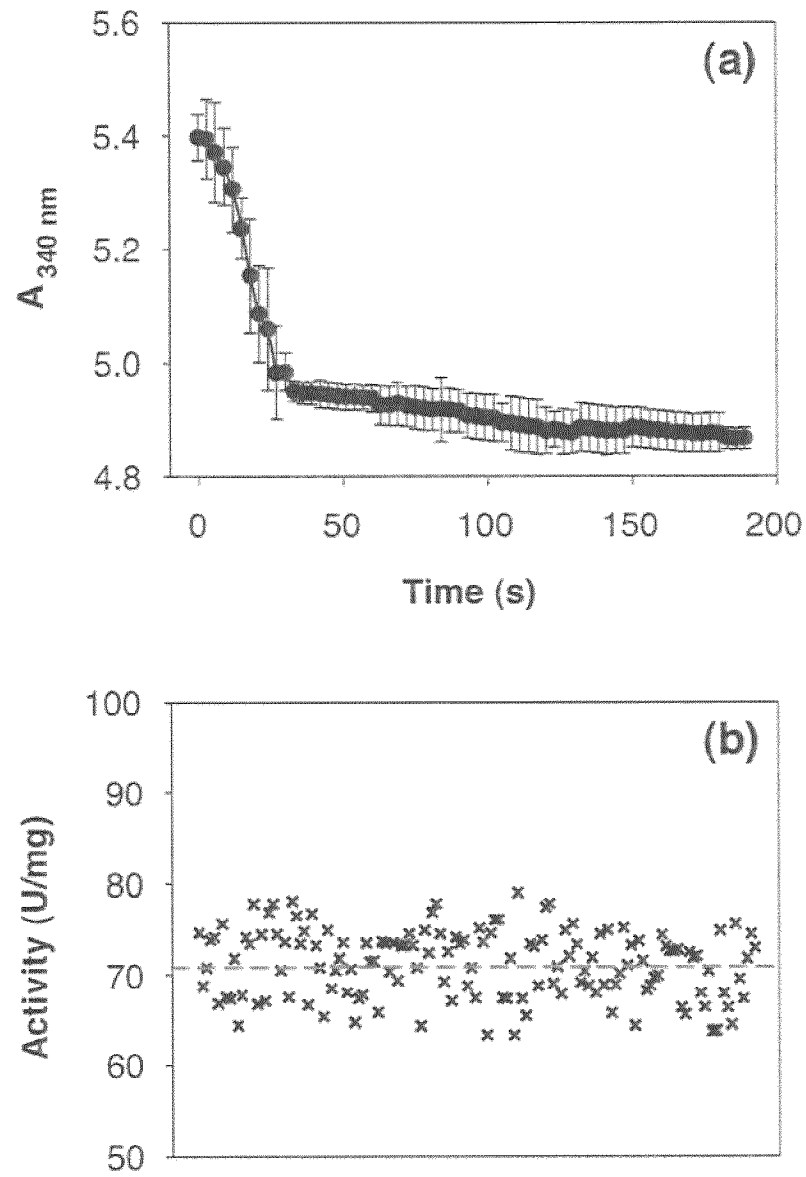


Figure 2.

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CAC2873      -MKEVVIASAVRTAIGSYGKSLKDVPVAVDLGATAIKEAVKKAGIKPEDVNEVILGNVLQA 59
Z.ram.       STPSIVIASAARTAVGSFNGAFANTPAHELGATVISAVLERAGVAAGEVNEVILGQVLPA 60
              .:*****.***:**. :. :.* :*****.*. .:::**. . :*****:** *

CAC2873      GLGQNPARQASFKAGLPVEIPAMTINKVCGSGLRTVSLAAQIIKAGDADVIIAGGMENMS 119
Z.ram.       GEGQNPARQAAMKAGVPQEATAWGMNQLCGSGLRAVALGMQQIATGDASIIVAGGMESMS 120
              * *****:***,* * .* :*:* *****:*,* * * :***:,*:*****,**

