(12) STANDARD PATENT (11) Application No. **AU 2010292910 B2**
(19) AUSTRALIAN PATENT OFFICE

(54) Title
Microorganisms and process for producing n-propanol

(51) International Patent Classification(s)
C12P 7/04 (2006.01)

(21) Application No: **2010292910** (22) Date of Filing: **2010.09.09**

(87) WIPO No: **WO11/029166**

(30) Priority Data

(31) Number	(32) Date	(33) Country
61/240,811	**2009.09.09**	**US**

(43) Publication Date: **2011.03.17**
(44) Accepted Journal Date: **2014.12.18**

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(56) Related Art
D1 : BR PI0604284 A (BRASKEM S.A.) 03 June 2008

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 March 2011 (17.03.2011)

PCT

(10) International Publication Number
WO 2011/029166 A1

(51) International Patent Classification:
C12P 7/04 (2006.01)

(21) International Application Number:
PCT/BR2010/000289

(22) International Filing Date:
9 September 2010 (09.09.2010)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/240,811 9 September 2009 (09.09.2009) US

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[Continued on next page]

(54) Title: MICROORGANISMS AND PROCESS FOR PRODUCING N-PROPANOL

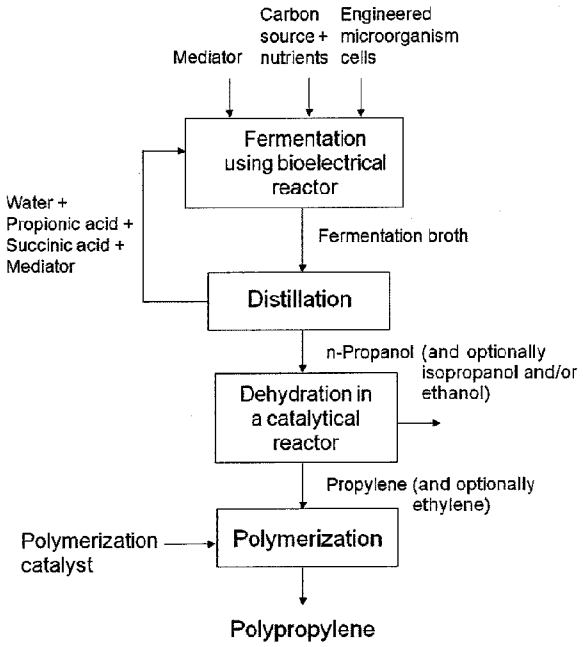

Figure 4

(57) Abstract: The invention provides fermentative methods for producing n-propanol. The methods of the invention involve providing a suitable carbon source, a microorganism expressing the dicarboxylic acid pathway, reducing equivalents, and at least one gene coding for an enzyme that catalyzes the conversion of propionate/propionyl-CoA into n-propanol. The methods further involve contacting the carbon source and reducing equivalents with the microorganism under conditions favorable for the production of n-propanol. Also provided are methods for producing propylene and polypropylene from the n-propanol and microorganisms suitable for use in the methods of the invention

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

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

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

MICROORGANISMS AND PROCESS FOR PRODUCING n-PROPANOL

FIELD OF THE INVENTION

The present invention relates to a process of bioconverting a biobased substrate (such as sugarcane juice, hydrolyzed starch, hydrolyzed cellulose or glycerol) into n-propanol using genetically modified microorganisms combined with a process for supplying reducing equivalents in the form of NAD(P)H during fermentation. The biobased n-propanol thus obtained could be dehydrated to propylene and polymerized to polypropylene to yield a bioplastic.

BACKGROUND OF THE INVENTION

n-Propanol (1-propanol, primary propyl alcohol, propan-1-ol) is a non-hazardous solvent that is freely miscible with water and other common solvents, with numerous applications in industry, such as printing inks, coatings, cleaners, adhesives, herbicides, insecticides, pharmaceuticals, de-icing fluids and as a chemical intermediate for the production of esters, propylamines, halides and thermoplastic resins. The use of n-propanol in fuel blends has also been suggested (U.S. Pat. No. 6,129,773), as this alcohol has the same capacity of ethanol to be used to increase as an antiknock additive and increase the octane number of gasoline according to Barannik V. P. et al. 2005, Chemistry and Technology of Fuels and Oils 41(6): 452-455.

n-Propanol is one of the main constituents of "fusel oils" or "potato oils", which are the higher-order alcohols by-products of ethanol fermentation by the yeast *Saccharomyces cerevisiae* (Hazelwood et al. 2008. The Ehrlich Pathway for Fusel Alcohol Production: a Century of Research on *Saccharomyces cerevisiae* Metabolism. Applied and Environmental Microbiology 74(8): 2259-2266). In the past, n-Propanol was obtained by fractional distillation of fusel oil, but nowadays it is manufactured from fossil feedstocks in a two-stage process known as Oxo Process, comprising ethylene hydroformylation at 80-120°C and 2.0 MPa in the presence of cobalt or rhodium carbonyl followed by hydrogenation of the resulting propionaldehyde on a copper-chromium, nickel-chromium or porous cobalt catalyst (U.S. Pat. No. 4,263,449 and U.S. Pat. No 5,866,725).

Worldwide interest in organic compounds produced from renewable feedstocks has increased considerably in recent years, especially for compounds that can be used as fuels or as bulk chemicals for the petrochemical industry. The latter are particularly interesting, since these compounds could be fixed in highly durable materials that can be recycled, thus effectively mitigating atmospheric CO_2 (Rincones et al. 2009. The golden bridge for nature: the new biology applied to bioplastics. Polymer Reviews 49: 85-106). Thus, the use of the chemical products obtained from renewable feedstocks is becoming increasingly accepted and widespread as a viable alternative aiming at decreasing our society's dependence on fossil carbon sources. Products obtained from green sources can be certified as to their renewable carbon content

according to the methodology described by the technical norm ASTM D 6866-06: "Standard Test Methods for Determining the Biobased Content of Natural Range Materials Using Radiocarbon and Isotope Ratio Mass Spectrometry Analysis".

The production of short-chain organic solvents (mainly reduced alcohols) through microorganism fermentation has been extensively studied. The most dramatic example is the production of ethanol as a commodity chemical, which is a major industrial process reaching nearly 90 million m^3/year and occurring by the fermentation of renewable carbon sources (mainly cornstarch and sugarcane juice) by the yeast *Saccharomyces cerevisiae*. This process is extremely efficient and has been refined to the point where ethanol distilled from the fermentation broth is obtained at 90-95% of the theoretical yield. The ethanol thus produced is used as an industrial solvent, as the main additive for gasoline in fuel blends and, in Brazil, is used as the sole fuel for small vehicles. Another use of a biobased ethanol is the manufacture of bio-ethylene to be used as a monomer in the polyethylene manufacture, through a dehydration reaction as described by Morschbacker A. L. 2009, Bio-Ethanol Based Ethylene, Journal of Macromolecular Science, Part C: Polymer Reviews, 49:79–84.

Other well-known examples of solvent production by fermentation are the Acetone-Butanol-Ethanol (ABE) and the Isopropanol-Butanol-Ethanol (IBE) fermentations performed by some bacterial species of the genus *Clostridium*, yielding more than 35% by weight of the solvent mixture (U.S. Pat. No.

