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(56) Related Art
Mermelstein LD et al. Metabolic engineering of Clostridium acetobutylicum ATCC 824 for Increased Solvent Production by Enhancement of Acetone Formation Enzyme Activities using a Synthetic Acetone Operon. Biotechnol. Bioeng. 1993; 42: 1053-1060
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(54) Title: CELLS AND METHOD FOR PRODUCING ACETONE

(54) Bezeichnung: ZELLEN UND VERFAHREN ZUR HERSTELLUNG VON ACETON

(57) Abstract: The object of the invention are cells and a method for the production of acetone.

(57) Zusammenfassung: Gegenstand der Erfindung sind Zellen und ein Verfahren zur Aceton-Produktion.



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Cells and method for producing acetoneField of the invention

The invention relates to cells and a method for producing acetone.

Prior art*ABE process in Clostridium*

The classical ABE fermentation process, i.e. the microbial production of acetone, butanol and ethanol, was for a long time the world's second-largest biotechnological process, directly after the fermentation of ethanol with yeasts. Commercial ABE fermentation began in 1916 in England, where *inter alia* Chaim Weizmann discovered that *Clostridium acetobutylicum* is able to form the solvents acetone, butanol and ethanol. The process was employed in the West until the late 1950s, and in South Africa even until 1981.

There are two main reasons why this process was abandoned: on the one hand, the chemical synthesis of acetone and butanol became more and more favorable, and on the other hand the price for the substrates for fermentation rose sharply. There was in particular a large increase in the price for molasses, on account of its use as a feed additive for cattle.

The increasing costs for petrochemical starting products, and new technological possibilities in the area of pathway engineering of microorganisms, now open up new options for the development of high-performance strains and commercial fermentation processes for the production of solvents such as acetone.

The classical ABE fermentation is based on the organisms *Clostridium acetobutylicum* and *Clostridium beijerinckii*. Both are Gram-positive and multiply under strictly anaerobic conditions. These organisms can convert mono-, di- and polysaccharides, and the substrates mainly used in fermentation are molasses and starch.

The fermentation process with *C. acetobutylicum* is divided into two phases. In the first phase, biomass formation is accompanied by the formation of acetate, butyrate and traces of ethanol ("acidogenic phase"). In the second phase, the so-called "solventogenic phase", the acids are then used for forming the fermentation products acetone, butanol and ethanol (ABE). The products acetone, butanol and ethanol are formed in wild-type *C. acetobutylicum* in the approximate proportions 3 : 6 : 1. These proportions of the products can vary widely, depending on the chosen culture conditions (e.g. pH or nutrient feed) or the substrates used.

The enzymes of solvent biosynthesis of acetone, butanol and ethanol have been extensively purified and characterized biochemically (cf. Fig. 1; Duerre, P., and Bahl, H. 1996. Microbial production of acetone/butanol/isopropanol. In: Biotechnology, Vol. 6, 2nd ed. M. Roehr, (ed.), VCH Verlagsgesellschaft mbH, Weinheim, Germany. p. 229-268. Duerre, P. 1998. New insights and novel developments in clostridial acetone/butanol/isopropanol fermentation. Appl. Microbiol. Biotechnol. 49: 639-648). The genome sequence of *C. acetobutylicum* is also available (Noelling, J., Breton, G., Omelchenko, M. V. & 16 other authors (2001). Genome sequence and comparative analysis of the solvent-producing bacterium *Clostridium acetobutylicum*. J Bacteriol 183, 4823-4838).

Clostridium acetobutylicum strains have already been generated in which the production of acetone has been decoupled from the production of butanol and ethanol, so that these strains only produce acetone (Mermelstein et al. (1993). Metabolic engineering of *Clostridium acetobutylicum* ATCC824 for increased solvent production by enhancement of acetone formation enzyme activities using a synthetic operon. Biotech. Bioeng. 42:1053-1060.; Nair, R.V., and Papoutsakis, E.T. 1994). The measured titers for acetone were always below its concentrations in the wild type.

Fig. 2 shows the classical metabolic pathway for acetone synthesis, characterized in *Clostridium*. This pathway starts with acetyl-CoA, a central metabolite that is formed in all microorganisms, regardless of the carbon source that is metabolized or which metabolic pathways are established. The required enzymes are: β -ketothiolase, the two subunits of acetyl-CoA/butyryl-CoA-transferase, and acetoacetate decarboxylase.

It has been shown that the heterologous expression of these enzymes from *C. acetobutylicum* in *Escherichia coli*, which catalyze acetone formation starting from acetyl-CoA (acetoacetate decarboxylase, acetyl-CoA/butyryl-CoA-transferase and thiolase) lead to acetone formation in this organism of approx. 150 mM, but there was the disadvantage that large amounts of acetate (50 mM) were also produced (Bermejo L. L., N. E. Welker, E. T. Papoutsakis. 1998. Expression of *Clostridium acetobutylicum* ATCC 824 Genes in *Escherichia coli* for Acetone Production and Acetate Detoxification. Appl. Env. Microbiol. 64:1079-1085).

Another disadvantage here is that acetone was only produced under aerobic conditions, as the redox equivalents that are formed during the metabolization of glucose to acetyl-CoA cannot be re-oxidized by *E. coli* under anaerobic conditions.

A disadvantage common to all the processes described is that they require complex carbon sources, for example sugars.

Acetogenic cells

Acetogenic cells, i.e. cells that are able to form acetate by means of anaerobic respiration, are known.

The acetogenic bacteria include e.g. species of the genus *Acetobacterium* such as *A. woodii* and *Clostridium aceticum*.

WO0068407 describes the use of acetogenic bacteria for the production of ethanol.

The genome sequence of *C. ljungdahlii* has also recently been made available. A genome sequence has not yet been published for *C. aceticum* and *C. carboxidivorans*. It is known, however, that *C. aceticum* additionally carries a plasmid (5.6 kbp, Lee et al., 1987). At present, no techniques have been published for genetically modifying these organisms.