CAC2873      RPYLANNARWGYRMGNAKFVDEMITDGLWDAFNDYHMGITAENIAERWNISREEQDEFA 179
Z.ram.       MAPHCAH-LRGGVKMGDFKMIDTMIKDGLTDAFYGYHMGTTAENVAKQWOLSRDEQDAFA 179
              **: * : * * :*: *: * * * * * * * * * * * * * * * * * * * * * *

CAC2873      LASQKKAEEAIKSGQFKDEIVPVVIKGRKGETVVDTEHPRFGSTIEGLAKLKPAFKKDG 239
Z.ram.       VASQNKAAEAQKDGFRFKDEIVPFIVKGRKGDITVDADEYIRHGATLDSMAKLRPAFDKEG 239
              :***:** * *.*:*****.:*****: .**:*: *.*:***:.*:*****:**

CAC2873      TVTAGNASGLNDCAAVLVIMSAEKAKELGVKPLAKIVSYGSAGVDPAIMGYGPFYATKAA 299
Z.ram.       TVTAGNASGLNDGAAAALLMSEAEASRRGIQPLGRIVSWATVGVDPKVMGTGPPIPASRKA 299
              ***** ** . :** :*.. *:***:***:..***** :** **: *: : *

CAC2873      IEKAGWTVDELDLIESNEAFAAQSLAVAKDLKFDMNKVN VNNGAIALGHPIGASGARILV 359
Z.ram.       LERAGWKIGDLDLVEANEAFAAQACAVNKDLGWDPSIVNVNNGAIAIGHPIGASGARILN 359
              :*:***:..:***:*:*****: ** *** :* , *****:*****

CAC2873      TLVHAMQKRDAKKGLATLCIGGGQGTAILLEKC 392
Z.ram.       TLLFEMKRRGARKGLATLCIGGGMGVAMCIESL 392
              **: . *:*.*:***** *.*: :*.

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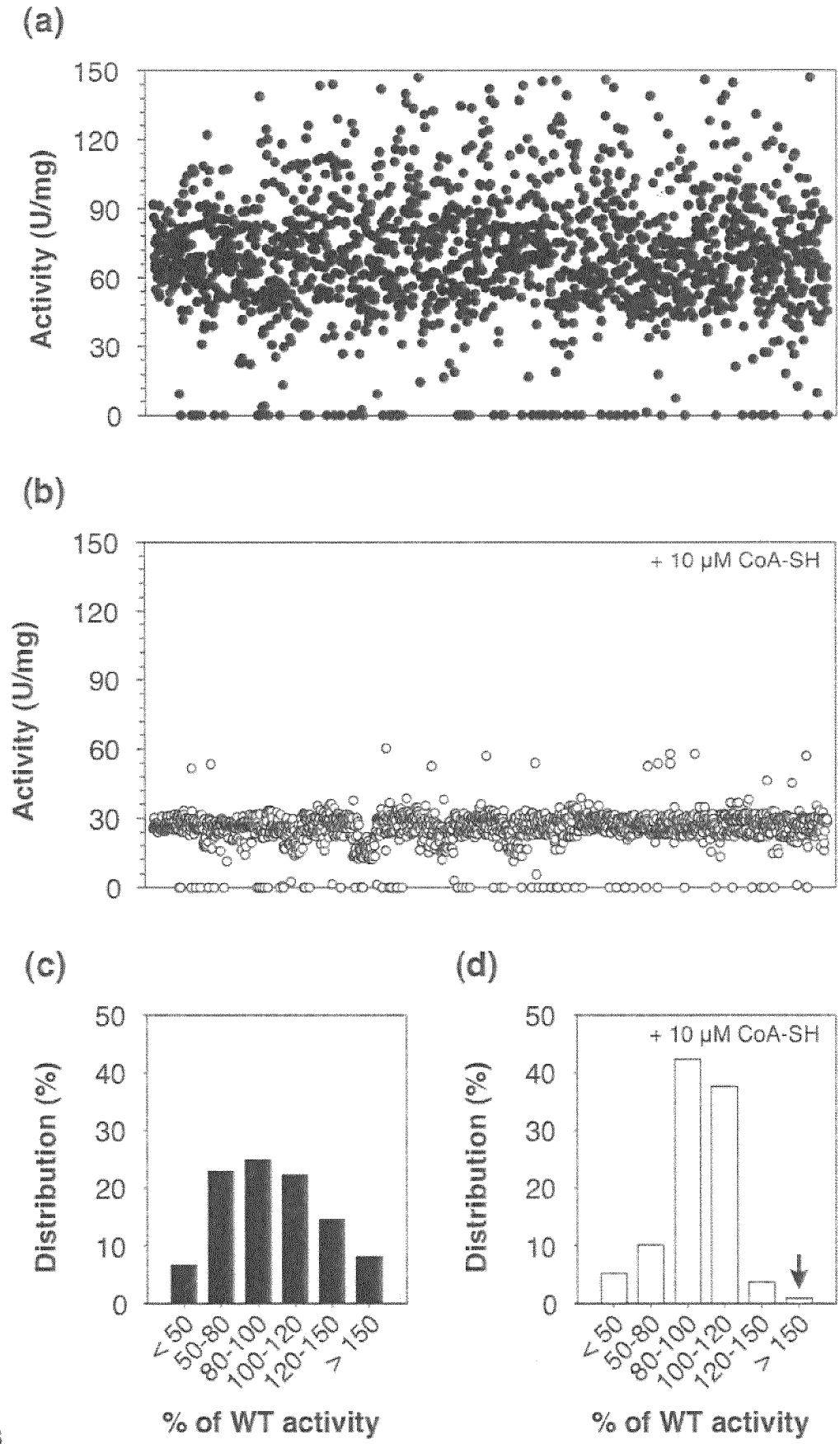


