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(54) Title: A GENETICALLY MODIFIED ACETOGENIC CELL

(57) Abstract: There is provided an acetogenic microbial cell which is capable of producing at least one higher alcohol from a carbon source, wherein the acetogenic microbial cell is genetically modified to comprise an increased expression relative to its wild type cell of at least one enzyme, E_8, a butyryl-CoA: acetate CoA transferase (cat3). There is also provided a method and use of the cell to produce higher alcohols.

A GENETICALLY MODIFIED ACETOGENIC CELL

FIELD OF THE INVENTION

The present invention relates to a recombinant cell for the production of a higher alcohol from a carbon source. In particular, the cell is an acetogenic microorganism. The present invention also relates to a method of producing at least one higher alcohol from a carbon source in the presence of the recombinant acetogenic cell.

BACKGROUND OF THE INVENTION

Butanol and higher alcohols have several uses including being used as fuel. For example, butanol in the future can replace gasoline as the energy contents of the two fuels are nearly the same. Further, butanol has several other superior properties as an alternative fuel when compared to ethanol. These include butanol having higher energy content, butanol being less “evaporative” than ethanol or gasoline and butanol being easily transportable compared to ethanol. For these reasons and more, there is already an existing potential market for butanol and/or related higher alcohols. Butanol and other higher alcohols are also used as industrial solvents. Higher alcohols are also used in the perfume and cosmetic industry. For example, hexanol is commonly used in the perfume industry.

Currently, butanol and other higher alcohols are primarily manufactured from petroleum. These compounds are obtained by cracking gasoline or petroleum which is bad for the environment. Also, since the costs for these starting materials will be linked to the price of petroleum, with the expected increase in petroleum prices in the future, butanol and other higher alcohol prices may also increase relative to the increase in the petroleum prices.

Historically (1900s-1950s), biobutanol was manufactured from corn and molasses in a fermentation process that also produced acetone and ethanol and was known as an ABE (acetone, butanol, ethanol) fermentation typically with certain butanol-producing bacteria such as *Clostridium acetobutylicum* and *Clostridium beijerinckii*. This method has recently gained popularity again with renewed interest in green energy. However, the “cornstarch butanol production” process requires a number of energy-consuming steps including agricultural corn-crop cultivation, corn-grain harvesting, corn-grain starch processing, and starch-to-sugar-to-butanol fermentation. The “cornstarch butanol production” process could also probably cost nearly as much energy as the energy value of its product butanol.

The Alfol® Alcohol Process is a method used to producing higher alcohols from ethylene using an organoaluminium catalyst. The reaction produces linear long chain primary alcohols (C_2-C_{28}). The process uses an aluminum catalyst to oligomerize ethylene and allow the resulting alkyl group to

be oxygenated. However, this method yields a wide spectrum of alcohols and the distribution pattern is maintained. This constant pattern limits the ability of the producer to make only the specific alcohol range that is in highest demand or has the best economic value. Also, the gases needed in the reaction have to be very clean and a distinct composition of the gases is needed for the reaction to be successfully carried out.

WO2009100434 also describes an indirect method of producing butanol and hexanol from a carbohydrate. The method includes a homoacetogenic fermentation to produce an acetic acid intermediate which is then chemically converted to ethanol. The ethanol and a remaining portion of the acetic acid intermediate are then used as a substrate in an acidogenic fermentation to produce butyric and caproic acid intermediates which are then chemically converted to butanol and hexanol. However, this method uses expensive raw material carbohydrates and has two additional process steps, the formation of the esters and the chemical hydrogenation of the esters which make the method not only longer but also results in loss of useful material along the way.

Perez, J.M., 2012 discloses a method of converting short-chain carboxylic acids into their corresponding alcohols in the presence of syngas with the use of *Clostridium ljungdahlii*. However, short-chain carboxylic acids have to be added as a substrate for the conversion to the corresponding higher alcohol.

The currently available methods of higher alcohol production thus has limitations in mass transfer of the gaseous substrates into fermentation broth, lower productivity, and lower concentrations of end products, resulting in higher energy costs for product purification.

Accordingly, it is desirable to find more sustainable raw materials, other than purely petroleum based or corn based sources, as starting materials for butanol and other higher alcohol production via biotechnological means which also cause less damage to the environment. In particular, there is a need for a simple and efficient one-pot biotechnological production of butanol and other higher alcohols from sustainable raw material.

DESCRIPTON OF THE INVENTION

The present invention provides a cell that has been genetically modified to produce at least one higher alcohol from a simple carbon source. In particular, the cell may be capable of converting CO and/or CO_2 to at least one higher alcohol. Namely, the cell may be genetically modified to express a butyryl-CoA: acetate CoA transferase (cat3) ($E_{8,}$) at an expression level higher relative to the wild type cell. This is advantageous as a single cell may be used to produce a higher alcohol from non-petroleum based sources. Also, using the recombinant cell make the method of producing higher alcohols more efficient.

According to one aspect of the present invention, there is provided an acetogenic microbial cell which is capable of producing at least one higher alcohol from a carbon source, wherein the

acetogenic microbial cell is genetically modified to comprise an increased expression relative to its wild type cell of at least one enzyme, E_8, butyryl-CoA: acetate CoA transferase (cat3).

The phrase “wild type” as used herein in conjunction with a cell or microorganism may denote a cell with a genome make-up that is in a form as seen naturally in the wild. The term may be applicable for both the whole cell and for individual genes. The term ‘wild type’ may thus also include cells which have been genetically modified in other aspects (i.e. with regard to one or more genes) but not in relation to the genes of interest. The term “wild type” therefore does not include such cells or such genes where the gene sequences have been altered at least partially by man using recombinant methods. A wild type cell according to any aspect of the present invention may thus refer to a cell that has no genetic mutation with respect to the whole genome and/or a particular gene. Therefore, in one example, a wild type cell with respect to enzyme E_8 may refer to a cell that has the natural/ non-altered expression of the enzyme E_8 in the cell. The wild type cell with respect to enzyme E_2, E_3, E_4, E_5, E_6, E_7, E_9, E_{10}, E_{11}, E_{12}, etc. may be interpreted the same way and may refer to a cell that has the natural/ non-altered expression of the enzyme E_2, E_3, E_4, E_5, E_6, E_7, E_9, E_{10}, E_{11}, E_{12},etc. respectively in the cell.

A skilled person would be able to use any method known in the art to genetically modify a cell or microorganism. According to any aspect of the present invention, the genetically modified cell may be genetically modified so that in a defined time interval, within 2 hours, in particular within 8 hours or 24 hours, it forms at least twice, especially at least 10 times, at least 100 times, at least 1000 times or at least 10000 times more higher alcohol than the wild-type cell. The increase in product formation can be determined for example by cultivating the cell according to any aspect of the present invention and the wild-type cell each separately under the same conditions (same cell density, same nutrient medium, same culture conditions) for a specified time interval in a suitable nutrient medium and then determining the amount of target product (higher alcohol e.g. butanol) in the nutrient medium.

The genetically modified cell or microorganism may be genetically different from the wild type cell or microorganism. The genetic difference between the genetically modified microorganism according to any aspect of the present invention and the wild type microorganism may be in the presence of a complete gene, amino acid, nucleotide etc. in the genetically modified microorganism that may be absent in the wild type microorganism. In one example, the genetically modified microorganism according to any aspect of the present invention may comprise enzymes that enable the microorganism to produce higher alcohols. The wild type microorganism relative to the genetically modified microorganism of the present invention may have none or no detectable activity of the enzymes that enable the genetically modified microorganism to produce the at least one higher alcohol. As used herein, the term ‘genetically modified microorganism’ may be used interchangeably with the term ‘genetically modified cell’. The genetic modification according to any aspect of the present invention is carried out on the cell of the microorganism.

The cells according to any aspect of the present invention are genetically transformed according to any method known in the art. In particular, the cells may be produced according to the method disclosed in WO/2009/077461.

The phrase 'the genetically modified cell has an increased activity, in comparison with its wild type, in enzymes' as used herein refers to the activity of the respective enzyme that is increased by a factor of at least 2, in particular of at least 10, more in particular of at least 100, yet more in particular of at least 1000 and even more in particular of at least 10000.

The phrase "increased activity of an enzyme", as used herein is to be understood as increased intracellular activity. Basically, an increase in enzymatic activity can be achieved by increasing the copy number of the gene sequence or gene sequences that code for the enzyme, using a strong promoter or employing a gene or allele that codes for a corresponding enzyme with increased activity and optionally by combining these measures. Genetically modified cells used in the method according to the invention are for example produced by transformation, transduction, conjugation or a combination of these methods with a vector that contains the desired gene, an allele of this gene or parts thereof and a vector that makes expression of the gene possible. Heterologous expression is in particular achieved by integration of the gene or of the alleles in the chromosome of the cell or an extrachromosomally replicating vector. For example, a cell with an increase in expression of an enzyme such as enzyme E_8 relative to a wild type cell, may refer to a cell that may comprise:
- an expression of a heterologous enzyme E_8,
- an increase in the copy number of the gene expressing enzyme E_8,
- an expression of enzyme E_8 with a heterologous promoter, or
- combinations thereof.