5,192,673). In addition, fermentation of 2,3-butanediol from carbohydrates by enteric bacteria of the genera *Klebsiella* and *Enterobacter* yields up to 47% by weight (Ji et al., 2009, Bioresource Technology 100:3410-3414). A recent success is the fermentative production of 1,3 propanediol from glucose in a single microorganism with high yield (35% w/w) and titer (129 g/L) (U.S. Pat. No. 7,169,588 B2; U.S. Pat. No. 7,067,300 B2; U.S. Pat. No. 5,686,276). The establishment of an industrial process for the production of this low cost biobased 1,3 propanediol from cornstarch and its subsequent use in the production of the polyester fiber polypropylene terephthalate constitutes one of the most significant advances to date in the production of biopolymers.

n-Propanol and isopropanol are interesting biobased intermediates for the production of propylene by dehydration and its subsequent polymerization into polypropylene. Up to date, the best yield for isopropanol has been obtained through a genetically engineered strain of *E. coli* containing genes coding for the enzymes of the acetone production pathway of *Clostridium acetobutylicum* plus the secondary alcohol dehydrogenase of the isopropanol production pathway of *Clostridium beijerinckii*, yielding 14% by weight of isopropanol from glucose (Int. Publ. No. WO 2008/131286 A1). This yield corresponds to approximately 50% of the theoretical maximum, since the proposed pathway for the production of isopropanol comprises the following conversions: a) cleavage of glucose into two molecules of pyruvate through glycolysis; b) oxidative decarboxylation of the molecules of pyruvate into acetyl-CoA; c) condensation of the two molecules of acetyl-CoA into acetoacetyl-CoA and CoA; d) conversion of

acetoacetyl-CoA and acetate into acetoacetate and acetyl-CoA; e) decarboxylation of acetoacetate into acetone; and f) reduction of acetone into isopropanol. As can be seen from the conversions above, involving three decarboxylation steps of intermediate metabolites, the maximum theoretical yield of isopropanol through this pathway is 1 mol of isopropanol from each mol of glucose (0.33 g/g).

In nature, microorganisms produce n-propanol in low amounts and as by-product of the main fermentation products. In the yeast *Saccharomyces cerevisiae*, n-propanol is produced as the degradation product of the amino acid 2-ketobutyrate through the Ehrlich pathway (Hazelwood et al., 2008, Appl. Env. Microbiol. 74:2259-2266). This pathway has been optimized in genetically engineered strains of the model microorganism *Escherichia coli* for the production of n-butanol and n-propanol from glucose, but with extremely low yields (4% by weight) (Shen & Liao, 2008, Met. Eng. 10:312-320). The production of iso-propanol or n-propanol via the degradation of the amino acid 2-ketobutyrate, from glucose through this pathway using genetically engineered microorganisms is also disclosed in a recent document, but similarly indicating very low yields (Intl. Pub. No. WO 2009/103026 A1). In bacterial species of the genus *Propionibacterium*, n-propanol has been observed as the by-product of propionic acid fermentation from glycerol, which is a more reduced substrate when compared to glucose or sucrose, but with low yields (4% by weight); no n-propanol is obtained when glucose, sucrose or lactate are used as substrates in the fermentation using *P. acidipropionici* American Type Culture Collection

(ATCC) No. 25562 (Barbirato et al., 1997, Appl. Microbiol. Biotechnol. 47: 441-446). Thus, the prior art fails to show fermentation processes for the production of n-propanol with high yields by fermentation of carbohydrates.

Propionic acid fermentation by several bacterial species, such as *Selenomonas ruminantium*, *Propionigenium* spp. and *Propionibacterium* spp. has been extensively studied. Propionic acid bacteria of the genus *Propionibacterium* have been the most studied due to their use in the production of cheese. These bacteria produce propionic acid as the main fermentation product from glucose and other substrates such as lactose, glycerol, and sucrose with high yields of propionic acid (65% w/w from glucose and 67% w/w from glycerol) (Suwannakham & Yang., 2005, Biotech. Bioeng 91:325-337; Barbirato et al., 1997, Appl. Microbiol. Biotechnol. 47: 441-446). The pathway for the production of propionic acid in *Propionibacterium* spp. is known as the dicarboxylic acid cycle, which begins by the trascarboxylation of pyruvate from methyl-malonyl-CoA to yield oxaloacetate followed by the subsequent transformations into malate, fumarate, succinate, succinyl-CoA and methyl-malonyl-CoA, which will be transcarboxylated to pyruvate to yield propionyl-CoA and oxaloacetate, thus closing the cycle (Boyaval and Corre, 1995, Lait 75:453-461). Therefore, no decarboxylation reactions are involved in this pathway, which would have a maximum theoretical yield of 2 mol of propionic acid for each mol of glucose (0.82 g/g). Nevertheless, the co-products acetic acid and succinic acid are usually formed in varying proportions depending on the substrate and growth conditions.

Several studies and patent applications are directed to method for increasing the yield of propionic acid, especially with regards to increase its yield in relation to co-products, such as acetic acid, and to improve the growth conditions and separation strategies ("Engineering *Propionibacterium acidipropionici* for Enhanced Propionic Acid Tolerance and Fermentation", Zhang and Yang, 2009, Biotechnology and bioengineering, in press" and "Construction and Characterization of ack Knock-Out Mutants of *Propionibacterium acidipropionici* for Enhanced Propionic Acid Fermentation", Suwannakham et al, 2006, Biotechnology and Bioengineering, Vol. 94, No. 2, June 5). However, no studies exist aiming at improving the formation of n-propanol using the propionic acid pathway as a metabolic intermediate.

No natural microorganisms are able to produce iso- or n-propanol with high yields from glucose and other sugars; in consequence, the correct combination of enzymes that would allow such bioconversion does not exist in nature. However, Holt et al. (1984, Appl. Env. Microbiol 48:1166-1170) have shown that the external supply of propionic acid to a growing culture of *Clostridium acetobutylicum* at acidic pH (5.0) yields n-propanol (50% w/w), suggesting that the alcohol/aldehyde dehydrogenase (ADH) enzymes of this bacterium are able to transform not only the organic acids it produces (butyrate and acetate) into the corresponding alcohols, but also propionate into n-propanol. However the experiments of this publication were conducted at a very low concentration and high levels of undesired by-products such as acetate,

butyrate, ethanol, butanol and acetone were obtained, thus indicating that there is still a problem to be solved in order to obtain propanol with high yields.

In addition, the metabolic pathways that lead to the production of industrially important compounds involve oxidation-reduction (redox) reactions. During fermentation, glucose is oxidized in a series of enzymatic reactions into smaller molecules with the concomitant release of energy. Since these reactions do not occur simultaneously, the electrons released are transferred from one reaction to another through universal electron carriers, such as Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP), which act as cofactors for oxidoreductase enzymes. In microbial catabolism, glucose is oxidized by enzymes using the oxidized form NAD(P)+ as cofactor and generating reducing equivalents in the form of the reduced form NAD(P)H. In order for fermentation to continue, the NAD(P)+ must be regenerated by the reduction of metabolic intermediates consuming NAD(P)H. Thus, it is very important for the microbial cell to maintain a balanced NAD(P)+/NAD(P)H ratio.

In general, reducing equivalents in the form of NAD(P)H are obtained in oxidative decarboxylation reactions, while NAD(P)+ is regenerated by the reduction of intermediates, such as the reduction of acetic acid into ethanol. As a consequence of the redox balance required for the catabolism of glucose into n-propanol, which has a lower oxidation state, this compound would be accompanied by the co-production of 2- and, possibly, 4-carbon compounds.