The group of the acetogenic bacteria belongs to the anaerobic prokaryotes that are able to utilize CO₂ as terminal electron acceptor, forming acetate. At present, 21 different genera have been assigned to the acetogens (Drake et al., 2006), including some Clostridia (Drake & Küsel, 2005). They are able to use carbon dioxide plus hydrogen or even carbon monoxide as the carbon and energy source (Wood, 1991). In addition, alcohols, aldehydes, carboxylic acids and numerous hexoses can also be used as the carbon source (Drake et al., 2004). The reductive

metabolic pathway that leads to the formation of acetate is called the acetyl-CoA or Wood-Ljungdahl pathway.

The problem to be solved by the invention was to provide a method by which acetone can be produced from ubiquitously available carbon sources.

Description of the invention

It was found, surprisingly, that the cells and the method described hereunder solve the problem of the invention.

The present invention therefore relates to cells as described in claim 1.

The invention also relates to a method for producing acetone with the cells according to the invention.

One advantage of the invention is that the cells can be anaerobic and therefore can be cultivated particularly favorably in energy terms.

Another advantage of the invention is that the cells according to the invention contribute to a decrease of the climate-damaging carbon dioxide.

Yet another advantage of the cells according to the invention is an increased yield through acetone production from carbon dioxide and hydrogen.

The present invention relates to an acetogenic cell, which is able to form acetone.

According to a first aspect of the present invention there is provided an acetogenic cell that is able to form acetone, wherein the acetogenic cell is genetically modified relative to its wild type, so that compared with its wild type, it has increased activity of each of the following enzymes E₁, E₂ and E₃:

enzyme E₁, which is an acetyl-CoA-C-acetyl transferase (EC 2.3.1.9);

enzyme E₂, which is a butyrate-acetoacetate-CoA-transferase (EC 2.8.3.9) or an acyl-CoA-hydrolase (EC 3.1.2.20);

enzyme E₃, which is an acetoacetate decarboxylase (EC 4.1.1.4);

and wherein the acetogenic cell is able to form more acetone compared with its wild type.

According to a second aspect of the present invention there is provided a method for producing acetone, comprising the process steps:

- A) contacting a cell as claimed according to the first aspect above with a nutrient medium comprising at least one carbon source selected from the group comprising carbon dioxide and carbon monoxide;
- B) cultivating the cell under conditions that enable the cell to form acetone;
- C) optionally isolating the acetone that has formed.

According to a third aspect of the present invention there is provided acetone obtained by the method according to the second aspect above.

The term “acetogenic cell” in the context of the present invention means cells that are able to form acetate by means of anaerobic respiration.

All percentages given are, unless stated otherwise, percentages by weight.

Preferably the acetogenic cell is an isolated, in particular a genetically modified cell.

According to the invention, a cell is preferred which, selected from at least one carbon source from the group comprising carbon dioxide and carbon monoxide, is able to form acetone. Especially preferably the acetogenic cell according to the invention is able to form acetone from carbon monoxide and carbon dioxide as the sole carbon source.

It is well known by a person skilled in the art that product yields in biological systems can be improved by means of recombinant gene technology. Therefore it is further preferred according to the invention for the acetogenic cell to be genetically modified relative to its wild type, so that it is able to form more acetone compared with its wild type.

The formulation "so that it is able to form more acetone compared with its wild type" also relates to the case when the wild type of the genetically modified cell is unable to form any acetone at all, or at least no detectable amounts of this compound, and it is only after the genetic modification that detectable amounts of this component can be formed.

A "wild type" of a cell preferably means a cell whose genome is in a state such as arose naturally by evolution. The term is used both for the whole cell and for individual genes. The term "wild type" therefore in particular does not include such cells or such genes whose gene sequences have been altered at least partially by human intervention by means of recombinant techniques.

These cells are preferably genetically modified so that they can form more acetone from a carbon source, compared with their wild type.

Moreover, it is preferable according to the invention that the acetogenic cell has been genetically modified so that in a defined time interval, preferably within 2 hours, more preferably within 8 hours and most preferably within 24 hours, it forms at least 2 times, especially preferably at least 10 times, more preferably at least 100 times, even more preferably at least 1000 times and most preferably at least 10 000 times more acetone than the wild-type cell. The increase in product formation can be determined for example by cultivating the cell according to the invention and the wild-type cell each separately under identical conditions (same cell density, same nutrient medium, same culture conditions) in a suitable nutrient medium for a specified time interval and then determining the amount of target product (acetone) in the nutrient medium.

In this connection it is preferable for the cell to have increased activity, compared with its wild type, of at least one of the following enzymes:

an enzyme E_1 , which catalyzes the reaction of acetyl-coenzyme A to acetoacetyl-coenzyme A;

an enzyme E_2 , which catalyzes the reaction of acetoacetyl-coenzyme A to acetoacetate;

an enzyme E_3 , which catalyzes the reaction of acetoacetate to acetone.

The formulation "an increased activity of an enzyme E_x " used in the foregoing and hereinafter preferably always means an activity of the respective enzyme E_x increased by a factor of at least 2, especially preferably of at least 10, more preferably of at least 100, even more preferably of at least 1000 and most preferably of at least 10 000. Furthermore, the cell according to the invention, which has "an increased activity of an enzyme E_x compared with its wild type", in particular also comprises a

cell whose wild type has no or at least no detectable activity of this enzyme E_x and only displays a detectable activity of this enzyme E_x after increasing the enzyme activity, for example by overexpression. In this connection, the term "overexpression" or the formulation "increase in expression" used hereinafter also comprises the case when a starting cell, for example a wild-type cell, has no or at least no detectable expression and it is only by recombinant techniques that a detectable expression of the enzyme E_x is induced.

In this connection, especially preferred cells are those in which the activity of the following enzyme or enzymes is increased: E_1 , E_2 , E_3 , $E_1 + E_2$, $E_1 + E_3$, $E_2 + E_3$, $E_1 + E_2 + E_3$, with $E_1 + E_2 + E_3$ being especially preferred.

Furthermore, it is preferable according to the invention for the enzyme E_1 to be an acetyl-CoA-C-acetyl transferase (EC 2.3.1.9); for the enzyme E_2 to be a butyrate-acetoacetate-CoA-transferase (EC 2.8.3.9) or an acyl-CoA-hydrolase (EC 3.1.2.20); for the enzyme E_3 to be an acetoacetate decarboxylase (EC 4.1.1.4).