Figure 3.

Figure 4.

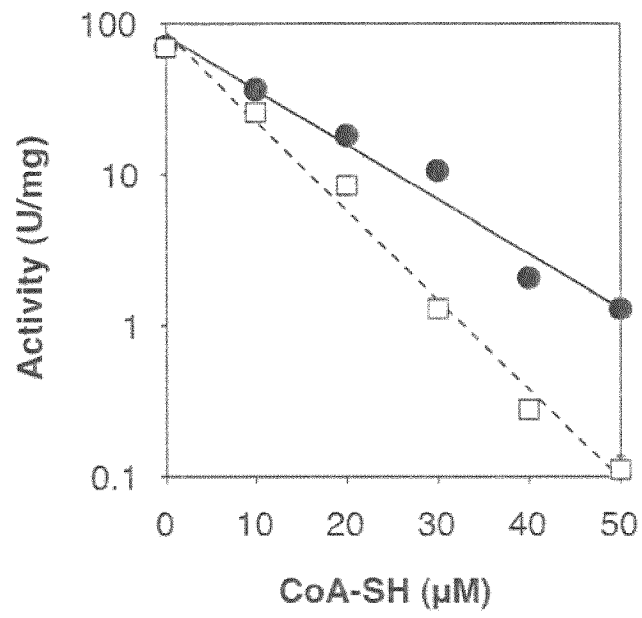
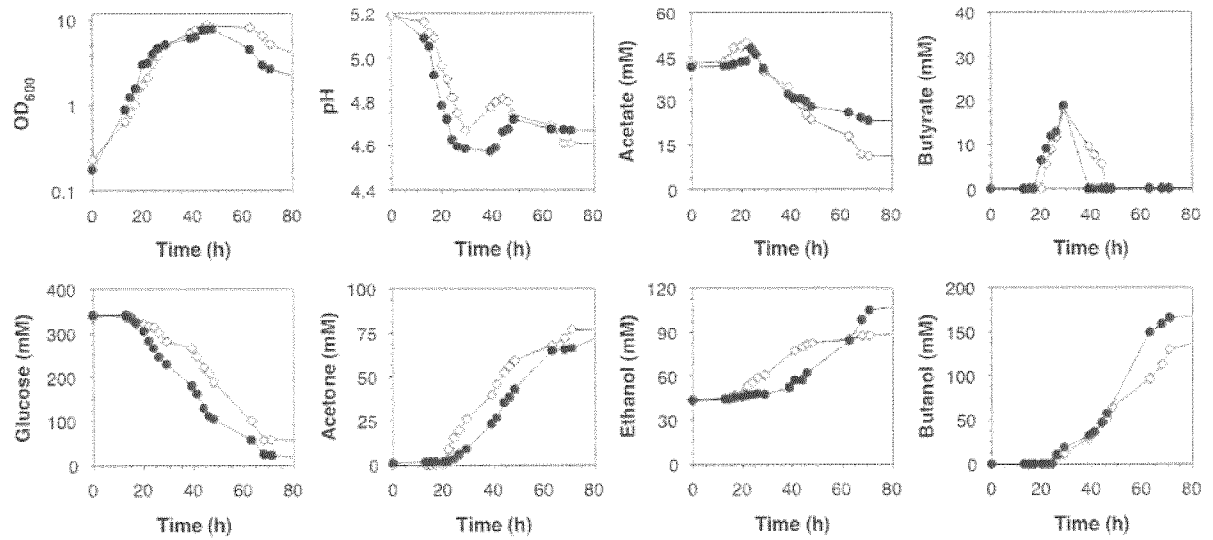