This fact suggests that low yields should be observed for the production of n-propanol, even when genetically engineered microorganisms are to be used due to the requirement of more reducing equivalents in the form of NAD(P)H than can be formed from the oxidation of glucose. Thus, this situation for n-propanol contrasts with the fermentative production of isopropanol from glucose disclosed in Int. Publ. No. WO 2008/131286 A1, in which the product results by a series of conversions involving three oxidative decarboxylation reactions from glucose, which generate enough reducing equivalents for the reduction of acetone into isopropanol, but at the expense of mass released as CO_2.

Previous studies have reported the use of electrical stimulation inside bioreactors in order to drive the redox balance to obtain different end-products. The application of an electrical current in *Clostridium acetobutylicum*, *Clostridium thermocellum* and *Saccharomyces cerevisiae* has been reported, resulting in a significant increase in ethanol production (Pequin et. al. 1994, Biotechnology letters 16(3): 269-274; Shin et al 2002, Appl. Microbial. Biotechnol. 58: 476-481). Also, there are works reporting the change in the end-products of fermentation by *Propionibacterium* spp. using electrical stimulation and mediators. Emde and Schink (D.E. Pat. No. 4,024,937-C1) enhanced propionate formation during glucose fermentation of *Propionibacterium freudenreichi* using a three-electrode system and cobalt sepulchrate as mediator. Results showed that this process increases propionate molar yield over acetate from 73 to 97%, respectively. In a similar work, Schuppert et al. (Appl. Microbiol. Biotechnol, 1992, 37:549-553) used thye three-electrode system and

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cobalt sepulchrate to shift the end-product ratio of *P. acidipropionici*. In this case, propionate was produced exclusively, thus increasing final yields and facilitating the downstream process. Finally, in a recent work, the end-product product profile of glucose fermentation by *P. freudenreichi* was modified by electrical stimulation without adding exogenous artificial mediators (Wang et. al. 2008, Biotechnol. Bioeng 101: 579-586). In this work, the authors reported that the molecule 1,4-dihydroxy-2-naphthoic acid produced and secreted by *P. freudenreichi* acts as the mediator and no improvement of the reaction was observed when other mediators were added. Overall, these results show that the metabolism and end-product profile of glucose fermentation by *Propionibacterium* spp. can be manipulated through the use of bioelectrical reactors.

The biobased n-propanol thus produced could be further used for the production of a bioplastic through its dehydration to propylene and its polymerization to polypropylene in a cost-effective manner.

Propylene is a chemical compound that is widely used to synthesize a wide range of petrochemical products. For instance, this olefin is the raw material used for the production of polypropylene, their copolymers and other chemicals such as acrylonitrile, acrylic acid, epichloridrine and acetone.

Propylene demand is growing faster than ethylene demand, mainly due to the growth of market demand for polypropylene. Propylene is polymerized to produce thermoplastics resins for innumerous applications such as rigid or flexible packaging materials, blow molding and injection molding.

Global interest for renewable material has been growing intensively in the last years especially in plastics production. Some available biopolymers are poly-(lactic acid) and poly-hydroxybutyrate which can be obtained from sugar sources. Another recent alternative is "green" polyethylene which is produced from sugarcane ethanol. These products generate no fossil carbon when incinerated.

Propylene is obtained mainly as a by-product of catalytical or thermal oil cracking, or as a co-product of ethylene production from natural gas. (Propylene, Jamie G. Lacson, CEH Marketing Research Report-2004, Chemical Economics Handbook-SRI International). The use of alternative routes for the production of propylene has been continuously evaluated using a wide range of renewable raw materials ("Green Propylene", Nexant, January 2009). These routes include propylene production by dimerization of ethylene to yield butylene followed by metathesis with additional ethylene to produce propylene. Another route is biobutanol production by sugar fermentation followed by dehydration and methatesis with ethylene. Some thermal routes are also being evaluated such as gasification of biomass to produce a syngas followed by

synthesis of methanol, which will then produce green propylene via methanol-to-olefin technology.

Propylene production by iso-propanol dehydration has been well-described in document EP00498573B1, wherein all examples show propylene selectivity higher than 90% with high conversions. Dehydration of n-propanol has also been studied in the following articles: "Mechanism and Kinetics of the Acid-Catalyzed Dehydration of 1- and iso-propanol in Hot Compressed Liquid Water" (Antal, M et al., Ind. Eng. Chem. Res. 1998, 37, 3820-3829) and "Fischer-Tropsch Aqueous Phase Refining by Catalytic Alcohol Dehydration" (Nel, R. et al., Ind. Eng. Chem. Res. 2007, 46, 3558-3565). The reported yield is higher than 90%.

BRIEF SUMMARY OF THE INVENTION

In spite of the innumerous developments achieved to date, there are still no teachings in the prior art that provide any description relative to the production of n-propanol with high yields through propionic acid metabolic pathway using genetically modified microorganisms combined with a process for supplying reducing equivalents in the form of NAD(P)H during fermentation of renewable carbon sources. The biobased n-propanol thus obtained could be dehydrated to propylene and polymerized to yield biobased polypropylenes. This thus produced bio-polypropylene, contrary to the majority of known biopolymers, have a low production cost and evidence clearly adequate properties for an immense variety of applications.

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The present invention as claimed herein is described in the following items 1 to 23:

1. A method for producing n-propanol comprising:

(a) providing a suitable carbon source for fermentation by a microorganism expressing the dicarboxylic acid pathway, reducing equivalents in the form of NAD(P)H, and at least one gene coding for an enzyme that catalyzes the conversion of propionate/propionyl-CoA into n-propanol;

(b) contacting the carbon source and reducing equivalents in the form of NAD(P)H with the microorganism under conditions favorable for the production of n-propanol by the microorganism; whereby a fermentation broth is produced; and

(c) recovering n-propanol from the fermentation broth.

2. The method of item 1, wherein the microorganism has been genetically engineered to express one or more enzymes, whereby the microorganism is capable of converting propionate/propionyl-CoA to n-propanol.

3. The method of item 2, wherein the microorganism is selected from the group consisting of: Propionigenium spp., Propionispira arboris, Propionibacterium spp., and Selenomonas.

4. The method of item 2, wherein the enzyme is selected from the group consisting of:
aldehyde dehydrogenases that are capable of using propionic acid as a substrate;
aldehyde dehydrogenases that are capable of using an acyl-CoA intermediateas a substrate;
alcohol dehydrogenases that catalyze the conversion of an aldehyde to its corresponding primary alcohol; and
multifunctional enzymes that posses both aldehyde/alcohol dehydrogenase domains.

5. The method of item 4, wherein the enzyme has alcohol dehydrogenase protein domain with e-value threshold below 1e-2.

6. The method of item 4, wherein the enzyme has aldehyde dehydrogenase protein domain with e-value threshold below 1e-2.

7. The method of item 4, wherein the aldehyde dehydrogenases are capable of using propionic acid as a substrate are selected from the group consisting of: Mus musculus (GenBank Accession No. AC162458.4); Clostridium botulinum A str. American Type Culture Collection (ATCC) No. 3502 (GenBank Accession No. AM412317.1); and Saccharomyces cerevisiae (GenBank Accession No. EU255273.1).

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8. The method of item 4, wherein the aldehyde dehydrogenases that are capable of using acyl-CoA intermediate as a substrate are selected from the group consisting of: Rhodococcus opacus (GenBank Accession No. AP011115.1); Entamoeba dispar (GenBank Accession No. DS548207.1); and Lactobacillus reuteri (GenBank Accession No. ACHG01000187.1).

9. The method of item 4, wherein the alcohol dehydrogenases that catalyze the conversion of an aldehyde to its corresponding primary alcohol are selected from the group consisting of: Aspergillus niger (GenBank Accession No. AM270229.1); Streptococcus pneumoniae Taiwan19F-14 (GenBank Accession No. CP000921.1); and Salmonella enterica (GenBank Accession No. CP001127.1).