A skilled person may be capable of measuring the activity of each of these enzymes using methods known in the art. The expression of the enzymes or genes according to any aspect of the present invention may be detected in a gel with the aid of 1- and 2-dimensional protein gel separation and subsequent visual identification of the protein concentration using suitable evaluation software. When the increase in an enzymatic activity is based exclusively on an increase in the expression of the gene in question, the quantification of the increase in the enzymatic activity can be determined in a simple manner by comparing the 1- or 2-dimensional protein separations between the wild type and the genetically modified cell. A conventional method of preparing the protein gels in coryneform bacteria, and of identifying the proteins, is the procedure described by Hermann et al. (*Electrophoresis,* 22: 1712.23 (2001)). The protein concentration can also be analysed by Western blot hybridization using an antibody which is specific for the protein to be detected (Sambrook et al., *Molecular Cloning: a laboratory manual*, 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA, 1989) followed by visual evaluation with suitable software for determining the concentration (Lohaus and Meyer (1989) *Biospektrum*, 5: 32-39; Lottspeich (1999), *Angewandte Chemie* 111: 2630-2647). The activity of DNA-binding proteins can be measured by means of DNA band shift assays (also referred to as gel retardation) (Wilson et al. (2001) *Journal*

of Bacteriology, 183: 2151-2155). The effect of DNA-binding proteins on the expression of other genes can be detected by various, extensively described methods of the reporter gene assay (Sambrook et al., *Molecular Cloning: a laboratory manual,* 2nd Ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y. USA, 1989). The intracellular enzymatic activities can be detected by various methods which have been described (Donahue et al. (2000) *Journal of Bacteriology* 182 (19): 5624-5627; Ray et al. (2000) *Journal of Bacteriology* 182 (8): 2277-2284; Freedberg et al. (1973) *Journal of Bacteriology* 115 (3): 816-823). In the event that no specific methods for determining the activity of a particular enzyme are detailed in what follows, the determination of the increase in the enzymatic activity, and also the determination of the reduction in an enzymatic activity, may be carried out by means of the methods described in Hermann et al., Electrophoresis, 22: 1712-23 (2001), Lohaus et al., Biospektrum 5 32-39 (1998), Lottspeich, *Angewandte Chemie* 111: 2630-2647 (1999) and Wilson et al. *Journal of Bacteriology*, 183: 2151-2155 (2001).

The term "acetogenic bacteria" as used herein refers to a microorganism which is able to perform the Wood-Ljungdahl pathway and thus is able to convert CO, CO_2 and/or hydrogen to acetate. These microorganisms include microorganisms which in their wild-type form do not have a Wood-Ljungdahl pathway, but have acquired this trait as a result of genetic modification. Such microorganisms include but are not limited to *E. coli* cells. These microorganisms may be also known as carboxydotrophic bacteria. Currently, 21 different genera of the acetogenic bacteria are known in the art (Drake et al., 2006), and these may also include some *clostridia* (Drake & Kusel, 2005). These bacteria are able to use carbon dioxide or carbon monoxide as a carbon source with hydrogen as an energy source (Wood, 1991). Further, alcohols, aldehydes, carboxylic acids as well as numerous hexoses may also be used as a carbon source (Drake et al., 2004). The reductive pathway that leads to the formation of acetate is referred to as acetyl-CoA or Wood-Ljungdahl pathway.

In particular, the acetogenic bacteria may be selected from the group consisting of *Acetoanaerobium notera (ATCC 35199), Acetonema longum (DSM 6540), Acetobacterium carbinolicum (DSM 2925), Acetobacterium malicum (DSM 4132), Acetobacterium species no. 446 (Morinaga et al., 1990, J. Biotechnol., Vol. 14, p. 187-194),Acetobacterium wieringae (DSM 1911), Acetobacterium woodii (DSM 1030), Alkalibaculum bacchi (DSM 22112), Archaeoglobus fulgidus (DSM 4304), Blautia producta (DSM 2950, formerly Ruminococcus productus, formerly Peptostreptococcus productus), Butyribacterium methylotrophicum (DSM 3468), Clostridium aceticum (DSM 1496), Clostridium autoethanogenum (DSM 10061, DSM 19630 and DSM 23693), Clostridium carboxidivorans (DSM 15243), Clostridium coskatii (ATCC no. PTA-10522), Clostridium drakei (ATCC BA-623), Clostridium formicoaceticum (DSM 92), Clostridium glycolicum (DSM 1288), Clostridium ljungdahlii (DSM 13528), Clostridium ljungdahlii C-01 (ATCC 55988), Clostridium ljungdahlii ERI-2 (ATCC 55380), Clostridium ljungdahlii O-52 (ATCC 55989), Clostridium mayombei (DSM 6539), Clostridium methoxybenzovorans (DSM 12182), Clostridium neopropionicum sp, Clostridium ragsdalei (DSM 15248), Clostridium scatologenes (DSM 757), Clostridium species ATCC 29797 (Schmidt et al., 1986, Chem. Eng. Commun., Vol. 45, p. 61-73),*

Desulfotomaculum kuznetsovii (DSM 6115), Desulfotomaculum thermobezoicum subsp. thermosyntrophicum (DSM 14055), Eubacterium limosum (DSM 20543), Methanosarcina acetivorans C2A (DSM 2834), Moorella sp. HUC22-1 (Sakai et al., 2004, Biotechnol. Let., Vol. 29, p. 1607-1612), Moorella thermoacetica (DSM 521, formerly Clostridium thermoaceticum), Moorella thermoautotrophica (DSM 1974), Oxobacter pfennigii (DSM 322), Sporomusa aerivorans (DSM 13326), Sporomusa ovata (DSM 2662), Sporomusa silvacetica (DSM 10669), Sporomusa sphaeroides (DSM 2875), Sporomusa termitida (DSM 4440) and Thermoanaerobacter kivui (DSM 2030, formerly Acetogenium kivui).

In particular, the acetogenic microbial cell used according to any aspect of the present invention may be selected from the group consisting of *Clostridium ljungdahlii* and *Clostridium autothenogenum*. In one example, suitable bacterium may be *Clostridium ljungdahlii*. In particular, strains selected from the group consisting of *Clostridium ljungdahlii* PETC, *Clostridium ljungdahlii* ERI2, *Clostridium ljungdahlii* COL and *Clostridium ljungdahlii* O-52 may be used in the conversion of synthesis gas to hexanoic acid. These strains for example are described in WO 98/00558, WO 00/68407, ATCC 49587, ATCC 55988 and ATCC 55989. In another example, the acetogenic bacteria selected bacteria may be *Clostridium autothenogenum*.

The enzyme, E_8, a butyryl-CoA: acetate CoA transferase (cat3), the expression of which is increased in the cell according to any aspect of the present invention, relative to a wild type cell, catalyses the following reaction amongst others:

acetate + butyryl-CoA ---> acetyl-CoA + Butyrate (CoA-Transferase).

This enzyme is especially advantageous in the acetogenic cell according to any aspect of the present invention as it has a broad substrate specificity (Stadtman ER (1953). J Biol Chem 203:501–512 and Stadtman ER (1953) Fed Proc 12:692–693.) and is capable of catalysing the conversion of acyl CoA to form at least one fatty acid (Seedorf et al., (2007) PNAS. 105 (6):2128-2133). The production of at least one acid from a carbon source comprising CO and/or CO_2 may be possible in the presence of the acetogenic cell according to any aspect of the present invention due to the presence of enzyme, E_8. The acid may be produced from the carbon source via acetate production where acetate may be used as the CoA acceptor. This may thus allow the cell according to any aspect of the present invention more efficient and effective in the production of a fatty acid.

In most acetogenic cells, butyrate may not be naturally produced. The production of butyrate may be introduced into an acetogenic cell by genetically modifying a cell to be capable of producing butyric acid from at least one carbon source comprising CO and/or CO_2. In one example, acetogenic cells already capable of producing butyrate may be used in the aspects of the present invention to introduce enzyme E_8 to enable the cell to produce at least one fatty acid from a carbon source comprising CO and/or CO_2. For example, *C. carboxidivorans* may be a cell like this.

In one example, the cell according to any aspect of the present invention may be genetically modified to comprise an increased expression relative to its wild type cell of at least one further enzyme selected from the group consisting of E_1 to E_7 and E_9 to E_{11}, wherein E_1 is an alcohol dehydrogenase (adh), E_2 is an acetaldehyde dehydrogenase (ald), E_3 is an acetoacetyl- CoA thiolase (thl), E_4 is a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5 is a 3-hydroxybutyryl-CoA dehydratase (crt), E_6 is a butyryl-CoA dehydrogenase (bcd), E_7 is an electron transfer flavoprotein subunit (etf), E_9 is an acetate kinase (ack), E_{11} is a transhydrogenase and E_{12} is a trans-2-enoyl-CoA reductase (TER) or crotonyl-CoA reductase (ccr). The cell may also comprise increased expression relative to the wild type cell of E_{10}, phosphotransacetylase (pta),

In particular, the activity of enzymes E_1 and E_2 may be measured using the assays taught at least in Hillmer P., 1972, Lurz R., 1979; the activity of enzyme E_2 may also be measured using the assay taught in Smith L.T., 1980; the activity of enzymes E_3 and E_4 may be measured using the assays taught at least in Sliwkowski M.X., 1984; the activity of E_4 may also be measured using the assay taught in Madan, V.K., 1972; the activity of E_5 may also be measured using the assay taught in Bartsch, R.G., 1961; the activity of enzymes E_6 and E_7 may be measured using the assay taught in Li, F., 2008; the activity of E_7 may also be measured using the assay taught in Chowdhury, 2013; the activity of E_8 may be measured using the assay taught in Stadman, 1953. In another example, the activity of E_8 may be measured using the assay taught in Barker, H. A., 1955. Methods Enzymol. 1:599–600; the activity of E_9 may be measured using the assay taught in Winzer, K., 1997; the activity of E_{10} may be measured using the assay taught in Smith L.T., 1976; and the activity of E_{11} may be measured using the assay taught in Wang S, 2010. E_{12} may be measured using the assay for TER activity taught in Inui et al. (1984) Eur. J. Biochem.142, 121–126 and/or Seubert et al. (1968) Biochim. Biophys. Acta 164, 498–517 and/or Hoffmeister, M. (2005), J. Biol. Chem., 280 (6), 4329–4338.

These methods amongst others known in the art may be used by a skilled person to confirm the increase in enzyme expression and/or activity relative to a wild type cell.

In one example, the cell according to any aspect of the present invention may be genetically modified to comprise an increased expression relative to its wild type cell of all the following enzymes E_3 an acetoacetyl- CoA thiolase (thl), E_4 a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5 a 3-hydroxybutyryl-CoA dehydratase (crt), and E_6 a butyryl-CoA dehydrogenase (bcd) In another example, the expression of E_7 an electron transfer flavoprotein subunit (etf) may also be increased relative to the wild type cell. The cell according to any aspect of the present invention may thus have increased expression relative of the wild type cell of enzymes E_3-E_6 and E_8. In another example, the cell according to any aspect of the present invention may have increased expression relative of the wild type cell of enzymes E_3-E_8.