Especially preferably, the enzyme used as enzyme E_1 is *thlA* from *Clostridium acetobutylicum*.

Especially preferably, butyrate-acetoacetate-CoA-transferase used as enzyme E_2 comprises *ctfA* and *ctfB* from *Clostridium acetobutylicum* and *atoD* and *atoA* from *Escherichia coli*.

Especially preferably, acyl-CoA hydrolase used as enzyme E_2 comprises *teII* from *B. subtilis* or *ybgC* from *Haemophilus influenzae*.

Especially preferably, the enzyme used as enzyme E_3 is *adc* from *Clostridium acetobutylicum*.

The acetogenic cell according to the invention is preferably a microorganism, preferably a bacterium and especially preferably an anaerobic bacterium, in particular a rod-shaped, Gram-positive bacterium.

Quite especially preferably, acetogenic cells are used that are selected from the group comprising *Thermoanaerobacter kivui*, *Acetobacterium woodii*, *Acetoanaerobium notera*, *Clostridium aceticum*, *Butyribacterium methylotrophicum*, *Moorella thermoacetica*, *Eubacterium limosum*, *Peptostreptococcus productus*, *Clostridium ljungdahlii* and *Clostridium carboxidivorans*. An especially suitable bacterium is *Clostridium carboxidivorans*, in particular strains such as "P7" and "P11". Said cells are described for example in US 2007/0275447 and US 2008/0057554.

Another especially suitable bacterium is *Clostridium ljungdahlii*, in particular strains selected from the group comprising *Clostridium ljungdahlii* PETC, *Clostridium ljungdahlii* ERI2, *Clostridium ljungdahlii* C01 and *Clostridium ljungdahlii* O-52 and are described in WO 98/00558 and WO 00/68407.

The invention also relates to a method for producing acetone, comprising the process steps: A) contacting a cell according to the invention with a nutrient medium comprising at least one carbon source selected from the group comprising carbon dioxide and carbon monoxide, B) cultivating the cell under conditions that enable the cell to form acetone and C) optionally isolating the acetone that formed.

The acetogenic cell according to the invention is able, preferably under anaerobic conditions, to form acetone from at least one carbon source selected from the group comprising carbon dioxide and carbon monoxide.

Regarding the source of these substrates, it is evident that there are many possible sources for providing CO or CO₂ as the carbon source. It can be seen that in practice, the carbon source used in the present invention can be any gas or gas mixture that is able to supply the acetogenic cell with sufficient amounts of carbon, so that it is able to perform its anaerobic respiration and form acetone.

In the method according to the invention it is preferable for the carbon source to be provided by waste gases, for example synthesis gas, flue gas, oil refinery waste gases, gases produced by yeast fermentation or clostridial fermentation, waste gases from the gasification of cellulose-containing materials or coal gasification.

These waste gases need not necessarily have been formed as side effects of other processes, but can be produced specially for use in the method according to the invention.

It can be seen that in practice the carbon source used for the present invention can be any waste gas that is able to supply the acetogenic cell with sufficient amounts of carbon, so that it can perform its anaerobic respiration.

In a preferred embodiment of the method according to the invention, the carbon source is synthesis gas.

Synthesis gas can for example be prepared from the by-product of coal gasification. The acetogenic cell therefore converts a substance that is a waste product into a valuable raw material. Alternatively synthesis gas can be provided for the method according to the invention by gasification of widely available, low-cost agricultural raw materials.

There are numerous examples of raw materials that can be converted to synthesis gas, as almost all forms of vegetation can be used for this purpose. Preferred raw materials are selected from the group comprising perennial grasses such as zebra grass, cereal residues, processing wastes such as sawdust.

Generally synthesis gas is obtained in a gasifier from dried biomass, mainly by pyrolysis, partial oxidation and steam reforming, wherein the primary products are CO, H₂ and CO₂. Normally a proportion of the product gas is reprocessed, in order to optimize product yields and avoid tar formation. Cracking of the unwanted tar into synthesis gas and CO can be carried out using lime and/or dolomite. These processes are described in detail in e.g. Reed, 1981 (Reed, T.B., 1981, Biomass gasification: principles and technology, Noves Data Corporation, Park Ridge, NJ). Mixtures of various sources can also be used as the carbon source.

The nutrient media used in the method according to the invention must suitably satisfy the requirements of the particular strains. Descriptions of nutrient media for various microorganisms are given in the manual "Manual of Methods for General Bacteriology" of the American Society for Bacteriology (Washington D. C., USA, 1981).

Besides the carbon sources, the nutrient medium contains in particular nitrogen and phosphorus sources, salts and pH control agents.

The nitrogen source used can be organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn-steep liquor, soybean flour and urea or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources can be used individually or as a mixture.

As the phosphorus source, the nutrient medium can contain phosphoric acid, potassium dihydrogen phosphate or dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must additionally contain metal salts, e.g. magnesium sulfate or iron sulfate, which are necessary for

growth. Finally, essential growth promoters such as amino acids and vitamins can be used in addition to the aforementioned substances.

The stated feed materials can be added to the culture in the form of a single preparation or can be supplied in a suitable manner during cultivation.

For controlling the pH of the culture, basic compounds such as sodium hydroxide, potassium hydroxide, ammonia or ammonia water or acidic compounds such as phosphoric acid or sulfuric acid can be used appropriately. To control the formation of foam, antifoaming agents such as fatty acid polyglycol esters can be used. To maintain the stability of plasmids, suitable substances with selective action, e.g. antibiotics, can be added to the medium.

In process step B) of the method according to the invention, the acetogenic cells are cultivated under conditions that allow the cell to form acetone. Preferably said culture takes place under anaerobic conditions.

The genetically modified cells according to the invention can be brought in contact with the nutrient medium continuously or discontinuously in a batch process or in a fed-batch process or repeated-fed-batch process, and therefore cultivated, for the purpose of producing acetone.