10. The method of item 4, wherein the multifunctional enzymes that posses both aldehyde/alcohol dehydrogenase domains are selected from the group consisting of: Lactobacillus sakei (GenBank Accession No. CR936503.1); Giardia intestinalis (GenBank Accession No. U93353.1); Shewanella amazonensis (GenBank Accession No. CP000507.1); Thermosynechococcus elongatus (GenBank Accession No. BA000039.2); Clostridium acetobutylicum (GenBank Accession No. AE001438.3); and Clostridium carboxidivorans ATCC No. BAA-624T (GenBank Accession No. ACVI01000101.1).

11. The method of item 1, wherein the fermentation broth further comprises ethanol and/or isopropanol.

12. The method of item 11, wherein ethanol and/or isopropanol are recovered from fermentation broth.

13. The method of item 1, wherein the microorganism has the expression of its gene encoding for an enzyme acetate kinase (E.C. 2.7.2.1) altered so as to diminish its activity.

14. The method of any one of items 1-13, wherein the reducing equivalents comprise NAD(P)H.

15. The method of item 14, wherein the NAD(P)+ is reduced to NAD(P)H comprising the use of electrodes and a mediator molecule, an overpressure of H_2, or a microorganism expressing a NAD^+-dependent formate dehydrogenase in the presence of formate.

16. The method of item 14, further comprising contacting the fermentation broth with electrodes and a mediator molecule.

17. The method of item 16, wherein mediator molecules are benzyl viologen, methyl viologen, anthraquinone 2,6-disulfonic acid, neutral red, cobalt sepulchrate, 1,4 dihydroxy-2-naphthoic acid (DHNA) and flavins.

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18. The method of item 16, wherein mediator molecules are compounds present in yeast extract and Propionibacterium spp. extract.

19. The method of any one of items 1-18, wherein the carbon source is sugarcane juice, sugarcane molasses, hydrolyzed starch, hydrolyzed ligno-cellulosic materials, glucose, sucrose, fructose, lactate, lactose, xylose or glycerol in any form or a mixture thereof.

20. A microorganism for using in the method as defined in any one of items 1 to 19.

21. A method of item 1 further comprising:
dehydrating the n-propanol produced by the method as defined in any one of items 1 to 19 to produce propylene.

22. A method of item 1 further comprising:
dehydrating in the same reactor n-propanol and isopropanol and/or ethanol produced by the method as defined in any of items 1 to 19 to produce propylene.

23. A method of item 1 further comprising:
polymerizing the propylene produced by the method as defined in any one of items 21 and 22 to produce polypropylene.

24. n-propanol produced by the method of any one of items 1 to 23.

The present invention provides an improved process for the bioconversion of a carbon source to n-propanol, and eventually additionally to iso-propanol and/or ethanol, with high yield by engineered microorganisms, having genes coding for the enzymes of the dicarboxylic acid pathway of propionate formation and at least one gene coding for an enzyme that catalyzes the conversion propionate/propionyl-CoA into n-propanol in the presence of externally supplied reducing equivalents in the form of NAD(P)H, either through the use of electrodes and a mediator molecule, or through the use of an overpressure of H_2, or through the use of a pathway, native or engineered, expressing a NAD^+-dependent formate dehydrogenase and the addition of formate to the culture medium.

The present invention provides methods for the biological production of n-propanol with high yields by microorganisms from an inexpensive carbon substrate such as glucose, sucrose, other sugars, glycerol, waste materials or a mixed of carbon sources, using the whole cell as catalyst and establishing an integrated process that may be upscaled to industry in a cost-effective manner. To this end, the present invention further provides engineered microorganisms capable of producing propionate/propionyl-CoA with high yields through the

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dicarboxylic acid cycle and that express the polypeptides corresponding to alcohol/aldehyde dehydrogenase enzymes capable of reducing propionate/propionyl-CoA into n-propanol.

The present invention provides a high yielding process for the fermentative production of n-propanol. In one embodiment of the invention, the processes or methods involve a balanced energy reaction in the conversion of glucose or other carbohydrates into n-propanol.

The present invention also comprises the product of the above process.

In certain embodiments, microorganisms that contain a native dicarboxylic acid cycle can be engineered to catalyze the further conversion into n-propanol by the addition of at least one heterologous gene coding for an aldehyde/alcohol dehydrogenase enzymes.

In certain embodiments, a suitable host with a native pathway for the conversion of propionyl-CoA/propionate into n-propanol is engineered for expression of the dicarboxylic acid cycle, where the expression of at least one enzyme is heterologous or has its expression pattern modified.

In certain embodiments, a suitable host, for which genetic manipulation techniques are well-established, is engineered for expression of the dicarboxylic acid cycle and the enzymes required for the reduction of propionate/propionyl-CoA into n-propanol, where the expression of at least one enzyme is heterologous or has its expression pattern altered.

In certain embodiments, microorganisms that contain a native or a modified dicarboxylic acid cycle and that contains a native or a modified

pathway for the conversion of propionyl-CoA/propionate into n-propanol can be further engineered to express the enzymes that catalyze the conversion of acetyl-CoA into isopropanol. This isopropanol would be used together with n-propanol for propylene synthesis by dehydration.

In certain embodiments, microorganisms that contain a native or a modified dicarboxylic acid cycle, a native or a modified pathway for the conversion of propionyl-CoA/propionate into n-propanol and a native or modified pathway for the conversion of acetyl-CoA into isopropanol may be engineered to present an altered expression (over or underexpression) of a defective enzyme involved in the acetic acid synthesis from acetyl-CoA, which would increase isopropanol synthesis. This isopropanol would be used together with n-propanol for propylene synthesis by dehydration.

The preferred method of externally supplying electrons is through the use of electrodes and a mediator molecule, which can be naturally produced by the microorganism or externally supplied in the culture medium.

In certain embodiments a fermentation media containing sugarcane juice as carbon source is preferentially used and a nitrogen source consisting of either yeast extract or N_2 is preferentially used. However, other combinations may be used and those skilled in the art recognize that these combinations are also considered within the scope of this invention.

In certain embodiments the culture media is supplied with pantothenic acid with the object of increasing yield and productivity. This pantothenic acid may be added in pure form or as a crude extract.

In certain embodiments, the n-propanol thus produced will be further dehydrated into propylene and polymerized to polypropylene to yield a bioplastic.

BRIEF DESCRIPTION OF THE FIGURES

Having thus described the invention in general terms, reference will now be made to the accompanying drawings, which are not necessarily drawn to scale, and wherein:

Figure 1. The production of propionic acid from glucose by several species of bacteria, such as *Propionigenium* spp., *Propionispira arboris*, *Propionibacterium* spp. and *Selenomonas ruminantium*, can be accomplished by the following series of steps. This series is representative of a number of pathways known to those skilled in the art. Glucose is converted in a series of steps by enzymes of glycolytic pathway to pyruvate. The pyruvate may be converted to Acetyl-Coa and then to acetate or to propionic acid through the dicarboxylic acid cycle. It has been reported that some species of the genus *Propionibacterium* may produce n-propanol when a reduced substrate such as glycerol is used; however, the pathway for the production of n-propanol has not

been described. The possible pathways and co-factors for the production of n-propanol are highlighted in gray.