In another example, the cell according to any aspect of the present invention may be genetically modified to comprise an increased expression relative to its wild type cell of enzymes E_3, an acetoacetyl- CoA thiolase (thl), E_4, a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5, a 3-hydroxybutyryl-CoA dehydratase (crt), E_6, a butyryl-CoA dehydrogenase (bcd) and E_8. In one example, E_3 may comprise the sequence of SEQ ID NO:2, E_4 may comprise the sequence of SEQ ID NO:3, E_5 may comprise the sequence of SEQ ID NO: 4, E_6, may comprise the sequence of SEQ ID NO: 5.

In a further example, the cell according to any aspect of the present invention may be genetically modified to comprise an increased expression relative to its wild type cell of the enzymes E_3, an acetoacetyl- CoA thiolase (thl), E_4, a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5, a 3-hydroxybutyryl-CoA dehydratase (crt), and E_8. In one example, E_3 may comprise the sequence of SEQ ID NO:2, E_4 may comprise the sequence of SEQ ID NO:3, E_5 may comprise the sequence of SEQ ID NO: 4, and E_6, may comprise the sequence of SEQ ID NO: 5.

In another example, the cell according to any aspect of the present invention may be further genetically modified to comprise an increased expression relative to its wild type cell of the enzymes E_1, an alcohol dehydrogenase (adh) and the enzyme E_{12}, trans-2-enoyl-CoA reductase or crotonyl-CoA reductase (TER). In particular, E_1 may be a butyrate-dehydrogenase from *C. acetobutylicum* or *E. coli*. More in particular, the butanol-dehydrogenase from *C. acetobutylicum* may comprise the sequence of SEQ OD NO:18 and the butanol-dehydrogenase from *E. coli* may comprise the sequence of SEQ ID NO: 19. More in particular, the enzyme E_{12} in the cell may be selected from the group consisting of SEQ ID NOs: 14, 15 and 16.

The cell may also comprise E_7 an electron transfer flavoprotein subunit (etf). More in particular, E_7 may be etfB and etfA from *C. acetobutylicum*. Even more in particular, E_7 may comprise the sequence of SEQ ID NOs:10 and 11.

In one example, the cell according to any aspect of the present invention may be further genetically modified to comprise an increased expression relative to its wild type cell of the enzyme E_6, a butyrate-dehydrogenase. In particular, E_6 may be from *C. kluyveri* and/or may comprise the sequence of SEQ ID NO:7. The cell may also comprise increased expression relative to the wild type cell of an electron-transfer protein ($E_{7)}$. In particular, E_7 may comprise the sequence of SEQ ID NOs: 12 and 13. The cell may also comprise an increased expression relative to the wild type cell of trans-2-enoyl-CoA reductase (TER) (E_{12}). In particular, the TER may be from *Treponema denticola*, *Euglena gracilis*, or *Caenorhabditis elegans*. Even more in particular, E_{12} may be a TER selected from the group consisting of SEQ ID NOs: 14, 15 and 16. In another example, E_{12} may be crotonyl-CoA reductase (ccr). In particular, the ccr may be from *Streptomyces collinus*. More in particular, the enzyme E_{12} may be a ccr comprising the sequence of SEQ ID NO: 17.

In one further example, the cell according to any aspect of the present invention may be further genetically modified to comprise an increased expression relative to its wild type cell of the enzyme E_6, a butyrate-dehydrogenase. In particular, E_6 may be from *C. kluyveri* and/or may comprise the sequence of SEQ ID NO:5. The cell may also comprise increased expression relative to the wild type cell of an electron-transfer protein ($E_{7)}$. In particular, E_7 may comprise the sequence of SEQ ID NOs: 8 and 9. The cell may also comprise an increased expression relative to the wild type cell of the phosphotransacetylase (pta) promoter and/or terminator.

In particular, the cell according to any aspect of the present invention may comprise an increased expression relative to the wild type cell of the following enzymes E_3E_8, E_4E_8, E_5E_8, E_6E_8, E_7E_8, $E_3E_4E_8$, $E_3E_5E_8$, $E_3E_6E_8$, $E_3E_7E_8$, $E_4E_5E_8$, $E_4E_6E_8$, $E_4E_7E_8$, $E_5E_6E_8$, $E_5E_7E_8$, $E_3E_4E_5E_8$, $E_3E_4E_6E_8$, $E_3E_4E_7E_8$, $E_4E_5E_6E_8$, $E_4E_5E_7E_8$, $E_5E_6E_7E_8$, $E_3E_4E_5E_6E_8$, $E_3E_4E_5E_7E_8$, $E_4E_5E_6E_7E_8$, $E_3E_4E_5E_6E_7E_8$, $E_3E_8E_{12}$, $E_4E_8E_{12}$, $E_5E_8E_{12}$, $E_6E_8E_{12}$, $E_7E_8E_{12}$, $E_3E_4E_8E_{12}$, $E_3E_5E_8E_{12}$, $E_3E_6E_8E_{12}$, $E_3E_7E_8E_{12}$, $E_4E_5E_8E_{12}$, $E_4E_6E_8E_{12}$, $E_4E_7E_8E_{12}$, $E_5E_6E_8E_{12}$, $E_5E_7E_8E_{12}$, $E_3E_4E_5E_8E_{12}$, $E_3E_4E_6E_8E_{12}$, $E_3E_4E_7E_8E_{12}$, $E_4E_5E_6E_8E_{12}$, $E_4E_5E_7E_8E_{12}$, $E_5E_6E_7E_8E_{12}$, $E_3E_4E_5E_6E_8E_{12}$, $E_3E_4E_5E_7E_8E_{12}$, $E_4E_5E_6E_7E_8E_{12}$, $E_3E_4E_5E_6E_7E_8E_{12}$, $E_3E_8E_1$, $E_4E_8E_1$, $E_5E_8E_1$, $E_6E_8E_1$, $E_7E_8E_1$, $E_3E_4E_8E_1$, $E_3E_5E_8E_1$, $E_3E_6E_8E_1$, $E_3E_7E_8E_1$, $E_4E_5E_8E_1$, $E_4E_6E_8E_1$, $E_4E_7E_8E_1$, $E_5E_6E_8E_1$, $E_5E_7E_8E_1$, $E_3E_4E_5E_8E_1$, $E_3E_4E_6E_8E_1$, $E_3E_4E_7E_8E_1$, $E_4E_5E_6E_8E_1$, $E_4E_5E_7E_8E_1$, $E_5E_6E_7E_8E_1$, $E_3E_4E_5E_6E_8E_1$, $E_3E_4E_5E_7E_8E_1$, $E_4E_5E_6E_7E_8E_1$, $E_3E_4E_5E_6E_7E_8E_1$, $E_3E_8E_{12}E_1$, $E_4E_8E_{12}E_1$, $E_5E_8E_{12}E_1$, $E_6E_8E_{12}E_1$, $E_7E_8E_{12}E_1$, $E_3E_4E_8E_{12}E_1$, $E_3E_5E_8E_{12}E_1$, $E_3E_6E_8E_{12}E_1$, $E_3E_7E_8E_{12}E_1$, $E_4E_5E_8E_{12}E_1$, $E_4E_6E_8E_{12}E_1$, $E_4E_7E_8E_{12}E_1$, $E_5E_6E_8E_{12}E_1$, $E_5E_7E_8E_{12}E_1$, $E_3E_4E_5E_8E_{12}E_1$, $E_3E_4E_6E_8E_{12}E_1$, $E_3E_4E_7E_8E_{12}E_1$, $E_4E_5E_6E_8E_{12}E_1$, $E_4E_5E_7E_8E_{12}E_1$, $E_5E_6E_7E_8E_{12}E_1$, $E_3E_4E_5E_6E_8E_{12}E_1$, $E_3E_4E_5E_7E_8E_{12}E_1$, $E_4E_5E_6E_7E_8E_{12}E_1$, $E_3E_4E_5E_6E_7E_8E_{12}E_1$, x and the like.

In particular, E_8 may be selected from the group consisting of butyryl-CoA: acetate CoA transferase, succinyl-CoA:coenzyme A transferase, 4-hydroxybutyryl-CoA: coenzyme A transferase and the like. More in particular, E_8 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_3595, CKL_3016, CKL_3018 and the like. More in particular, E_8 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_3595, CKL_3016 and CKL_3018. E_8 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 1. In particular, E_8 may be from *Clostridium kluyveri* or *Clostridium carboxidivorans*. More in particular, E_8 may be from *Clostridium kluyveri*. Even more in particular, E_8 may be from *Clostridium kluyveri* strain ATCC 8527.

In particular, E_1 may be selected from the group consisting of alcohol dehydrogenase 1, alcohol dehydrogenase 2, alcohol dehydrogenase 3, alcohol dehydrogenase B and combinations thereof. More in particular, E_1 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_1075, CKL_1077, CKL_1078, CKL_1067, CKL_2967, CKL_2978, CKL_3000, CKL_3425, and CKL_2065. Even more in particular, E_1 may comprise a

polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_1075, CKL_1077, CKL_1078 and CKL_1067. E_1 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 18 or SEQ ID NO: 19. In particular, E_1 may be selected from the group consisting of *C. acetobutylicum* and *E. coli*.

In particular, E_2 may be selected from the group consisting of acetaldehyde dehydrogenase 1, alcohol dehydrogenase 2 and combinations thereof. In particular, E_2 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_1074, CKL_1076 and the like. More in particular, E_2 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_1074 and CKL_1076.

E_3 may be selected from the group consisting of acetoacetyl-CoA thiolase A1, acetoacetyl-CoA thiolase A2, acetoacetyl-CoA thiolase A3 and combinations thereof. In particular, E_3 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_3696, CKL_3697, CKL_3698 and the like. More in particular, E_3 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_3696, CKL_3697 and CKL_3698. More in particular, E_3 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 2. In particular, E_3 may be from *C. acetobutylicum*.

E_4 may be 3-hydroxybutyryl-CoA dehydrogenase 1, 3-hydroxybutyryl-CoA dehydrogenase 2 and the like. In particular, E_4 may comprise sequence identity of at least 50 % to a polypeptide CKL_0458, CKL_2795 and the like. More in particular, E_4 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to the polypeptide CKL_0458 or CKL_2795. More in particular, E_4 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 3. In particular, E_4 may be from *Clostridium kluyveri*.