A semi-continuous process is also conceivable, as described in GB-A-1009370. A summary of known culture techniques is described in Chmiel's textbook ("Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik" ["Bioprocess technology 1. Introduction to bioprocess technology"] (Gustav Fischer Verlag, Stuttgart, 1991)) or in the textbook by Storhas ("Bioreaktoren und periphere Einrichtungen" ["Bioreactors and peripheral equipment"], Vieweg Verlag, Brunswick/Wiesbaden, 1994).

Other very suitable methods for cultivation of the acetogenic cell in process step b) are described e.g. in the DOE report "Bench-scale Demonstration of Biological Production of Ethanol from Coal Synthesis Gas", Topical Report 5, November 1995 (DOE Contract Number DE-AC22-92PC92118) and in documents WO 98/00558, WO 00/68407 and WO 02/08438.

In step C) of the method according to the invention, the acetone formed by the cells can optionally be isolated from the cells and/or the nutrient medium, wherein all the methods for isolating low-molecular substances from complex compositions known by a person skilled in the art may be considered for isolation.

As examples we may mention at this point precipitation by means of suitable solvents, extraction by means of suitable solvents, complexation, for example by means of cyclodextrins or cyclodextrin derivatives, crystallization, purification or isolation by chromatographic methods or transformation of the acetone into derivatives that can be separated easily. In particular, methods of separation by distillation are suitable for use in process step C).

The acetone obtainable from the method according to the invention also forms part of the present invention.

In the examples presented below, the present invention is described by way of illustration, but the invention, the scope of which follows from the complete description and the claims, is not to be limited to the embodiments presented in the examples.

The following figures form part of the disclosure:

Fig. 1: Biosynthetic pathway of the classical, clostridial ABE process

Fig. 2: Biosynthetic pathway of acetone in *C. acetobutylicum*

Fig. 3: Plasmid map pUC_adc_ctfAB_thlA:

Fig. 4: Plasmid map pIMP_adc_ctfAB_thlA:

Examples:

Example 1. Cloning of the expression vectors

For acetone production in acetogenic cells, clonings into *E. coli* XL2 blue were carried out. For this, the genes *ctfA* and *ctfB* from *Clostridium acetobutylicum*, or *atoA* and *atoD* from *E. coli*, or *teII* from *B. subtilis*, and/or *ybgC* from *Haemophilus influenzae* together with the genes *thlA* and *adc* from *C. acetobutylicum* were arranged on the plasmid pIMP1 (Mermelstein et al., 1992). An overview of the corresponding expression plasmids is presented in Table 1.

Table 1: Plasmids

Plasmid	CoA-transferase/thioesterase from	Seq ID No
pIMP_adc_ctfAB_thlA	<i>C. acetobutylicum</i>	13
pIMP_adc_atoDA_thlA	<i>E. coli</i>	14
pIMP_adc_teII_thlA	<i>B. subtilis</i>	15
pIMP_adc_ybgC_thlA	<i>H. influenzae</i>	16

The genes were cloned sequentially. For this, firstly oligonucleotides (Table 2) were designed for amplification of

the genes, introducing corresponding cleavage sites, and then all fragments were amplified.

Table 1: Oligonucleotides

Name	Sequence (5' → 3') *)	Cleavage site	Seq ID No
adc fw	GGAA GGTACC TTTTATG	Acc65I	1
adc rev	GTAAC TCTGAATTC TATTACTTAAG	EcoRI	2
atoDA fw	CACAACGGT GGATCC AAGAG	BamHI	3
atoDA rev	CGCGATAT GGTACCA ATCAT	Acc65I	4
ctfAB fw	GAATTTAAAAGGAG GGATCC AAATGAAC	BamHI	5
ctfAB rev	GTTTCATAGTATT GGTACCT AAACAGC	Acc65I	6
thl fw	CTCAG GTCGACTT CAAGAAG	SalI	7
thl rev	CAGAGTTATTTTAA GGATCCT TTTCTAGC	BamHI	8
tell fw	CAATT GGATCC GATAACAATTTACACAG	BamHI	9
tell rev	GAGATCT GGTACCC GTTAAATGATCGGA	Acc65I	10
ybgc fw	CTCTAGAA GGATCCT GTTTAACTTTAAG	BamHI	11
ybgc rev	ATT GGTACCT CATTGCATACTCCG	Acc65I	12

Corresponding fragments were amplified by genomic DNA using conventional PCR techniques, separated electrophoretically and purified.

Generation of pUC_adc_ctfAB_thlA:

In the first step, *adc* was cloned via the cleavage sites Acc65I and EcoRI into the vector pUC18 and then *thlA* was added via SalI and BamHI. In the last step, *ctfA* and *ctfB* were cloned in one step, as it is organized as operon in *C. acetobutylicum*, via the cleavage sites BamHI and Acc65I. In the resultant vector pUC_adc_ctfAB_thlA, the genes required for acetone production are now organized in an operon.

Fig. 3 shows the resultant pUC plasmid.

Generation of pIMP_adc_ctfAB_thlA:

Next, the gene cassette is recloned into the vector pIMP1 via the restriction endonucleases SalI and EcoRI, resulting in the expression plasmid pIMP_adc_ctfAB_thlA, cf. Fig. 4.

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Generation of pIMP_adc_atoDA_thlA, pIMP_adc_teII_thlA and pIMP_adc_ybgC_thlA:

For generating the vectors pIMP_adc_atoDA_thlA, pIMP_adc_teII_thlA and pIMP_adc_ybgC_thlA, the genes *ctfA* and *ctfB* were cut out of the vector pIMP_adc_ctfAB_thlA via the cleavage sites *Bam*HI and *Acc*65I. *atoD* and *atoA*, which are organized in one operon in *E. coli*, were amplified, as well as the genes *teII* from *B. subtilis* and *ybgC* from *H. influenzae*, generating the cleavage sites *Bam*HI and *Acc*65I. These fragments were first cloned via this into the vector pDrive (*atoDA*) or pUC19 (*teII* and *ybgC*). Then these genes were recloned from the pDrive or pUC vectors, for which the gene cassettes were cut via the cleavage sites *Bam*HI and *Acc*65I, purified and ligated into the vector backbone of pIMP_adc_ctfAB_thlA that had been restricted as described above (*Acc*65I and *Bam*HI) and purified.