Figure 5.



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/064302

A. CLASSIFICATION OF SUBJECT MATTER

INV. C12N9/10 C07K14/33 C12P1/00 C12P7/16
ADD.

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N C07K C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

EPO-Internal, Sequence Search, BIOSIS, EMBASE, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2012/088281 A1 (KIM KYUNG JIN [KR]) 12 April 2012 (2012-04-12) the whole document	1-11, 14-16
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Further documents are listed in the continuation of Box C.



See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search

8 November 2013

Date of mailing of the international search report

15/11/2013

Name and mailing address of the ISA/

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Roscoe, Richard

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/064302

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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X	----- DATABASE UniProt [Online] 5 October 2010 (2010-10-05), "SubName: Full=Acetyl-CoA acetyltransferase; EC=2.3.1.9;", XP002687427, retrieved from EBI accession no. UNIPROT:D9TPZ6 Database accession no. D9TPZ6 sequence	11,13-16
A	----- RYAN SILLERS ET AL: "Aldehyde-alcohol dehydrogenase and/or thiolase overexpression coupled with CoA transferase downregulation lead to higher alcohol titers and selectivity in Clostridium acetobutylicum fermentations", BIOTECHNOLOGY AND BIOENGINEERING, vol. 102, no. 1, 1 January 2009 (2009-01-01), pages 38-49, XP055044193, ISSN: 0006-3592, DOI: 10.1002/bit.22058 the whole document	1-16
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INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2013/064302

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	GITTE MERILÄINEN ET AL: "The sulfur atoms of the substrate CoA and the catalytic cysteine are required for a productive mode of substrate binding in bacterial biosynthetic thiolase, a thioester-dependent enzyme", FEBS JOURNAL, vol. 275, no. 24, 4 December 2008 (2008-12-04), pages 6136-6148, XP055085766, ISSN: 1742-464X, DOI: 10.1111/j.1742-4658.2008.06737.x the whole document -----	1-16
A	WIESENBORN D P ET AL: "Thiolase from Clostridium acetobutylicum ATCC 824 and Its Role in the Synthesis of Acids and Solvents.", APPLIED AND ENVIRONMENTAL MICROBIOLOGY NOV 1988, vol. 54, no. 11, November 1988 (1988-11), pages 2717-2722, XP055086063, ISSN: 0099-2240 the whole document -----	1-16
A	KURSULA P ET AL: "High Resolution Crystal Structures of Human Cytosolic Thiolase (CT): A Comparison of the Active Sites of Human CT, Bacterial Thiolase, and Bacterial KAS I", JOURNAL OF MOLECULAR BIOLOGY, ACADEMIC PRESS, UNITED KINGDOM, vol. 347, no. 1, 18 March 2005 (2005-03-18), pages 189-201, XP004759229, ISSN: 0022-2836, DOI: 10.1016/J.JMB.2005.01.018 the whole document -----	1-16

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

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