E_5 may be 3-hydroxybutyryl-CoA dehydratase 1, 3-hydroxybutyryl-CoA dehydratase 2 and combinations thereof. In particular, E_5 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_0454, CKL_2527 and the like. More in particular, E_5 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_0454 and CKL_2527. More in particular, E_5 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 4. In particular, E_5 may be from *Clostridium kluyveri*.

E_6 may be selected from the group consisting of butyryl-CoA dehydrogenase 1, butyryl-CoA dehydrogenase 2 and the like. In particular, E_6 may comprise sequence identity of at least 50 % to

a polypeptide selected from the group consisting of CKL_0455, CKL_0633 and the like. More in particular, E_6 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_0455 and CKL_0633. More in particular, E_6 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 5, SEQ ID NO:6 or SEQ ID NO: 7. In particular, E_6 may be selected from the group consisting of *Clostridium kluyveri*, and *C. acetobutylicum*.

E_7 may be selected from the group consisting of electron transfer flavoprotein alpha subunit 1, electron transfer flavoprotein alpha subunit 2, electron transfer flavoprotein beta subunit 1 and electron transfer flavoprotein beta subunit 2. In particular, E_7 may comprise sequence identity of at least 50 % to a polypeptide selected from the group consisting of CKL_3516, CKL_3517, CKL_0456, CKL_0457 and the like. More in particular, E_7 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide selected from the group consisting of CKL_3516, CKL_3517, CKL_0456 and CKL_0457. More in particular, E_7 may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 8, SEQ ID NO:9, SEQ ID NO:10, SEQ ID NO:11. SEQ ID NO:12 or SEQ ID NO: 13. In particular, E_7 may be selected from the group consisting of *Clostridium kluyveri*, and *C. acetobutylicum*.

E_9 may be an acetate kinase A (ack A). In particular, E_9 may comprise sequence identity of at least 50 % to a polypeptide sequence of CKL_1391 and the like. More in particular, E_9 may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide of CKL_1391.

E_{10} may be phosphotransacetylase (pta). In particular, E_{10} may comprise sequence identity of at least 50 % to a polypeptide sequence of CKL_1390 and the like. More in particular, E_{10} may comprise a polypeptide with sequence identity of at least 50, 60, 65, 70, 75, 80, 85, 90, 91, 94, 95, 98 or 100 % to a polypeptide of CKL_1390. In particular, E_{10} may be selected from *C. acetobutylicum*.

E_{11} may be a tranhydrogenase. In particular, E_{11} may be the transhydrogenase disclosed in Hatefi, Y., (1977) Proc. Natl. Acad. Sci. USA 74 (3). 846-850 and/or Anderlund M. (1999), Appl Environ Microbiol., 65(6): 2333–2340

E_{12} may comprise an amino acid sequence that has 50, 55, 60, 65, 70, 75, 80, 85, 90, 95 or 100 % sequence identity to SEQ ID NO: 14, SEQ ID NO:15, SEQ ID NO:16 or SEQ ID NO: 17. In particular, E_{12} may be selected from the group consisting of *Treponema denticola*, *Euglena gracilis*, *Caenorhabditis elegans*, and *Streptomyces collinus*.

Throughout this application, any data base code, unless specified to the contrary, refers to a sequence available from the NCBI data bases, more specifically the version online on 12 June 2014, and comprises, if such sequence is a nucleotide sequence, the polypeptide sequence obtained by translating the former.

According to another aspect of the present invention there is provided a method of producing a higher alcohol, the method comprising
- contacting a recombinant microbial cell according to any aspect of the present invention with a medium comprising a carbon source.

The term “contacting”, as used herein, means bringing about direct contact between the cell according to any aspect of the present invention and the medium comprising the carbon source. For example, the cell, and the medium comprising the carbon source may be in different compartments. On particular, the carbon source may be in a gaseous state and added to the medium comprising the cells according to any aspect of the present invention.

The term "acetate" as used herein, refers to both acetic acid and salts thereof, which results inevitably, because as known in the art, since the microorganisms work in an aqueous environment, and there is always a balance between salt and acid present.

The term ‘about’ as used herein refers to a variation within 20 percent. In particular, the term "about" as used herein refers to +/- 20 %, more in particular, +/-10 %, even more in particular, +/- 5 % of a given measurement or value.

All percentages (%) are, unless otherwise specified, volume percent.

The carbon source used according to any aspect of the present invention may be any carbon source known in the art. In particular, the carbon source may be selected from the group consisting of carbohydrates such as, for example, glucose, sucrose, lactose, fructose, maltose, molasses, starch and cellulose. In one example, hydrocarbons such as methane, amino acids such as L-glutamate or L-valine, or organic acids such as, for example, acetic acid may be used as a carbon source. These substances may be used singularly or as a mixture. It is especially preferred to employ carbohydrates, in particular monosaccharides, oligosaccharides or polysaccharides, as described in U.S. Pat. No. 601,494 and U.S. Pat. No. 6,136,576, or C5-sugars, or glycerol. In one example, the carbon source may comprise carbon dioxide and/or carbon monoxide. A skilled person would understand that many possible sources for the provision of CO and/or CO_2 as a carbon source exist. It can be seen that in practice, as the carbon source according to any aspect of the present invention any gas or any gas mixture can be used which is able to supply the microorganisms with sufficient amounts of carbon, so that acetate and/or ethanol, may be formed from the source of CO and/or CO_2.

Generally, for the mixed culture according to any aspect of the present invention the carbon source comprises at least 50 % by volume, at least 70 % by volume, particularly at least 90 % by volume of CO and / or CO_2, wherein the percentages by volume - % relate to all carbon sources that are available to the first microorganism in the mixed culture. In one example, the carbon source may be a gas mixture comprising 5 - 25 % by volume of CO, 25 -35 % by volume CO_2 and 50 - 65 H_2 gas. In another example, the carbon source may be a gas mixture comprising 22 % by volume of CO, 6 % by volume CO_2 and 44 % H_2 gas. In a further example, the carbon source may be a gas mixture comprising 33 % by volume CO_2 and 67 % H_2 gas. In a particular example, the carbon source may be a gas mixture comprising 25 % by volume of CO, 25 % by volume CO_2 and 50 % H_2 gas.
In the mixed culture according to any aspect of the present invention, the carbon material source may be provided. Examples of carbon sources in gas forms include exhaust gases such as synthesis gas, flue gas and petroleum refinery gases produced by yeast fermentation or clostridial fermentation. These exhaust gases are formed from the gasification of cellulose-containing materials or coal gasification. In one example, these exhaust gases may not necessarily be produced as by-products of other processes but can specifically be produced for use with the mixed culture according to any aspect of the present invention.

According to any aspect of the present invention, the carbon source may be synthesis gas. Synthesis gas can for example be produced as a by-product of coal gasification. Accordingly, the microorganism of the mixed culture according to any aspect of the present invention may be capable of converting a substance which is a waste product into a valuable resource. In another example, synthesis gas may be a by-product of gasification of widely available, low-cost agricultural raw materials for use with the mixed culture of the present invention to produce at least one higher alcohol.

There are numerous examples of raw materials that can be converted into synthesis gas, as almost all forms of vegetation can be used for this purpose. In particular, raw materials are selected from the group consisting of perennial grasses such as miscanthus, corn residues, processing waste such as sawdust and the like.

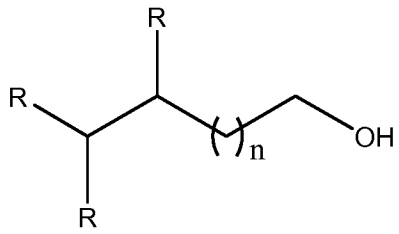
In general, synthesis gas may be obtained in a gasification apparatus of dried biomass, mainly through pyrolysis, partial oxidation and steam reforming, wherein the primary products of the synthesis gas are CO, H_2 and CO_2. Syngas may also be a product of electrolysis of CO_2. A skilled person would understand the suitable conditions to carry out electrolysis of CO_2 to produce syngas comprising CO in a desired amount.

Usually, a portion of the synthesis gas obtained from the gasification process is first processed in order to optimize product yields, and to avoid formation of tar. Cracking of the undesired tar and CO in the synthesis gas may be carried out using lime and/or dolomite. These processes are described in detail in for example, Reed, 1981.

Mixtures of sources can be used as a carbon source.

According to any aspect of the present invention, a reducing agent, for example hydrogen may be supplied together with the carbon source. In particular, this hydrogen may be supplied when the C and/or CO_2 is supplied and/or used. In one example, the hydrogen gas is part of the synthesis gas present according to any aspect of the present invention. In another example, where the hydrogen gas in the synthesis gas is insufficient for the method of the present invention, additional hydrogen gas may be supplied.

'Higher alcohols' as used herein refers to alcohols that contain 4 to 12 carbon atoms, in particular, 4 to 10 carbon atoms, 4 to 8 carbon atoms, 6 to 10 carbon atoms and may be somewhat viscous, or oily, and have heavier fruity odours. More in particular, the 'higher alcohol' may be comprise the formula I below and has 4 to 10 carbon atoms



Formula I

R= H, CH_3,

n=1- 6

Higher alcohols may include but are not limited to hexanol, heptanol, octanol, nonanol, decanol and the like. More in particular, the higher alcohol may be selected from the group consisting of 1-butanol, 2-methyl-1-butanol, isobutanol, 3-methyl-1-butanol, 1-hexanol, 1-octanol, 1-pentanol, 1-heptanol, 3-methyl-1-pentanol, 4-methyl-1-hexanol, 5-methyl-1-heptanol, 4-methyl-1-pentanol, 5-methyl-1-hexanol, 6-methyl-1-heptanol and combinations thereof.

A skilled person would understand the other conditions necessary to carry out the method according to any aspect of the present invention. In particular, the conditions in the container (e.g. fermenter) may be varied depending on the first and second microorganisms used. The varying of the conditions to be suitable for the optimal functioning of the microorganisms is within the knowledge of a skilled person.

In one example, the method according to any aspect of the present invention may be carried out in an aqueous medium with a pH between 5 and 8, 5.5 and 7. The pressure may be between 1 and 10 bar.