Example 2: Acetone synthesis in *E. coli*

To check for functionality, all plasmid variants obtained (see Table 1) were investigated for acetone formation in the *E. coli* cloning strain XL2-blue. The analyses were performed at the 100-ml scale in TY medium with ampicillin (100 µg/ml). After inoculation from corresponding precultures to an optical density (600 nm) of 0.1, incubation was carried out at 37°C and 150 rpm. The optical density was monitored photometrically and at specified timepoints, over a period of approx. 50 h, samples were taken and the concentration of acetone and acetate in the

cell-free supernatant was determined by gas chromatography. It was found that with the combination of clostridial genes (*thlA* and *adc*) with *atoDA* from *E. coli*, up to 80 mM acetone was produced. With purely clostridial genes (*thlA*, *ctfAB*, *adc*), 5 mM acetone was produced, and with the combinations of clostridial genes (*thlA* and *adc*) with *teII* from *B. subtilis* or *ybgC* from *H. influenzae*, 1 mM acetone was produced.

Example 3: Acetogenic acetone production

Different media were used, depending on the Clostridia strain employed:

For preparation of the media for *C. carboxidivorans* or *C. ljungdahlii*, the chemicals were weighed, dissolved in water and then the pH was adjusted. In addition, the redox indicator reszurin (1 mg/l) was added, to permit later testing of the redox potential and therefore of the oxygen content. Then the media were brought to the boil in a heating mantle and cooled in an ice bath. During this, gassing was carried out with nitrogen, to remove the dissolved oxygen. Then the media were transferred into the anaerobic chamber, the final volume was adjusted with anaerobic water, it was filled and sealed hermetically. If a gas phase other than nitrogen was to be used, gas exchange was carried out, wherein the medium was gassed with the corresponding gas by means of a long cannula and finally a slight excess pressure of approx. 0.8 bar was applied.

For the medium for *C. aceticum*, all components were weighed, dissolved and filled. In addition the redox indicator reszurin (1 mg/l) was added, to allow subsequent testing for the redox potential and therefore the oxygen content. Then gassing was carried out via cannulas with a mixture of 80% N₂ and 20% CO₂, until a pH of 7.4 was reached. Once again, a slight excess pressure was applied. After autoclaving, sterile Na₂CO₃ was added in the form of an anaerobic 5% Na₂CO₃ solution added, to obtain a

pH of 8.2. Additionally, fructose was added in sterile conditions to a final concentration of 1%. For autotrophic growth, a gas atmosphere of 80% H₂ and 20% CO₂ was produced. All media were autoclaved for 15 min at 121°C and 1.2 bar. Some constituents of the media were autoclaved separately, to prevent chemical reactions of the components with one another. Heat-labile components were dissolved, sterile-filtered and were added to the cooled, autoclaved medium before use. For the production of solid media, 1.5% (w/v) agar was added before autoclaving and directly thereafter they were poured into Petri dishes in the anaerobic chamber. After pouring, the plates were dried for a few days and were stored at 4°C until use.

Medium for *C. aceticum*

NH ₄ Cl	1.00	g	18.7	mM
KH ₂ PO ₄	0.33	g	2.4	mM
K ₂ HPO ₄	0.45	g	2.6	mM
MgSO ₄ x 7 H ₂ O	0.10	g	0.4	mM
Trace-element solution (s.u.)	20.00	ml	2	% (v/v)
Wolfe's vitamin solution (s.u.)	20.00	ml	2	% (v/v)
Yeast extract	2.00	g	0.2	% (w/v)
NaHCO ₃	10.00	g	0.1	M
Cysteine-HCl x H ₂ O	0.50	g	2.8	mM
Na ₂ S x 9 H ₂ O	0.50	g	2.1	mM
Water	to 1000	ml		

After autoclaving, 25 ml l-1 of a 5 wt.% Na₂CO₃ solution was added in sterile conditions, to obtain a pH of 8.2.

Additionally, fructose was added in sterile conditions to a final concentration of 1 wt.% relative to the total medium. For autotrophic growth, a gas atmosphere of 80 vol.% H₂ and 20 vol.% CO₂ was produced before autoclaving.

Medium for *C. carboxidivorans* - Wilkins-Chalgren Medium

Wilkins-Chalgren anaerobic broth (OXOID CM0643)	33	g	3.3	%
NaHCO ₃	1	g	12	mM

Water

to 1000 ml

The pH was adjusted to 5.6 before boiling and anaerobization and after autoclaving, 10 ml of reducing agent 1 (see below) was added, after which the pH should be 6.0.

Medium for *C. ljungdahlii* - ATCC Medium 1754 (PETC medium)

NH ₄ Cl	1.0 g	19 mM
KCl	0.1 g	1.35 mM
MgSO ₄ * 7 H ₂ O	0.2 g	0.8 mM
NaCl	0.8 g	14 mM
KH ₂ PO ₄	0.1 g	0.7 mM
CaCl ₂ * 2 H ₂ O	20.0 mg	0.15 mM
Yeast extract	1.0 g	0.1 % (w/v)
Trace-element solution	10.0 ml	1 % (v/v)
Wolfe's vitamin solution	10.0 ml	1 % (v/v)
NaHCO ₃	2.0 g	24 mM
Water	to 1000 ml	pH 5.5

Before boiling and anaerobization, the pH was adjusted to 5.5. After autoclaving, 20 ml of a sterile fructose solution (250 g/l) and 5 ml each of reducing agent 1 and 2 (see below) were added, after which the pH should be 5.9.

Reducing agent 1

1.8 g NaOH is dissolved in 200 ml water, boiled and cooled under nitrogen gassing. In the anaerobic chamber, first 4 g L-cysteine-HCl and then 4 g Na₂S * 9 H₂O are dissolved in 100 ml anaerobic NaOH and then autoclaved.

Reducing agent 2

1.8 g NaOH is dissolved in 200 ml water, boiled and cooled under nitrogen gassing. In the anaerobic chamber, 4 g of L-cysteine-HCl is dissolved in 100 ml anaerobic NaOH and then autoclaved.