Figure 2. The production of alcohols by species of *Clostridium* may be described by the following steps. Glucose is converted in a series of steps by enzymes of glycolytic pathway to pyruvate. From pyruvate may be formed lactate or acetyl-CoA which is the precursor of acetate and ethanol. In addition, acetyl-CoA can be converted to acetoacetyl-CoA and then to acetone, which is finally reduced to isopropanol. Another possibility is the conversion of acetoacetyl-CoA in butyryl-Coa through a series of steps known by those skilled in the art. The butyryl-CoA may be converted to either butanol or butyrate.

Figure 3. Schematic representation of a stirred-tank bioelectrical reactor with a three-electrode system.

Figure 4. Schematic representation of the integrated processes wherein an engineered microorganism is used to produce n-propanol in the presence of reducing equivalents externally supplied through the use of a bioelectrical reactor. The resulting n-propanol is distilled and dehydrated in a catalytic reactor in order to produce polymer grade propylene, which is then subjected to a polymerization step to produce polypropylene.

Figure 5. Schematic representation of expression vector pBK1T1 containing a synthetic construct designed to express an aldehyde alcohol

dehydrogenase from *Clostridium carboxidivorans* in *Propionibacterium acidipropionici*. This bifunctional enzyme catalyzes the conversion of propionyl-CoA into n-propanol.

Figure 6. Schematic representation of expression vector pBK1T2 containing a synthetic construct designed to express an aldehyde alcohol dehydrogenase from *Clostridium acetobutylicum* in *Propionibacterium acidipropionici*. This bifunctional enzyme catalyzes the conversion of propionyl-CoA into n-propanol.

Figure 7. Thiostrepton resistance positive selection marker cassette for *Propionibacterium acidipropionici*, synthetic construct. NcoI site (underlined), controlling regions (bold) and initiation and stop codons of the resistance gene ORF (in parenthesis) are highlighted.

Figure 8. Expression cassette for heterologous bifunctional aldehyde/alcohol dehydrogenase of *Clostridium carboxidivorans* in *Propionibacterium acidipropionici*, synthetic construct. *Xba*I and *Hind*III sites (underlined), controlling regions (bold) and initiation and stop codons of the gene ORF (in parenthesis) are highlighted.

Figure 9. Expression cassette for heterologous bifunctional aldehyde/alcohol dehydrogenase of *Clostridium acetobutylicum* in *Propionibacterium acidipropionici*, synthetic construct. *Xba*I and *Hind*III sites

(underlined), controlling regions (bold) and initiation and stop codons of the gene ORF (in parenthesis) are highlighted.

Figure 10. Expression plasmid pBK1T1, synthetic construct. A schematic view of the plasmid vector is presented in Figure 5.

Figure 11. Expression plasmid pBK1T2, synthetic construct. A schematic view of the plasmid vector is presented in Figure 6.

Figure 12. HPLC spectra obtained after 36 hrs of (a) control fermentation and (b) fermentation supplemented with 1.0 mM cobalt sepulchrate as a mediator molecule. Chromatogram (a): Sucrose (11.437 min); succinic acid (17.782 min); acetic acid (22.610 min); propionic acid (26.515 min); Chromatogram (b): Sucrose (11.420 min); succinic acid (17.714 min); acetic acid (22.586 min); propionic acid (26.493 min); n-propanol (39.199). The undefined peaks are corresponding to compounds from yeast extract.

Figure 13. GC-MS chromatogram corresponding to fermentation using 1.0 mM cobalt sepulchrate. The intensity of the peaks are not corresponding to the real concentration of the products in the fermentation medium.

Figure 14. Time course for cell growth of a control fermentation and a fermentation supplemented with 1.0 mM cobalt sepulchrate as a mediator molecule

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a novel integrated approach that takes advantage of the high propionic acid fermentation yields from renewable feedstocks through the dicarboxylic acid cycle, the aldehyde/alcohol dehydrogenase genes of alcohol-producing microbial species, such as clostridia, yeasts and enteric bacteria, and the external supply of reducing equivalents in the form of NAD(P)H in order to produce n-propanol from fermentation with high yield. Therefore, the present invention provides a novel and inventive integrated process using microorganisms combined with the use of externally supplied reducing equivalents for the production of n-propanol with high yield, and as an option, a complementary production of iso-propanol and/or ethanol with the aim to maximize the carbon yield in molecules of interest.

A process is disclosed herein for the bioconversion of a carbon source to n-propanol with high yield in engineered microorganisms expressing genes coding for the enzymes of the dicarboxylic acid pathway of propionate formation and at least one gene coding for an enzyme that catalyzes the conversion propionate/propionyl-CoA into n-propanol in the presence of externally supplied reducing equivalents in the form of NAD(P)H, either through the use of electrodes and a mediator molecule, or through the use of an overpressure of H_2, or through the use of a pathway, native or engineered, expressing a NAD^+-dependent formate dehydrogenase and the addition of formate to the culture medium.

The term "microorganism" as used herein includes prokaryotic and eukaryotic species from the domains Archaea, Bacteria and Eukarya, the latter limited to filamentous fungi, yeasts, algae, protozoa or higher Protista. "Cell", "microbial cell" or "microbe" are used interchangeably with microorganism. The term "organism" as used herein refers to any self-replicating entity.

The term "carbon source" generally refers to a substrate or compound suitable for sustaining microorganism growth. Carbon sources may be in various forms, including, but not limited to polymers, carbohydrates, alcohols, acids, aldehydes, ketones, amino acids, peptides, etc. For example, these may include monosaccharides (such as glucose, fructose, and xylose), oligosaccharides (i.e. sucrose, lactose), polysaccharides (i.e. starch, cellulose, hemicellulose), lignocellulosic materials, fatty acids, succinate, lactate, acetate, glycerol, etc. or a mixture thereof. The carbon source may be a product of photosynthesis, such as glucose or cellulose. Monosaccharides used as carbon sources may be the product of hydrolysis of polysaccharides, such as acid or enzymatic hydrolysates of cellulose, starch and pectin. The term "energy source" may be used here interchangeably with carbon source since in chemoorganotrophic metabolism the carbon source is used both as an electron donor during catabolism and as a carbon source during cell growth.

The term "nucleic acid" refers to an organic polymer composed by more than two monomers of nucleotides of nucleosides, including, but not limited to, single-stranded or double-stranded, sense or anti-sense, deoxyribonucleic acid

(DNA) of any length, and, where appropriate, single-stranded or double-stranded, sense or anti-sense, ribonucleic acid (RNA) of any length. The term "nucleotide" refers to any or several compounds that consist of a ribose or deoxyribose sugar joined to a purine or pyrimidine base and to a phosphate group, and that are the basic structural units of nucleic acids. The term "nucleoside' refers to a compound (as guanosine or adenosine) that consists of a purine or pyrimidine base combined with deoxyribose or ribose and is found especially in nucleic acids. A nucleic acid containing from three to 200 nucleotides may also called "oligonucleotide".

The term "protein" or "polypeptide" is used here to indicate an organic polymer composed of two or more amino acid monomers and/or analogs thereof. As used herein, the term "amino acid" refers to any natural and/or synthetic amino acids. Accordingly, the term polypeptide includes amino acid polymers of any length, including full length proteins and peptides, as well as analogs and fragments thereof.

The term "enzyme" refers to any substance that catalyzes of promotes any chemical or biochemical reaction. Enzymes are totally or partially composed by polypeptides, but can include molecules composed of a different molecule, including nucleic acids.

The term "domain", "protein domain" or "enzyme domain" refers to a distinct structural unit of a protein or polypeptide, where a specific reaction

takes place or where a specific function can be attributed. A protein or enzyme may possess one or more domains that may have separate functions and may fold as independent compact units.