In particular, the aqueous medium may comprise a carbon source comprising CO and/or CO_2. More in particular, the carbon source comprising CO and/or CO_2 is provided to the aqueous

medium in a continuous gas flow. Even more in particular, the continuous gas flow comprises synthesis gas. In one example, the gases are part of the same flow/stream. In another example, each gas is a separate flow/stream provided to the aqueous medium. These gases may be divided for example using separate nozzles that open up into the aqueous medium, frits, membranes within the pipe supplying the gas into the aqueous medium and the like.

According to another aspect of the present invention, there is provided a use of the cell according to any aspect of the present invention for the production of a higher alcohol.

In the reaction mixture according to any aspect of the present invention, there may be oxygen present. Accordingly, the microorganisms according to any aspect of the present invention may be grown aerobically. In particular, oxygen may be provided to the aqueous medium according to any aspect of the present invention in a continuous gas flow. More in particular, the O_2 concentration in the gas flow may be may be present at less than 1 % by volume of the total amount of gas in the gas flow. In particular, the oxygen may be present at a concentration range of 0.000005 to 2 % by volume, at a range of 0.00005 to 2 % by volume, 0.0005 to 2 % by volume, 0.005 to 2 % by volume, 0.05 to 2 % by volume, 0.00005 to 1.5 % by volume, 0.0005 to 1.5 % by volume, 0.005 to 1.5 % by volume, 0.05 to 1.5 % by volume, 0.5 to 1.5 % by volume, 0.00005 to 1 % by volume, 0.0005 to 1 % by volume, 0.005 to 1 % by volume, 0.05 to 1 % by volume, 0.5 to 1 % by volume, 0.55 to 1 % by volume, 0.60 to 1 % by volume, particularly at a range of 0.60 to 1.5 %, 0.65 to 1 %, and 0.70 to 1 % by volume. In particular, the acetogenic microorganism is particularly suitable when the proportion of O_2 in the gas phase/flow is about 0.00005, 0.0005, 0.005, 0.05, 0.5, 0.6, 0.7, 0.8, 0.9, 1, 1.5, 2 % by volume in relation to the volume of the gas in the gas flow. A skilled person would be able to use any one of the methods known in the art to measure the volume concentration of oxygen in the gas flow. In particular, the volume of oxygen may be measured using any method known in the art. In one example, a gas phase concentration of oxygen may be measured by a trace oxygen dipping probe from PreSens Precision Sensing GmbH. Oxygen concentration may be measured by fluorescence quenching, where the degree of quenching correlates to the partial pressure of oxygen in the gas phase. Even more in particular, the first and second microorganisms according to any aspect of the present invention are capable of working optimally in the aqueous medium when the oxygen is supplied by a gas flow with concentration of oxygen of less than 1 % by volume of the total gas, in about 0.015 % by volume of the total volume of gas in the gas flow supplied to the reaction mixture.

The aqueous medium according to any aspect of the present invention may comprise oxygen. The oxygen may be dissolved in the medium by any means known in the art. In particular, the oxygen may be present at 0.5mg/L. In particular, the dissolved concentration of free oxygen in the aqueous medium may at least be 0.01mg/L. In another example, the dissolved oxygen may be about 0.01, 0.02, 0.03, 0.04, 0.05, 0.1, 0.2, 0.3, 0.4, 0.5 mg/L. In particular, the dissolved oxygen concentration may be 0.01-0.5mg/L, 0.01-0.4mg/L, 0.01-0.3mg/L, 0.01-0.1mg/L. In particular, the oxygen may be provided to the aqueous medium in a continuous gas flow. More in particular, the aqueous medium

may comprise oxygen and a carbon source comprising CO and/or CO_2. More in particular, the oxygen and a carbon source comprising CO and/or CO_2 is provided to the aqueous medium in a continuous gas flow. Even more in particular, the continuous gas flow comprises synthesis gas and oxygen. In one example, both gases are part of the same flow/stream. In another example, each gas is a separate flow/stream provided to the aqueous medium. These gases may be divided for example using separate nozzles that open up into the aqueous medium, frits, membranes within the pipe supplying the gas into the aqueous medium and the like. The oxygen may be free oxygen. According to any aspect of the present invention, 'a reaction mixture comprising free oxygen' refers to the reaction mixture comprising elemental oxygen in the form of O_2. The O_2 may be dissolved oxygen in the reaction mixture. In particular, the dissolved oxygen may be in the concentration of ≥ 5ppm (0.000005 % vol; $5x10^{-6}$). A skilled person may be capable of using any method known in the art to measure the concentration of dissolved oxygen. In one example, the dissolved oxygen may be measured by Oxygen Dipping Probes (Type PSt6 from PreSens Precision Sensing GmbH, Regensburg, Germany.

In one example according to any aspect of the present invention, the carbon source is synthesis gas and the carbon source may be blended with the oxygen gas before being supplied into the aqueous medium. This blending step may improve the efficiency and the production of higher alcohols in the reaction. The overall efficiency, alcohol productivity and/or overall carbon capture of the method of the present invention may be dependent on the stoichiometry of the CO_2, CO, H_2 and O_2 in the continuous gas flow. The continuous gas flows applied may be of composition O_2, CO_2 and H_2. In particular, in the continuous gas flow, concentration range of O_2 may be within 0.000005 to 1 % by volume, CO/CO_2 about 10 – 50 %, in particular 33 % by volume and H_2 would be within 44 % to 84 %, in particular, 64 to 66.04 % by volume. More in particular, the concentration of gases in the continuous gas flow may be 0.15 % by volume of O_2, 32 % by volume of CO/CO_2 and 64 % by volume of H_2. In another example, the continuous gas flow can also comprise inert gases like N_2, up to a N_2 concentration of 50 % by volume.

A skilled person would understand that it may be necessary to monitor the composition and flow rates of the streams at relevant intervals. Control of the composition of the stream can be achieved by varying the proportions of the constituent streams to achieve a target or desirable composition. The composition and flow rate of the blended stream can be monitored by any means known in the art. In one example, the system is adapted to continuously monitor the flow rates and compositions of at least two streams and combine them to produce a single blended substrate stream in a continuous gas flow of optimal composition, and means for passing the optimised substrate stream to the mixed culture according to any aspect of the present invention.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is the vector pSOS95

EXAMPLES

The foregoing describes preferred embodiments, which, as will be understood by those skilled in the art, may be subject to variations or modifications in design, construction or operation without departing from the scope of the claims. These variations, for instance, are intended to be covered by the scope of the claims.

All the sequences within the examples are of the genes connected together and does not include the actual vector pSOS95 backbone sequence.

Example 1

Generation of a genetically modified acetogenic bacteria for the formation of Butanol

- Vectors pATh-LEM-04 and pATh-LEM-14

The genes Thiolase from *C. acetobutylicum* ATTC 824 (thl_Ca) (SEQ ID NO:28), hydroxybutyryl-CoA dehydrogenase from *C. kluyveri* (hbd1_Ck) (SEQ ID NO: 29), crotonase from *C. klyuveri* (crt1_Ck) (SEQ ID NO:30) and butyryl-CoA dehydrogenase form *C. kluyveri* (bcd1_Ck) (SEQ ID NO:31) are amplified from the corresponding genome and were inserted into the vector pEmpty by using *Kas*I and *Bam*HI. This plasmid (pEmpty) was based on the plasmid backbone pSOS95 (Figure 1). To use pSOS95, it was digested with *BamHI* and *KasI*. This removed the operon ctfA-ctfB-adc, but leaves the thl promoter and the rho-independent terminator of adc. The newly generated vector, which bore the named genes, was called pATh-LEM-02 (SEQ ID NO:51 refers to the sequences of the genes connected together in pATh-LEM-02 without the sequence of the actual vector).

In a second cloning step, the vector pATh-LEM-02 was digested with *EcoRI* and *KasI* and the CoA-Transferase from *C. kluyveri* (cat3_Ck) (SEQ ID NO:26) was amplified from genomic DNA and integrated into the vector. The newly designed vector was named pATh-LEM-04. To create the vector pATh-LEM-14, the vector pATH-LEM-04 was digested with *KasI* and *BspEI*. The genes *etfBA* were amplified from genomic DNA of *Clostridium kluyveri* by using the oligonucleotides of SEQ ID NOs: 46 and 47.

A fragment of cat3 was amplified from pATh-LEM-04 by using the oligonucleotides of SEQ ID NOs: 48 and 49. The resultant fragment has sequence of SEQ ID NO:52. The two fragments of *cat3* and *etfBA* were then fused using PCR with primers of SEQ ID NO: 50 and 49. This fusion insert of *cat3* and *etfBA* was then added to the *KasI* and *BspEI* opened vector pATH-LEM-04. The resultant vector was called pATH-LEM-14 (SEQ ID NO:20 is the sequence of the target genes fused together that can be easily inserted into the vector).

- Vectors pATh-Syn4-03 and pATh-LEM-23

To generate a vector named pATh-Syn4-03 a cassette with SEQ ID NO:53 was first formed. This cassette comprised the genes: Thiolase from *C. acetobutylicum* ATTC 824 (thl_Ca) (SEQ ID

NO:28), hydroxybutyryl-CoA dehydrogenase from *C. kluyveri* (hbd1_Ck) (SEQ ID NO: 29), and crotonase from *C. klyuveri* (crt1_Ck) (SEQ ID NO:30). The cassette with SEQ ID NO:53 was then inserted into the vector pEmpty by using *Kas*I and *Bam*HI.

This plasmid (pEmpty) was based on the plasmid backbone pSOS95 (Figure 1). To use pSOS95, it was digested with *Bam*HI and *Kas*I. This removes the operon ctfA-ctfB-adc, but leaves the thl promoter and the rho-independent terminator of adc. In a second step, the thl promoter was removed from the vector by digesting it with *Sbf*I and *Bam*HI. The pta promotor fragment (SEQ ID NO: 25 (Ueki et al. (2014) mBio. 585): 1636-14) was synthesized and was ligated to the *Bam*HI/*Sbf*I digested vector. The newly generated vector, which bears the named genes and the pta promotor, was called pATh-Syn4-14.