Trace-element solution for ATCC Medium 1754 and for the *C. aceticum* medium

Nitrilotriacetic acid	2 g	10.5 mM
MnSO ₄ * H ₂ O	1 g	6 mM
Fe(SO ₄) ₂ (NH ₄) ₂ * 6 H ₂ O	0.8 g	2 mM
CoCl ₂ * 6 H ₂ O	0.2 g	0.86 mM
ZnSO ₄ * 7 H ₂ O	0.2 mg	0.7 μM
CuCl ₂ * 2 H ₂ O	20 mg	0.12 mM
NiCl ₂ * 6 H ₂ O	20 mg	80 μM
Na ₂ MoO ₄ * 2 H ₂ O	20 mg	80 μM
Na ₂ SeO ₄	20 mg	80 μM
Na ₂ WO ₄	20 mg	60 μM
Water	to 1000 ml	

First, the nitrilotriacetic acid was dissolved completely in water, the pH was adjusted to 6.0 with potassium hydroxide and then the other components were dissolved.

Wolfe's vitamin solution for ATCC Medium 1754 and for the *C. aceticum* medium

Biotin (vitamin H)	2.0 mg	8 μM
Folic acid (vitamin B9)	2.0 mg	4.5 μM
Pyridoxine-HCl (vitamin B6)	10.0 mg	49 μM
Thiamin-HCl (vitamin B1)	5.0 mg	15 μM
Riboflavin (vitamin B2)	5.0 mg	13 μM
Nicotinamide (vitamin PP)	5.0 mg	41 μM
Calcium D-(+)-pantothenate	5.0 mg	10.5 μM
Cyanocobalmin (vitamin B12)	0.1 mg	74 μM
p-Aminobenzoic acid	5.0 mg	36 μM
Lipoic acid	5.0 mg	24 μM
Water	to 1000 ml	pH 4.3

The plasmids constructed in *E. coli* XL2-blue were then introduced into acetogenic Clostridia by conjugation (Purdy et al., 2002) or transformation (Zhu et al., 2004), so that the recombinant strain acquires the capacity to produce acetone.

For the conjugation experiments, the *E. coli* donor strain CA434 with the plasmid to be transferred was grown aerobically overnight in LB medium. A 1-ml aliquot was centrifuged for 1 min at $10000 \times g$ and the cell sediment was carefully suspended, in the anaerobic chamber, in 1 ml of sterile, anaerobic PBS buffer (1.5 mM KH_2PO_4 , 4.2 mM Na_2HPO_4 , 137 mM NaCl , 2.7 mM KCl), to prevent shearing-off of the conjugative pili. The cells were centrifuged again and were taken up in 200 μl of a *Clostridium* culture grown overnight in an appropriate medium. In the anaerobic chamber, this mixture was distributed on well-dried agar plates in 10- μl drops and incubated anaerobically at 37°C for 6 h. Then the cells were washed from the agar plate 2-3 times with in each case 0.5 ml of sterile, anaerobic PBS buffer. The conjugation mixture was plated out on selective agar plates (clarithromycin) and incubated anaerobically at 37°C .

For the transformation, the clostridial cells were grown in 50 ml of *C. acetivum* medium with 40 mM of DL-threonine at 30°C to an optical density of 0.3-0.4. The next steps were carried out in the anaerobic chamber. Here, the cells were harvested (6000 rpm, 10 min, RT), washed twice with SMP buffer (270 mM sucrose, 1 mM MgCl_2 , 7 mM NaH_2PO_4) and finally taken up in 500 to 700 μl SMP buffer and used in the transformation. For this, the cells were transferred to electroporation cuvettes (4 mm) and 0.5 to 1.5 μg plasmid-DNA was added. After incubation for 5 minutes, electroporation was carried out at 25 μF , 600 Ω and 2.5 kV in a Gene-Pulser (Bio-Rad Laboratories GmbH; Munich) with 4 mm cuvettes (Biozym Scientific GmbH). Then the cells were added immediately to 5 ml of preheated medium. This was followed by incubation for resistance expression at 37°C overnight for up to four days, and then 5 ml of medium was inoculated with clarithromycin (5 $\mu\text{g ml}^{-1}$) and incubated for 3 to 5 days at 37°C .

To verify the transformation, this was followed by plasmid isolation using a "peqGOLD® Plasmid Miniprep Kit II" (Peqlab, Erlangen). Preparation was carried out according to the manufacturer's instructions, carrying out all optional steps. Then plasmid recovery was carried out, using the *E. coli* strain XL2-blue, followed by restriction digestion.

All plasmid variants obtained (see Table 1) were investigated for acetone formation in the autotrophic Clostridia. The analyses were carried out at the 50-ml scale in the corresponding medium with clarithromycin (5 µg/ml). After inoculation from corresponding precultures, incubation was carried out at 37°C. The necessary gassing of the medium was carried out during preparation of the medium. Either synthesis gas or a CO₂/H₂ mixture in 1:2 ratio was used for this. The optical density was monitored photometrically and samples were taken at specified timepoints over a period of approx. 100 to 200 h and the concentration of acetone and acetate in the cell-free supernatant was determined by gas chromatography. It is found that with the combination clostridial genes (*thlA* and *adc*) with *atoDA* from *E. coli* and with the combinations clostridial genes (*thlA* and *adc*) with *teII* from *B. subtilis* or *ybgC* from *H. influenzae*, up to 1 mM acetone is produced. With purely clostridial genes (*thlA*, *ctfAB*, *adc*) up to 0.24 mM acetone was produced.

CLAIMS

1. An acetogenic cell that is able to form acetone, wherein the acetogenic cell is genetically modified relative to its wild type, so that compared with its wild type, it has increased activity of each of the following enzymes E₁, E₂ and E₃:

enzyme E₁, which is an acetyl-CoA-C-acetyl transferase (EC 2.3.1.9);

enzyme E₂, which is a butyrate-acetoacetate-CoA-transferase (EC 2.8.3.9) or an acyl-CoA-hydrolase (EC 3.1.2.20);

enzyme E₃, which is an acetoacetate decarboxylase (EC 4.1.1.4);

and wherein the acetogenic cell is able to form more acetone compared with its wild type.