The term "E-value" or "expected value" refers to a parameter that describes the number of hits one can expect to see by chance when searching a Conserved Domain Database from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/cdd).

The term "pathway" or "metabolic pathway" is used here to refer to a biological process including one or more enzymatically controlled chemical reactions by which a substrate is converted into a product. Accordingly, a pathway for the convertion of a carbon source into n-propanol is a biological process including one or more enzymatically controlled reactions by which the carbon source is converted to n-propanol. A "heterologous pathway" refers to a pathway in which at least one or more chemical reactions of the pathway is catalyzed by at least one heterologous enzyme. On the other hand, a "native pathway" refers to a pathway wherein all chemical reactions are catalyzed by a native enzyme.

The term "reducing equivalents in the form of NAD(P)H", refers to the coenzymes nicotinamine adenine dinucleotide (NAD) or nicotinamine adenine dinucleotide phosphate (NADP) in their reduced forms. In the reduced forms, these coenzymes are able to donate their electrons, or reducing equivalents, for

reduction reactions catalyzed by enzymes that use these coenzymes as co-factors, such as the enzymes of the class of oxidoreductases.

The term "microorganism extract" or "yeast extract" or "*Propionibacterium* spp. extract" are used here to refer a water-soluble portion of autolyzed microorganism cell culture, like yeast or *Propionibacterium* spp. The microorganism extract is typically prepared by growing the microorganism in a carbohydrate-rich medium. After that the microorganism is harvested, washed, resuspended in water and submit to an autolysis process (self-digestion of the cell wall using the enzymes). The microorganism extract is the total soluble portion of this autolytic action.

The terms "heterologous" or "exogenous" are used here to refer to enzymes and nucleic acids that are expressed in other organism different than that from which they were originated, independently on the level of expression, which can be lower, equal, or higher than the level of expression of the molecule found in the native microorganism.

The terms "endogenous" or "native" are used here to refer to enzymes and nucleic acids that are expressed in the organism in which they are found in nature, independently of their level of expression.

The terms "host" or "host cells" are used here interchangeably to refer to microorganisms, native or wild type, eukaryotic or prokaryotic, that can be

engineered for the conversion of a carbon source to n-propanol. The terms host and host cell refers not only to the particular subject cell but also to the progeny or potential progeny of such cell, carrying the genetic modifications. Since certain modifications may occur in this progeny due to mutation or environmental difference, it is possible that such progeny may not be identical to the parent cell, but are still included within the scope of the term as used here.

The term "yield" as used herein refers to the amount of product obtained from the amount of substrate in g/g.

The microorganisms disclosed herein can be wild-type microorganisms or engineered using genetic engineering techniques to provide microorganisms that utilize heterologously or endogenously expressed enzymes to produce n-propanol and, optionally, iso-propanol and/or ethanol at high carbon yield. The terms "modified" or "modification" as used here refer to the state of a metabolic pathway being altered in which at least one step or process in the pathway is either increased (upregulated) or decreased (downregulated), such as an activity of an enzyme or expression of a nucleic acid. In a specific embodiment, the modification is the result of an alteration in a nucleic acid sequence which encodes as enzyme in the pathway, an alteration in expression of a nucleic acid sequence which encodes an enzyme in the pathway, or an alteration in translation or proteolysis of an enzyme in the pathway (i.e. alcohol dehydrogenase), or a combination thereof. A skilled artisan recognizes that there

are commonly used methods in the art to obtain alterations, such as by deletion or superexpression.

The term "mediator" includes any molecules with the characteristics of being lipid or water soluble, pH-independent, stable and holding a redox potential for driving the electron transfer process.

The term "electrode" includes any electrically conductive material, preferably graphite or a noble metal. One or more reference electrodes can be included in the system.

The production of propionic acid from glucose by several species of bacteria, such as *Propionigenium* spp., *Propionispira arboris*, *Propionibacterium* spp. and *Selenomonas ruminantium*, can be accomplished by the following series of steps. This series is representative of a number of pathways known to those skilled in the art. Glucose is converted in a series of steps by enzymes of glycolytic pathway to pyruvate. The pyruvate may be converted to Acetyl-CoA and then to acetate or to propionic acid through the dicarboxylic acid cycle, which may include the following conversion steps:

Conversion a) Pyruvate and Methylmalonyl-CoA to Oxaloacetate and Propionyl-CoA through the action of the enzyme methylmalonyl-CoA carboxytransferase (E.C. 2.1.3.1);

Conversion b) Oxaloacetate and NADH to Malate and NAD^+ through the action of the enzyme malate dehydrogenase (E.C. 1.1.1.37);

Conversion c) Malate to Fumarate and H_20 through the action of the enzyme fumarate hydratase (E.C. 4.2.1.2);

Conversion d) Fumarate and FPH_2 to Succinate and FP through the action of the enzyme succinate dehydrogenase (E.C. 1.3.99.1);

Conversion e) Succinate and Propionyl-CoA to Succinyl-CoA and Propionate through the action of the enzyme propionyl-CoA: succinate CoA transferase (E.C. 2.8.3.1);

Conversion f) Succinyl-CoA to (S)Methylmalonyl-CoA through the action of the enzyme methylmalonyl-CoA mutase (E.C. 5.4.99.1);

Conversion g) (S)Methylmalonyl-CoA to (R)Methylmalonyl-CoA through the action of the enzyme methylmalonyl-CoA epimerase (E.C. 5.1.99.1); and

Conversion h) (R)Methylmalonyl-CoA and Pyruvate to Propionyl-CoA and Oxaloacetate through the action of the enzyme methylmalonyl-CoA carboxytransferase (E.C. 2.1.3.1), thus closing the cycle.

Natural or recombinant microorganisms containing the genes coding for the enzymes catalyzing the conversions a, b, c, d, e, f, g and h may be isolated or constructed using techniques such as heterologous DNA insertion, differential expression or deletion of genes well known by those skilled in the art. Alternatively, any genes encoding the enzymes catalyzing the conversions a, b, c, d, e, f, g and h that are known in the art can be used in the methods disclosed herein.

In some organisms, the production of alcohols from their corresponding organic acids or acyl-CoA intermediates occurs in a two-step process through the sequential action of an aldehyde dehydrogenase and an alcohol dehydrogenase, with both steps being dependent on reducing equivalents in the form of NAD(P)H. Examples of aldehyde dehydrogenases that act on the organic acid include, but are not limited to the ones found in *Mus musculus* (GenBank Accession No. AC162458.4); *Clostridium botulinum* A str. ATCC No. 3502 (American Type Culture Collection or "ATCC", P.O. Box 1549, Manassas, VA USA, (GenBank Accession No. AM412317.1) *Saccharomyces cerevisiae* (GenBank Accession No. EU255273.1) Yet in other microorganisms, the production of alcohols occurs only through the acyl-CoA intermediate of the organic acid in two sequential steps catalyzed by similar aldehyde and alcohol dehydrogenase enzymes, dependent on reducing equivalents in the form of NAD(P)H. Examples of aldehyde dehydrogenase that act on acyl-CoA intermediates include, but are not limited to, *Rhodococcus opacus* (GenBank Accession No. AP011115.1), *Entamoeba dispar* (GenBank Accession No.