The vector pATh-Syn4-14 was opened with *Kas*I and *Eco*RI and ligated with SEQ ID NO:54 which was synthesized from CoA-Transferase from *C. kluyveri* . The generated vector was named pATh-LEM-23 (SEQ ID NO:21)

- Vectors pATh-LEM-15, pATh-LEM-16, pATh-LEM-24, pATh-LEM-25, pATh-LEM-26

A cassette containing Thiolase from *C. acetobutylicum* ATTC 824 (thl_Ca) (SEQ ID NO:29), hydroxybutyryl-CoA dehydrogenase from *C. kluyveri* (hbd1_Ck) (SEQ ID NO: 29), and crotonase from *C. klyuveri* (crt1_Ck) (SEQ ID NO:30) were synthesized and were inserted into the vector pEmpty by using *Kas*I and *Bam*HI. This plasmid (pEmpty) is based on the plasmid backbone pSOS95 (Figure 1). To use pSOS95, it was digested with *Bam*HI and *Kas*I. This removes the operon ctfA-ctfB-adc, but leaves the thl promoter and the rho-independent terminator of adc. The newly generated vector, which bears the named genes, was called pATh-Syn4-03.

The vector pATh-Syn4-03 was opened with *Kas*I and a cassette containing butyrate-dehydrogenase from *C. acetobutylicum* (bcd_Ca) (SEQ ID NO:34), electron-transfer protein from *C. acetobutylicum* (etfBA_Ca) (SEQ ID NOs: 35 and 36) and CoA-transferase from *C. kluyveri* (cat3_Ck) (SEQ ID NO:26) was ligated by *in vitro* cloning. The newly constructed vector is named pATh-LEM-15 (SEQ ID NO:55).

The vector pATh-Syn4-03 was opened with *Kas*I/*Eco*RI and ligated with a cassette (SEQ ID NO:56 without the full sequence of the vector containing butyrate-dehydrogenase from *C. kluyveri* (bcd1_Ck) (SEQ ID NO:5), electron-transfer protein from *C. klyuveri* (etfBA1_Ck) (SEQ ID NOs:8 and 9) and CoA-transferase from *C. kluyveri* (cat3_Ck) (SEQ ID NO:1). The newly constructed vector is named pATh-LEM-16.

The vector pATh-Syn4-03 was opened with *Kas*I and *Eco*RI. A DNA fragment of CoA-Transferase from *C. kluyveri* (SEQ ID NO:57) was synthesized and ligated to the prepared vector. The generated vector was named pATh-LEM-24 (SEQ ID NO:22).

To generate the vector pATh-LEM-25, the plasmid pATh-Syn4-24 was opened with *Asi*SI and *Eco*RI. A DNA fragment containing the Butanol dehydrogenase B from *C. acetobutylicum* (bdhB_Ca) (SEQ ID NO:44) was synthesized and ligated to the prepared vector. The generated vector was named pATh-LEM-25 (SEQ ID NO:23).

To generate the vector pATh-LEM-26, the plasmid pATh-Syn4-25 (SEQ ID NO:23) was opened with *Asi*SI and *Asc*I. The Butanol dehydrogenase from *E. coli* codon optimized for *C. ljungdahlii* (YghD_E(coCl)) (SEQ ID NO:58) was amplified, fused with a ribosome binding site and ligated to the prepared vector. The generated vector was named pATh-LEM-26 (SEQ ID NO:24).

- Vectors pATh-LEM-17, pATh-LEM-18, pATh-LEM-19, pATh-LEM-20, pATh-LEM-21

The vector pATh-LEM-16 was opened with *Kas*I and *Not*I. A DNA fragment of SEQ ID NO: 59 containing butyrate-dehydrogenase 2 from *C. kluyveri* (bcd2_Ck) (SEQ ID NO:37) and electron-transfer protein 2 from *C. klyuveri* (etfBA2_Ck) (SEQ ID NOs: 39 and 38) was ligated. The newly constructed vector was named pATh-LEM-17.

To create pATh-LEM-18 the vector pATh-LEM-16 was opened with *Kas*I and *Not*I. The DNA fragment containing the codon optimized trans-2-enoyl-CoA reductase from *Treponema denticola* (TER_Td(coCl)) (SEQ ID NO:41) was ligated. The newly constructed vector is named pATh-LEM-18.

To create pATh-LEM-19 the vector pATh-LEM-16 was opened with *Not* and AarI. The DNA fragment containing the codon optimized trans-2-enoyl-CoA reductase from *Euglena gracilis* (TER_Eg(coCl)) (SEQ ID NO:40) was ligated. The newly constructed vector was named pATh-LEM-19.

The vector pATh-LEM-16 was opened with *Aar*I and *Not*I. The DNA fragment containing the codon optimized trans-2-enoyl-CoA reductase from *Caenorhabditis elegans* (TER_Ce(coCl)) (SEQ ID NO:42) was ligated. The newly constructed vector was named pATh-LEM-20.

The vector pATh-LEM-16 was opened with *FseI* and *Not*I. The synthetic DNA fragment containing the codon optimized crotonyl-CoA reductase from *Streptomyces collinus* (Ccr_Sc(coCl)) (SEQ ID NO:43) was ligated. The newly constructed vector was named pATh-LEM-21.

- Vector pATh-LEM-22

A DNA fragment (SEQ ID NO: 60) containing the butyryl-CoA dehydrogenase from *C. klyveri* (bcd1_Ck) (SEQ ID NO:31), electron-transfer protein from *C. kluyveri* (etfBA1_Ck) (SEQ ID NOs:32 and 33), the CoA-transferase from *C. kluyveri* (cat3_Ck) (SEQ ID NO:26) and transcriptional elements (pta-Promotor and a Terminator) . The parental vector pATh-Syn4-03 was opened with *Eco*RI / *XhoI* and the DNA fragment (SEQ ID NO: 60) ligated in to produce the vector pATh-LEM-22.

Transformation of Acetogens:

The transformation of *C. ljungdahlii* DSMZ 13528 and *C. autoethanogenum* DSMZ 10061 was done as disclosed in Leang et al. (2013) Applied and Environmental Microbiology 79(4): 1102-1109.

Example 2

Fermentation of genetically modified strains on mixtures of H_2, CO_2 and CO showing acid and higher alcohol formation.

For cell culture of

C. ljungdahlii pATh-LEM-04

C. ljungdahlii pATh-LEM-14

C. ljungdahlii pATh-LEM-15

C. ljungdahlii pATh-LEM-16

C. ljungdahlii pATh-LEM-17

C. ljungdahlii pATh-LEM-18

C. ljungdahlii pATh-LEM-19

C. ljungdahlii pATh-LEM-20

C. ljungdahlii pATh-LEM-21

C. ljungdahlii pATh-LEM-22

C. ljungdahlii pATh-LEM-23

C. ljungdahlii pATh-LEM-24

C. ljungdahlii pATh-LEM-25

C. ljungdahlii pATh-LEM-26

C. ljungdahlii pEmpty

C. autoethanogenum pATh-LEM-04

C. autoethanogenum pATh-LEM-14

C. autoethanogenum pATh-LEM-15

C. autoethanogenum pATh-LEM-16

C. autoethanogenum pATh-LEM-17

C. autoethanogenum pATh-LEM-18

C. autoethanogenum pATh-LEM-19

C. autoethanogenum pATh-LEM-20

C. autoethanogenum pATh-LEM-21

C. autoethanogenum pATh-LEM-22

C. autoethanogenum pATh-LEM-23

C. autoethanogenum pATh-LEM-24

C. autoethanogenum pATh-LEM-25

C. autoethanogenum pATh-LEM-26

C. autoethanogenum pEmpty

5 mL of the culture will be anaerobically grown in 500 ml of LM33-medium with 100 mg/L of erythromycin.

LM 33 media was prepared at pH 5.5 as follows in tables 1-3. All ingredients with the exception of cysteine HCL were mixed in dH_2O to a total volume of 1 L. This solution was made anaerobic by heating to boiling point and allowing it to cool to room temperature under a constant flow of N_2 gas. Once cool, the cysteine HCL (0,5 g/L) was added and the pH of the solution adjusted to 5.5; anaerobicity was maintained throughout the experiments.

Media component	concentration	
$MgCl_2$ x 6 H_2O	0,5	g/L
NaCl	0,2	g/L
$CaCl_2$ x 2 H_2O	0,135	g/L
NaH_2PO_4 x 2 H_2O	2,65	g/L
KCl	0,5	g/L
NH_4Cl	2,5	g/L
MES	20,0	g/L
LS06-trace element solution	10	mL/L
LS03-vitamin solution	10	mL/L
$FeCl_3$-Solution	2	mL/L

Table 1. Media component (LM-33) used in Example 1

components	concentration	
Nitriloacetic acid	1,5	g/L
$MgSO_4$ x 7 H_2O	3	g/L
$MnSO_4$ x H_2O	0,5	g/L
NaCl	1	g/L
$FeSO_4$ x 7 H_2O	0,1	g/L
$Fe(SO_4)_2(NH4)_2$ x 6 H_2O	0,8	g/L
$CoCl_2$ x 6 H_2O	0,2	g/L
$ZnSO_4$ x 7 H_2O	0,2	g/L
$CuCl_2$ x 2 H_2O	0,02	g/L
$KAl(SO_4)_2$ x 12 H_2O	0,02	g/L
H_3BO_3	0,3	g/L
Na_2MoO_4 x 2 H_2O	0,03	g/L
Na_2SeO_3	0,02	g/L
$NiCl_2$ x 6 H_2O	0,02	g/L
Na_2WO_4 x 6 H_2O	0,02	g/L

Table 2. LS06-trace element solution

component	concentration	
Biotin	20	mg/L
Folic Acid	20	mg/L
Pyridoxine HCl	10	mg/L
Thiamin HCl	50	mg/L
Riboflavin	50	mg/L
Nicotinic Acid	50	mg/L
CalciumD-(+)-pantothenate	50	mg/L
Vitamin B12	50	mg/L
p-Aminobenzoic acid	50	mg/L
Lipoic Acid	50	mg/L

Table 3 LS03-vitamin-solution

Cultivation is carried out in duplicate into 1 L glass bottles with a premixed gas mixture composed of around H_2, CO_2 and CO in an open water bath shaker at 37°C, 150 rpm and aeration of 3 L/h for 70 h. The gas will enter the medium through a filter with a pore size of 10 microns, which will mount in the middle of the reactor, at a gassing tube. When sampling each 5 ml sample will be removed for determination of OD600 nm, pH and the product range. The determination of the product concentration will be performed by semi-quantitative 1 H-NMR spectroscopy. As an internal quantification standard sodium trimethylsilylpropionate will be used. In contrast to the negative controls *C. ljungdalii* pEmpty and *C. autoethanogenum* pEmpty the modified strains will produce butyrate, butanol, hexanoate, hexanol, octanoate and octanol.