2. The acetogenic cell as claimed in claim 1, wherein the acetogenic cell is able to form acetone from at least one carbon source selected from the group comprising carbon dioxide and carbon monoxide.

3. The acetogenic cell as claimed in claim 1 or claim 2, wherein the enzyme E₁ is thlA from *Clostridium acetobutylicum*.

4. The acetogenic cell as claimed in any one of claims 1 to 3, wherein the enzyme E₂ is a butyrate-acetoacetate-CoA-transferase comprising ctfA and ctfB from *Clostridium acetobutylicum* and atoD and atoA from *Escherichia coli*.

5. The acetogenic cell as claimed in any one of claims 1 to 3, wherein the enzyme E₂ is an acyl-CoA-hydrolase comprising telI from *B. subtilis* or ybgC from *Haemophilus influenzae*.

6. The acetogenic cell as claimed in any one of claims 1 to 5, wherein the enzyme E₃ is adc from *Clostridium acetobutylicum*.

7. The acetogenic cell as claimed in any one of claims 1 to 6, wherein the acetogenic cell is a microorganism, selected from the group comprising *Thermoanaerobacter kivui*, *Acetobacterium woodii*, *Acetoanaerobium notera*, *Clostridium aceticum*, *Butyribacterium methylotrophicum*, *Moorella thermoacetica*, *Eubacterium limosum*, *Peptostreptococcus productus*, *Clostridium ljungdahlii* and *Clostridium carboxidivorans*.

8. A method for producing acetone, comprising the process steps:

- A) contacting a cell as claimed in any one of claims 1 to 7 with a nutrient medium comprising at least one carbon source selected from the group comprising carbon dioxide and carbon monoxide;
 - B) cultivating the cell under conditions that enable the cell to form acetone;
 - C) optionally isolating the acetone that has formed.
9. Acetone obtained by the method as claimed in claim 8.

Evonik Degussa GmbH
Patent Attorneys for the Applicant/Nominated Person
SPRUSON & FERGUSON