DS548207.1) and *Lactobacillus reuteri* (GenBank Accession No. ACHG01000187.1). Examples of alcohol dehydrogenases that catalyze the conversion of an aldehyde to its corresponding primary alcohol include, but are not limited to, *Aspergillus niger* (GenBank Accession No. AM269994.1), *Streptococcus pneumoniae* Taiwan19F-14 (GenBank Accession No. CP000921.1) and *Salmonella enterica* (GenBank Accession No. CP001127.1). Yet in other microorganisms, both reactions can occur sequentially by the action of a single enzyme possessing both aldehyde/alcohol dehydrogenase domains, independently of the enzyme having only these two domains or more. Examples of such multifunctional enzymes include, but are not limited to, *Lactobacillus sakei* (GenBank Accession No. CR936503.1), *Giardia intestinalis* (GenBank Accession No. U93353.1), *Shewanella amazonensis* (GenBank Accession No. CP000507.1), *Thermosynechococcus elongatus* (GenBank Accession No. BA000039.2), *Clostridium acetobutylicum* (GenBank Accession No. AE001438.3) and *Clostridium carboxidivorans* ATCC No. BAA-624T (GenBank Accesion No. ACVI01000101.1).

Examples of enzymes that can be used in the present inventions include, but not limited to, those enzymes listed in the Tables 1-4.

Table 1. Aldehyde Dehydrogenases that Can Use an Organic Acid as a Substrate

Organism	GenBank Accession No.	GI number
Mus musculus	AC162458.4	7106242
Clostridium botulinum A str. ATCC No. 3502	AM412317.1	148288571
Saccharomyces cerevisiae	EU255273.1	160415767

Table 2. Aldehyde Dehydrogenases that Can Use Acyl-CoA Intermediates as a Substrate

Organism	GenBank Accession No.	GI number
Rhodococcus opacus	AP011115.1	226243131
Entamoeba dispar	DS548207.1	165903565
Lactobacillus reuteri	ACHG01000187.1	227184849

Table 3. Aldehyde Dehydrogenases that Catalyze the Conversion of an Aldehyde to its Corresponding Primary Alcohol

Organism	GenBank Accession No.	GI number
Aspergillus niger	AM269994.1	145231224
Streptococcus pneumoniae Taiwan19F-14	CP000921.1	225726676
Salmonella enterica	CP001127.1	194712950

Table 4. Aldehyde/Alcohol Dehydrogenases Multifunctional Enzymes

Organism	GenBank Accession No.	GI number
Lactobacillus sakei	CR936503.1	78609634
Giardia intestinalis	U93353.1	2052472
Shewanella amazonensis	CP000507.1	119767329
Thermosynechococcus elongatus	BA000039.2	22293948
Clostridium acetobutylicum	AE001438.3	14994351
Clostridium carboxidivorans ATCC No. BAA-624T	ACVI01000101.1	255508861

Natural or recombinant organisms containing the gene that encodes the enzyme alcohol/aldehyde dehydrogenase capable of reducing an acyl-CoA or an organic acid and then the aldehyde or a ketone to the corresponding primary alcohol may be isolated or constructed using techniques such as heterologous DNA insertion, differential expression or deletion of genes well known in the art.

Conversion ia) Acyl-CoA + NAD(P)H + H^+ ⇆ Aldehyde + $NAD(P)^+$ or

Conversion ib) Organic acid + NAD(P)H + H^+ ⇆ Aldehyde + $NAD(P)^+$ + H_2O and

Conversion j) Aldehyde or ketone + NAD(P)H + H^+ ⇆ alcohol + $NAD(P)^+$

In order to maximize the production of n-propanol, it is of great importance that the carbon flux of our engineered microorganism flows preferentially from pyruvate to propionic acid through the dicarboxylic acid cycle. However, the present invention realizes that due to cellular requirements for ATP and NAD(P)H some of the carbon might flow to the production of acetate from pyruvate through an irreversible oxidative decarboxylation reaction. The acetate or acetyl-CoA intermediate thus formed are of no economic interest. However, this acetate or its acetyl-CoA intermediate may be further metabolized into ethanol by the action of the enzymes aldehyde/alcohol dehydrogenases described above, or alternatively, these intermediates could be

further metabolized into isopropanol by the condensation of two molecules of acetyl-CoA into acetoacetyl-CoA and CoA, followed by another oxidative decarboxylation reaction into acetone and final reduction into isopropanol, through the action of the enzymes from the isopropanol production pathway of *Clostridium beijerinckii*, as disclosed in International Application No. WO 2008/131286 A1.

Conversion k) condensation of the two molecules of acetyl-CoA into acetoacetyl-CoA and CoA through the action of the enzyme thiolase (E.C. 2.3.1.19);

Conversion l) acetoacetyl-CoA into acetoacetate and CoA through the action of the enzyme acetoacetyl-CoA hydrolase (E.C. 3.1.2.11);

Conversion m) decarboxylation of acetoacetate into acetone through the action of the enzyme acetoacetate decarboxylase (E.C. 4.1.1.4);

Conversion n) reduction of acetone into isopropanol through the action of the enzyme primary-secondary alcohol dehydrogenase (E.C. 1.1.1.1) found in microorganisms such as *Clostridium beijerinckii*, *Burkholderia* spp. and *Thermoanaerobacter brockii*.

In certain embodiments, the engineered microorganism will express the enzymes corresponding to the conversions a, b, c, d, e, f, g, h, ia, ib and j, in

which at least one of the conversions is carried out by an heterologous gene, and the final end alcohol products of the fermentation are either n-propanol or ethanol or a mixture of both.

In certain embodiments, the engineered microorganisms will express the enzymes corresponding to the conversions a, b, c, d, e, f, g, h, ia, ib, j, k, l , m, and n, in which at least one of the conversions is carried out by an heterologous gene, and the final end alcohol products of the fermentation are either n-propanol, ethanol or isopropanol or a mixture thereof.

In certain embodiments, the gene encoding for an enzyme acetate kinase (E.C. 2.7.2.1) of the host organism, catalyzing the conversion of acetyl-CoA into acetate, will have its expression altered so as to diminish its activity and thus increase availability of acetyl-CoA for isopropanol production. For example, the acetate kinase encoding gene of *P. acidipropionici* (GenBank Accession No. AY936474.1) may be altered, deleted or underexpressed using techniques known by those skilled in the art.

The invention encompasses the use of isolated or substantially purified polynucleotide and enzyme or protein compositions. An "isolated" or "purified" polynucleotide or enzyme, or biologically active portion thereof, is substantially or essentially free from components that normally accompany or interact with the polynucleotide or protein as found in its naturally occurring environment. Thus, an isolated or purified polynucleotide or enzyme is substantially free of

other cellular material or culture medium when produced by recombinant techniques, or substantially free of chemical precursors or other chemicals when chemically synthesized. Optimally, an "isolated" polynucleotide is free of sequences (optimally protein encoding sequences) that naturally flank the polynucleotide (*i.e.*, sequences located at the 5' and 3' ends of the polynucleotide) in the genomic DNA of the organism from which the polynucleotide is derived. For example, in various embodiments, the isolated polynucleotide can contain less than about 5 kb, 4 kb, 3 kb, 2 kb, 1 kb, 0.5 kb, or 0.1 kb of nucleotide sequence that naturally flank the polynucleotide in genomic DNA of the cell from which the polynucleotide is derived. An enzyme or protein that is substantially free of cellular material includes preparations of protein having less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of contaminating protein. When the protein of the invention or biologically active portion thereof is recombinantly produced, optimally culture medium represents less than about 30%, 20%, 10%, 5%, or 1% (by dry weight) of chemical precursors or non-protein-of-interest chemicals.