Example 3

Materials and Methods

In the following examples, genetically modified *Clostridium ljungdahlii* or *Clostridium autoethanogenum* were cultivated in order to produce butanol and/or the precursors 3-hydroxybutyrate and/or butyrate. A complex medium with 5 g/L fructose was used, consisting of 1 g/L NH_4Cl, 0.1 g/L KCl, 0.2 g/L $MgSO_4$ x 7 H_2O, 0.8 g/L NaCl, 0.1 g/L KH_2PO_4, 20 mg/L $CaCl_2$ x 2 H_2O, 20 g/L MES, 1 g/L yeast extract, 0.4 g/L L-cysteine-HCl, 0.4 g/L Na_2S x 9 H_2O, 20 mg/L nitrilotriacetic acid, 10 mg/L $MnSO_4$ x H_2O, 8 mg/L $(NH_4)_2Fe(SO_4)_2$ x 6 H_2O, 2 mg/L $CoCl_2$ x 6 H_2O, 2 mg/L $ZnSO_4$ x 7 H_2O, 0.2 mg/L $CuCl_2$ x 2 H_2O, 0.2 mg/L Na_2MoO_4 x 2 H_2O, 0.2 mg/L $NiCl_2$ x 6 H_2O, 0.2 mg/L Na_2SeO_4, 0.2 mg/L Na_2WO_4 x 2 H_2O, 20 µg/L biotin, 20 µg/L folic acid, 100 µg/L pyridoxine-HCl, 50 µg/L thiamine-HCl x H_2O, 50 µg/L riboflavin, 50 µg/L nicotinic acid, 50 µg/L Ca-pantothenoic acid, 1 µg/L vitamin B12, 50 µg/L p-aminobenzoic acid, 50 µg/L lipoic acid.
The heterotrophic cultivations were performed in 50 mL medium in a 250 mL serum bottle. The serum bottle was continuously shaken in an open water bath Innova 3100 from New Brunswick Scientific at 37 °C and a shaking rate of 150 min^{-1}.
The experiments were inoculated with 5 mL cell suspension grown in Hungate tubes in above described medium. During the experiment samples of 5 mL were taken for the determination of OD_{600}, pH and product concentrations. The latter were determined by quantitative ^{1}H-NMR-spectroscopy.

Results and Discussion:

Example 3a

Cultivation of genetically modified Clostridium ljungdahlii pATh-LEM-14

Genetically modified *C. ljungdahlii* pATh-LEM-14 as shown in Examples 1 and 2, was heterotrophically cultivated under above described conditions.
After inoculation, cells grew up to a maximal optical density of 1.82 after 56.6 hours. Besides the natural products acetate and ethanol a maximal butanol concentration of 59 mg/L was measured

after 56.6 h. Butyrate was produced up to a concentration of 200 mg/L. The results are shown in Table 4.

			NMR-analytics				
Process time, h	**pH**	**OD_{600}**	**Acetate, mg/L**	**Ethanol, mg/L**	**3-Hydroxy-butyrate, mg/L**	**n-Butanol, mg/L**	**Butyrate, mg/L**
0.0	5.96	0.14	160	17	n.d.	n.d.	41
56.6	5.01	1.82	2650	500	n.d.	59	190
117.7	5.03	1.21	2700	490	n.d.	59	200

Table 4. Results of *C. ljungdahlii* pATh-LEM-14 fermentation (n.d. = not detected)

Example 3b

Cultivation of genetically modified Clostridium ljungdahlii pATh-LEM-23

Genetically modified *C. ljungdahlii* pATh-LEM-23 was heterotrophically cultivated under above described conditions. After inoculation, cells grew up to a maximal optical density of 1.21 after 113.6 hours. Besides the natural products acetate and ethanol a maximal butanol concentration of 8 mg/L was measured after 113.6 h. 3-hydroxybutyrate and butyrate were produced up to concentrations of 230 mg/L and 15 mg/L respectively. The results are shown in Table 6.

			NMR-analytics				
Process time, h	**pH**	**OD_{600}**	**Acetate, mg/L**	**Ethanol, mg/L**	**3-Hydroxy-butyrate, mg/L**	**n-Butanol, mg/L**	**Butyrate, mg/L**
0.0	5.91	0.14	210	25	25	n.d.	n.d.
113.6	4.99	1.21	2950	520	230	8	15

Table 5. Results of *C. ljungdahlii* pATh-LEM-23 fermentation (n.d. = not detected)

Example 3c

Cultivation of genetically modified Clostridium ljungdahlii pATh-LEM-24

Genetically modified C. ljungdahlii pATh-LEM-24 was heterotrophically cultivated under above described conditions. After inoculation, cells grew up to a maximal optical density of 1.92 after 113.6 hours. Besides the natural products acetate and ethanol a maximal butanol concentration of 7 mg/L was measured after 113.6 h. 3-hydroxybutyrate and butyrate were produced up to concentrations of 170 mg/L and 12 mg/L respectively. The results are shown in Table 7.

			NMR-analytics

Process time, h	pH	OD_{600}	Acetate, mg/L	Ethanol, mg/L	3-Hydroxy-butyrate, mg/L	n-Butanol, mg/L	Butyrate, mg/L
0.0	5.94	0.06	91	15	17	n.d.	n.d.
113.6	4.95	1.92	3000	580	170	7	12

Table 6. Results of *C. ljungdahlii* pATh-LEM-24 fermentation (n.d. = not detected)

Example 3d

Cultivation of genetically modified Clostridium ljungdahlii pATh-LEM-25

Genetically modified *C. ljungdahlii* pATh-LEM-25 was heterotrophically cultivated under above described conditions. After inoculation, cells grew up to a maximal optical density of 1.52 after 117.4 hours. Besides the natural products acetate and ethanol no butanol was detected. Butyrate had a peak of 13 mg/L after 51.1 hours, but was consumed again thereafter. The precursor 3-hydroxybutyrate was produced up to a concentration of 73 mg/L. The results are shown in Table 8.

			NMR-analytics				
Process time, h	pH	OD_{600}	Acetate, mg/L	Ethanol, mg/L	3-Hydroxy-butyrate, mg/L	n-Butanol, mg/L	Butyrate, mg/L
0.0	6.01	0.07	88	19	17	n.d.	n.d.
51.1	5.82	0.61	730	320	55	n.d.	13
117.4	5.04	1.52	2800	640	73	n.d.	n.d.

Table 7. Results of *C. ljungdahlii* pATh-LEM-25 fermentation (n.d. = not detected)

Example 3e

Cultivation of genetically modified Clostridium autoethanogenum pATh-LEM-23

In this example, genetically modified *C. autoethanogenum* pATh-LEM-23 was heterotrophically cultivated under above described conditions.
After inoculation, cells grew to a maximal optical density of 0.98 after 117.4 hours. Besides the natural products acetate and ethanol no butanol was detected. The precursor butyrate had a peak of 6 mg/L after 51.1 hours, but was consumed again thereafter. The precursor 3-hydroxybutyrate was produced up to a concentration of 140 mg/L. The results are shown in Table 9.

			NMR-analytics				
Process time, h	pH	OD_{600}	Acetate, mg/L	Ethanol, mg/L	3-Hydroxy-butyrate, mg/L	n-Butanol, mg/L	Butyrate, mg/L
0.0	5.98	0.08	120	31	12	n.d.	n.d.

51.1	5.93	0.27	350	180	16	n.d.	6
117.4	5.26	0.98	2200	760	140	n.d.	n.d.

Table 8. Results of *C. autoethanogenum* pATh-LEM-23 fermentation (n.d. = not detected)

Example 3f

Cultivation of wildtype Clostridium ljungdahlii DSM 13528 (wildtype)

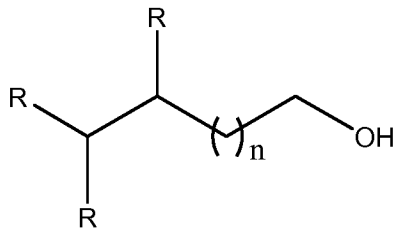
The wildtype of *C. ljungdahlii* (DSM 13528) was heterotrophically cultivated under above described conditions. After inoculation, cells began to grow up to a maximal optical density of 1.20 after 68.5 hours. Only the natural products acetate and ethanol were measured after 68.5 h to maximal concentrations of 1197 mg/L and ethanol respectively.

			NMR-analytics				
Process time, h	**pH**	**OD_{600}**	**Acetate, mg/L**	**Ethanol, mg/L**	**3-Hydroxy-butyrate, mg/L**	**n-Butanol, mg/L**	**Butyrate, mg/L**
0.0	6.00	0.11	156	15	n.d.	n.d.	n.d.
68.5	5.61	1.20	1197	402	n.d.	n.d.	n.d.

Table 9. Results of *C. ljungdahlii* wt fermentation (n.d. = not detected)

CLAIMS

1. An acetogenic microbial cell which is capable of producing at least one higher alcohol from a carbon source, wherein the acetogenic microbial cell is genetically modified to comprise an increased expression relative to its wild type cell of at least one enzyme, E_8, a butyryl-CoA: acetate CoA transferase (cat3), and wherein the higher alcohol comprises the formula I below and has 4 to 10 carbon atoms



Formula I

R= H, CH_3,

n=1- 6

2. The cell according to claim 1, wherein the cell is genetically modified to comprise an increased expression relative to its wild type cell of at least one further enzyme selected from the group consisting of E_1 to E_7 and E_9 to E_{11}, wherein E_1 is an alcohol dehydrogenase (adh), E_2 is an acetaldehyde dehydrogenase (ald), E_3 is an acetoacetyl-CoA thiolase (thl), E_4 is a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5 is a 3-hydroxybutyryl-CoA dehydratase (crt), E_6 is a butyryl-CoA dehydrogenase (bcd), E_7 is an electron transfer flavoprotein subunit (etf), E_9 is an acetate kinase (ack), E_{11} is a transhydrogenase and E_{12} is a trans-2-enoyl-CoA reductase or crotonyl-CoA reductase.