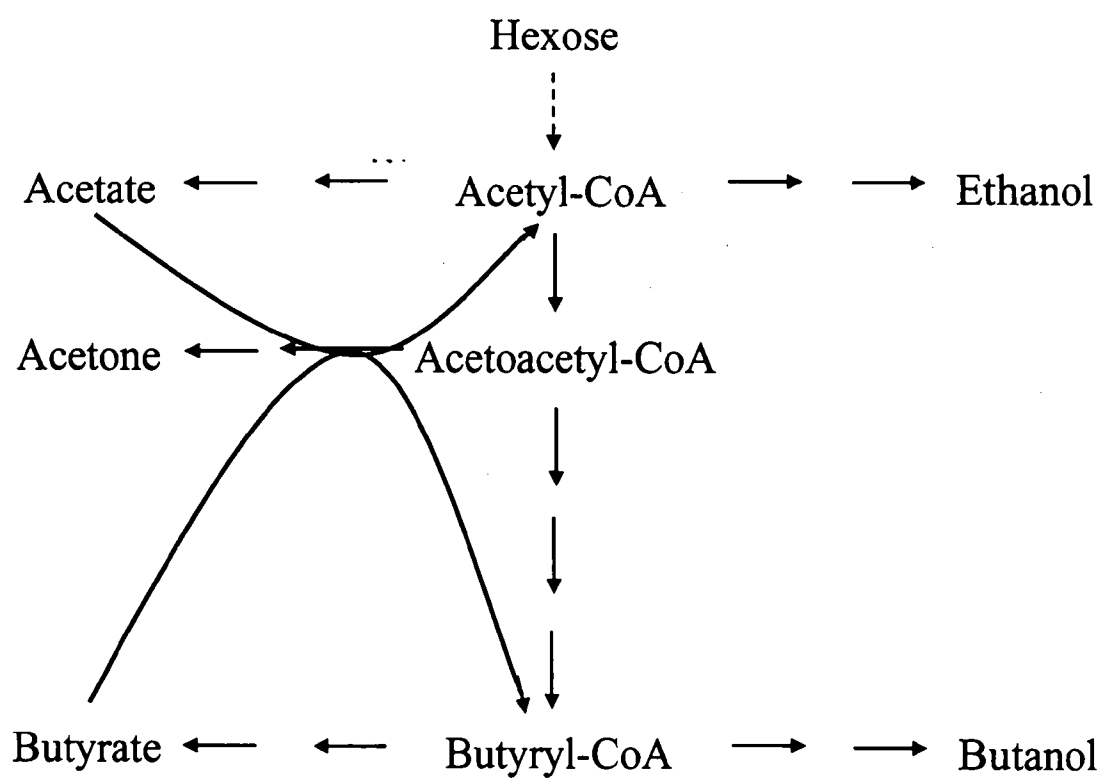


Fig. 1

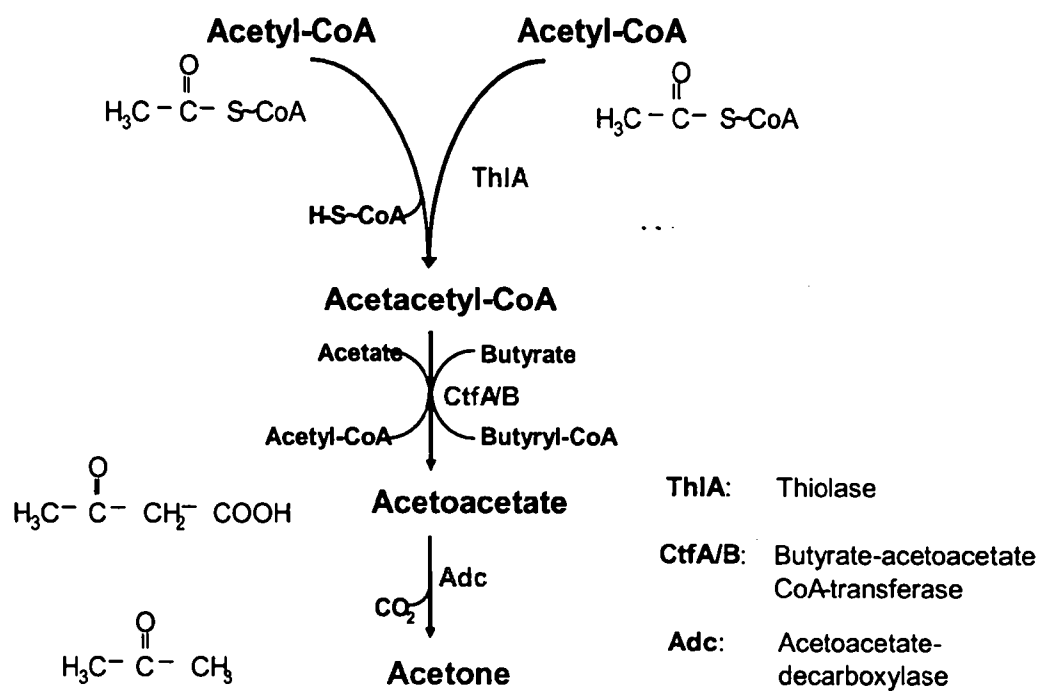


Fig. 2

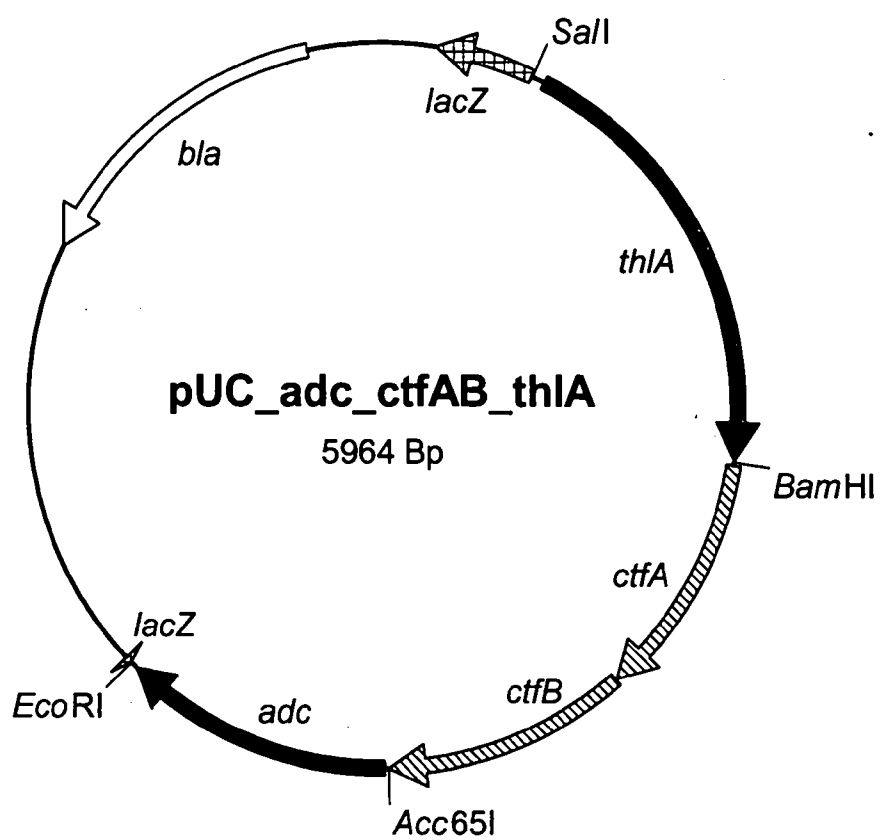


Fig. 3

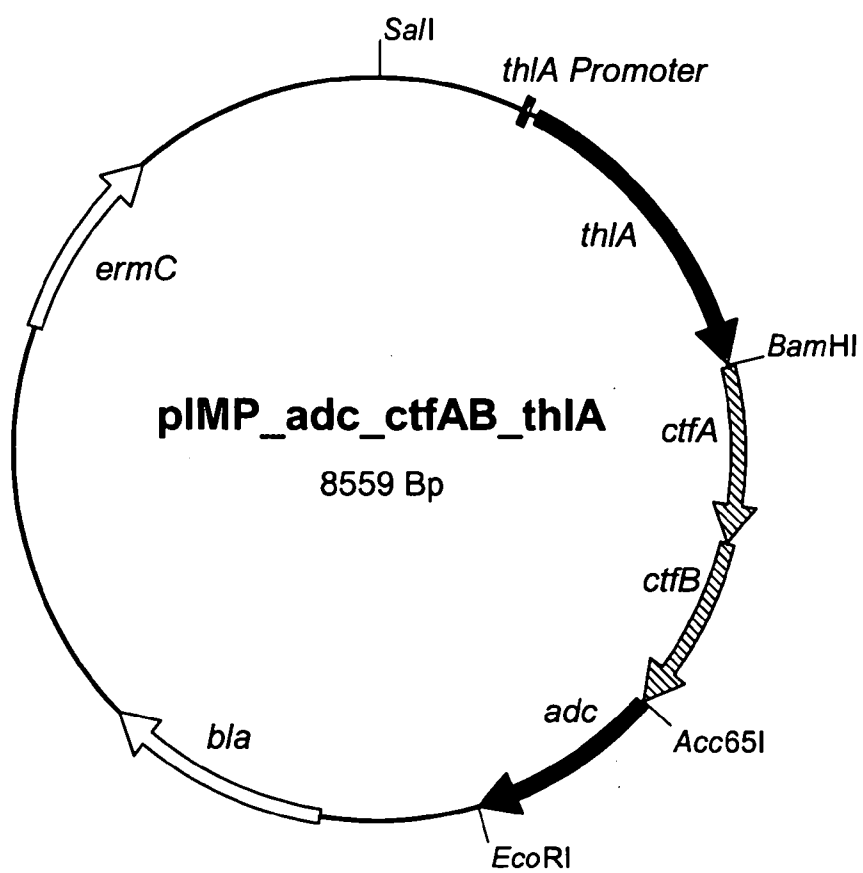


Fig. 4

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