3. The cell according to either claim 1 or 2, wherein E_8 comprises 60 % sequence identity with SEQ ID NO: 1.

4. The cell according to any one of the preceding claims, wherein E_8 is from *Clostridium kluyveri*.

5. The cell according to any one of the preceding claims, wherein the cell is genetically modified to comprise an increased expression relative to its wild type cell of the enzymes E_3, an acetoacetyl- CoA thiolase (thl), E_4, a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5, a 3-hydroxybutyryl-CoA dehydratase (crt) and E_6, a butyryl-CoA dehydrogenase (bcd).

6. The cell according to any one of claims 1 to 4, wherein the cell is genetically modified to comprise an increased expression relative to its wild type cell of the enzymes E_3, an acetoacetyl- CoA thiolase (thl), E_4, a 3-hydroxybutyryl-CoA dehydrogenase (hbd), and E_5, a 3-hydroxybutyryl-CoA dehydratase (crt).

7. The cell according to any one of claims 1 to 4, wherein the cell is genetically modified to comprise an increased expression relative to its wild type cell of the enzymes E_3, an acetoacetyl- CoA thiolase (thl), E_4, a 3-hydroxybutyryl-CoA dehydrogenase (hbd), E_5, a 3-hydroxybutyryl-CoA dehydratase (crt) E_6, a butyryl-CoA dehydrogenase (bcd) and E_7 is an electron transfer flavoprotein subunit (etf).

8. The cell according to any one of claims 5 to 7, wherein the cell is further genetically modified to comprise an increased expression relative to its wild type cell of at least one of the enzymes selected from the group consisting of E_1, an alcohol dehydrogenase (adh) and the enzyme E_{12}, trans-2-enoyl-CoA reductase or crotonyl-CoA reductase.

9. The cell according to any one of claims 2 to 8, wherein the enzymes
   - E_1 is selected from the group consisting of *C. acetobutylicum* and *E. coli* and/or E_1 comprises 60 % sequence identity with SEQ ID NO: 18 or SEQ ID NO: 19;
   - E_3 is from *C. acetobutylicum* and/or E_3 comprises 60 % sequence identity with SEQ ID NO: 2;
   - E_4 is from *Clostridium kluyveri* and/or E_4 comprises 60 % sequence identity with SEQ ID NO: 3;
   - E_5 is from *Clostridium kluyveri* and/or E_5 comprises 60 % sequence identity with SEQ ID NO: 4;
   - E_6 is selected from the group consisting of *Clostridium kluyveri*, and *C. acetobutylicum* and/or E_6 comprises 60 % sequence identity with at least one amino acid sequence selected from the group consisting of SEQ ID NOs: 5- 7;
   - E_7 is selected from the group consisting of *Clostridium kluyveri*, and *C. acetobutylicum*, and/or E_7 comprises 60 % sequence identity with an amino acid sequence selected from the group consisting of SEQ ID NOs: 8- 13;
   - E_{12} is selected from the group consisting of *Treponema denticola*, *Euglena gracilis*, *Caenorhabditis elegans*, and *Streptomyces collinus* and/or E_{12} comprises 60 % sequence identity with an amino acid sequence selected from the group consisting of SEQ ID NO: 15- 17.

10. The cell according to any one of the preceding claims, wherein the acetogenic microbial cell is selected from the group consisting of *Acetoanaerobium notera (ATCC 35199), Acetonema longum (DSM 6540), Acetobacterium carbinolicum (DSM 2925), Acetobacterium malicum (DSM 4132), Acetobacterium species no. 446, Acetobacterium wieringae (DSM 1911), Acetobacterium woodii (DSM 1030), Alkalibaculum bacchi (DSM 22112), Archaeoglobus fulgidus (DSM 4304), Blautia producta (DSM 2950), Butyribacterium methylotrophicum (DSM 3468), Clostridium aceticum (DSM 1496), Clostridium autoethanogenum (DSM 10061, DSM 19630 and DSM 23693), Clostridium carboxidivorans (DSM 15243), Clostridium coskatii (ATCC no. PTA-10522), Clostridium drakei (ATCC BA-623), Clostridium formicoaceticum (DSM 92), Clostridium glycolicum*

(DSM 1288), Clostridium ljungdahlii (DSM 13528), Clostridium ljungdahlii C-01 (ATCC 55988), Clostridium ljungdahlii ERI-2 (ATCC 55380), Clostridium ljungdahlii O-52 (ATCC 55989), Clostridium mayombei (DSM 6539), Clostridium methoxybenzovorans (DSM 12182), Clostridium neopropionicum sp, Clostridium ragsdalei (DSM 15248), Clostridium scatologenes (DSM 757), Clostridium species ATCC 29797, Desulfotomaculum kuznetsovii (DSM 6115), Desulfotomaculum thermobezoicum subsp. thermosyntrophicum (DSM 14055), Eubacterium limosum (DSM 20543), Methanosarcina acetivorans C2A (DSM 2834), Moorella sp. HUC22-1, Moorella thermoacetica (DSM 521), Moorella thermoautotrophica (DSM 1974), Oxobacter pfennigii (DSM 322), Sporomusa aerivorans (DSM 13326), Sporomusa ovata (DSM 2662), Sporomusa silvacetica (DSM 10669), Sporomusa sphaeroides (DSM 2875), Sporomusa termitida (DSM 4440) and Thermoanaerobacter kivui (DSM 2030).

11. The cell according to any one of the preceding claims, wherein the acetogenic microbial cell is *Clostridium ljungdahlii* or *Clostridium autothenogenum*.

12. The cell according to any one of the preceding claims, wherein the higher alcohol is selected from the group consisting of 1-butanol, 2-methyl-1-butanol, isobutanol, 3-methyl-1-butanol, 1-hexanol, 1-octanol, 1-pentanol, 1-heptanol, 3-methyl-1-pentanol, 4-methyl-1-hexanol, 5-methyl-1-heptanol, 4-methyl-1-pentanol, 5-methyl-1-hexanol, 6-methyl-1-heptanol and combinations thereof.

13. A method of producing at least one higher alcohol, the method comprising
- contacting a recombinant microbial cell according to any one of claims 1 to 12 with a medium comprising a carbon source.

14. The method according to claim 13, wherein the carbon source comprises CO and/or CO_2.

15. Use of the cell according to any one of the claims 1 to 12 for the production of at least one higher alcohol.

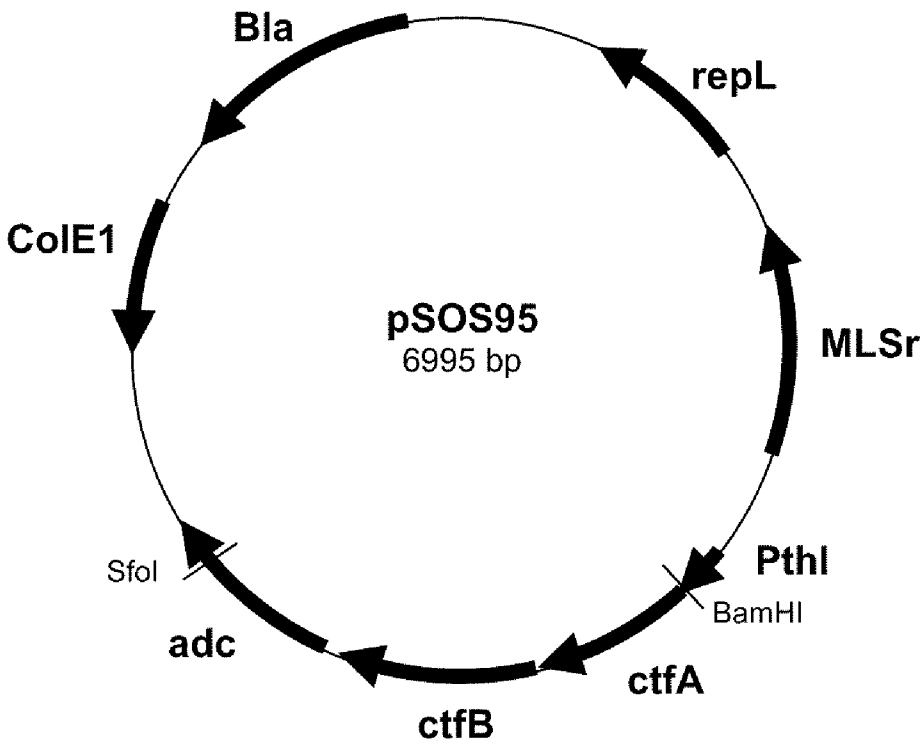

Bla
repL
ColE1
pSOS95
6995 bp
MLSr
PthI
SfoI
BamHI
adc
ctfA
ctfB

FIGURE 1

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/081202

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/10 C12P7/04
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 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, BIOSIS, EMBASE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 2012/177943 A1 (GENOMATICA INC [US]; BURGARD ANTHONY P [US]; OSTERHOUT ROBIN E [US]; S) 27 December 2012 (2012-12-27) Whole doc., in particular para. [0093], 0240, 0440, 0650, 0689, 0722]	1-15
A	WO 2009/078973 A2 (GLYCOS BIOTECHNOLOGIES INC [US]; CAMPBELL PAUL [US]; GONZALEZ RAMON [U) 25 June 2009 (2009-06-25) Whole doc., in particular p.22-23, p.36, l.1-3	1-15
A	US 2010/137641 A1 (IIDA AYA [JP]) 3 June 2010 (2010-06-03) para. [0005]	1-15

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier application or patent but published on or after the international filing date

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

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"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
23 February 2017	03/03/2017

Name and mailing address of the ISA/	Authorized officer
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1

Form PCT/ISA/210 (second sheet) (April 2005)

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

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1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2016/081202

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