Fragments and variants of the disclosed polynucleotides and enzymes encoded thereby are also encompassed by the present invention. By "fragment" is intended a portion of the polynucleotide or a portion of the amino acid sequence and hence enzyme or protein encoded thereby. Fragments of polynucleotides comprising coding sequences may encode enzyme or protein fragments that retain biological activity of the native enzyme. Alternatively, fragments of a polynucleotide that are useful as hybridization probes generally

do not encode proteins that retain biological activity or do not retain promoter activity. Thus, fragments of a nucleotide sequence may range from at least about 20 nucleotides, about 50 nucleotides, about 100 nucleotides, and up to the full-length polynucleotide of the invention.

A fragment of a polynucleotide that encodes a biologically active portion of an enzyme of the invention will encode at least 15, 25, 30, 50, 100, 150, 200, 300, 400, 500, 750. or 1000 contiguous amino acids, or up to the total number of amino acids present in a full-length enzyme of the invention. Fragments of a polynucleotide encoding an enzyme of the present invention that are useful as hybridization probes or PCR primers generally need not encode a biologically active portion of the enzyme.

Thus, a fragment of polynucleotide of the present invention may encode a biologically active portion of an enzyme, or it may be a fragment that can be used as a hybridization probe or PCR primer using methods disclosed below. A biologically active portion of an enzyme protein can be prepared by isolating a portion of one of the polynucleotides of the invention, expressing the encoded portion of the enyzme or protein (e.g., by recombinant expression *in vivo*), and assessing the enzyme activity of the encoded portion of the enzyme. Polynucleotides that are fragments of a nucleotide sequence comprise at least 16, 20, 50, 75, 100, 150, 200, 250, 300, 350, 400, 450, 500, 550, 600, 650, 700, 800, 900, 1000, 1100, 1200, 1300, 1400, 1500, 2000, 2500, or 3000 contiguous

nucleotides, or up to the number of nucleotides present in a full-length polynucleotide disclosed herein.

"Variants" is intended to mean substantially similar sequences. For polynucleotides, a variant comprises a polynucleotide having deletions (*i.e.*, truncations) at the 5' and/or 3' end; deletion and/or addition of one or more nucleotides at one or more internal sites in the native polynucleotide; and/or substitution of one or more nucleotides at one or more sites in the native polynucleotide. As used herein, a "native" polynucleotide or polypeptide comprises a naturally occurring nucleotide sequence or amino acid sequence, respectively. For polynucleotides, conservative variants include those sequences that, because of the degeneracy of the genetic code, encode the amino acid sequence of one of the polypeptides of the invention. Naturally occurring allelic variants such as these can be identified with the use of well-known molecular biology techniques, as, for example, with polymerase chain reaction (PCR) and hybridization techniques as outlined below. Variant polynucleotides also include synthetically derived polynucleotides, such as those generated, for example, by using site-directed mutagenesis but which still encode an enzyme of the invention. Generally, variants of a particular polynucleotide of the invention will have at least about 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity to that particular polynucleotide as determined by sequence alignment programs and parameters as described elsewhere herein.

Variants of a particular polynucleotide of the invention (*i.e.*, the reference polynucleotide) can also be evaluated by comparison of the percent sequence identity between the polypeptide encoded by a variant polynucleotide and the polypeptide encoded by the reference polynucleotide. Percent sequence identity between any two polypeptides can be calculated using sequence alignment programs and parameters described elsewhere herein. Where any given pair of polynucleotides of the invention is evaluated by comparison of the percent sequence identity shared by the two polypeptides they encode, the percent sequence identity between the two encoded polypeptides is at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, 99% or more sequence identity.

"Variant" protein is intended to mean a protein derived from the native protein by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the native protein; deletion and/or addition of one or more amino acids at one or more internal sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein. The biological activity of variant proteins of the invention can be assayed by methods known in the art. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native enzyme of the invention will have at least about 60%, 65%, 70%, 75%, 80%, 85%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%,

98%, 99% or more sequence identity to the amino acid sequence for the native protein as determined by sequence alignment programs and parameters described elsewhere herein. A biologically active variant of a protein of the invention may differ from that protein by as few as 1-15 amino acid residues, as few as 1-10, such as 6-10, as few as 5, as few as 4, 3, 2, or even 1 amino acid residue.

"Variant" protein is intended to mean a protein derived from the native protein by deletion (so-called truncation) of one or more amino acids at the N-terminal and/or C-terminal end of the native protein; deletion and/or addition of one or more amino acids at one or more internal sites in the native protein; or substitution of one or more amino acids at one or more sites in the native protein. Variant proteins encompassed by the present invention are biologically active, that is they continue to possess the desired biological activity of the native protein. The biological activity of variant proteins of the invention can be assayed by methods known in the art. Such variants may result from, for example, genetic polymorphism or from human manipulation. Biologically active variants of a native enzyme aldehyde dehydrogenase and alcohol dehydrogenase of the invention will have an E-value threshold below 1e-2 when compared with conserved domain protein database (CDD) from National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/cdd).

The enzymes or proteins of the invention may be altered in various ways including amino acid substitutions, deletions, truncations, and insertions.

Methods for such manipulations are generally known in the art. For example, amino acid sequence variants and fragments of the enzymes can be prepared by mutations in the DNA. Methods for mutagenesis and polynucleotide alterations are well known in the art. See, for example, Kunkel (1985) *Proc. Natl. Acad. Sci. USA* 82:488-492; Kunkel *et al.* (1987) *Methods in Enzymol.* 154:367-382; U.S. Patent No. 4,873,192; Walker and Gaastra, eds. (1983) *Techniques in Molecular Biology* (MacMillan Publishing Company, New York) and the references cited therein. Guidance as to appropriate amino acid substitutions that do not affect biological activity of the protein of interest may be found in the model of Dayhoff *et al.* (1978) *Atlas of Protein Sequence and Structure* (Natl. Biomed. Res. Found., Washington, D.C.), herein incorporated by reference. Conservative substitutions, such as exchanging one amino acid with another having similar properties, may be optimal.

Thus, the genes and polynucleotides of the invention include both the naturally occurring sequences as well as mutant forms. Likewise, the proteins of the invention encompass both naturally occurring proteins as well as variations and modified forms thereof. Such variants will continue to possess the desired enzyme activity. Obviously, the mutations that will be made in the DNA encoding the variant must not place the sequence out of reading frame and optimally will not create complementary regions that could produce secondary mRNA structure. See, EP Patent Application Publication No. 75,444.

The deletions, insertions, and substitutions of the protein sequences encompassed herein are not expected to produce radical changes in the characteristics of the protein. However, when it is difficult to predict the exact effect of the substitution, deletion, or insertion in advance of doing so, one skilled in the art will appreciate that the effect will be evaluated by routine screening assays. That is, enzyme activity can be evaluated by routine assays known in the art.

Variant polynucleotides and enzymes also encompass sequences and enzymes derived from a mutagenic and recombinogenic procedure such as DNA shuffling. Strategies for such DNA shuffling are known in the art. See, for example, Stemmer (1994) *Proc. Natl. Acad. Sci. USA* 91:10747-10751; Stemmer (1994) *Nature* 370:389-391; Crameri *et al.* (1997) *Nature Biotech.* 15:436-438; Moore *et al.* (1997) *J. Mol. Biol.* 272:336-347; Zhang *et al. (1997) Proc. Natl. Acad. Sci. USA* 94:4504-4509; Crameri *et al.* (1998) *Nature* 391:288-291; and U.S. Patent Nos. 5,605,793 and 5,837,458.

It is recognized that the methods of the present invention encompass the use of polynucleotide molecules and proteins comprising a nucleotide or an amino acid sequence that is sufficiently identical to a nucleotide or amino acid sequence disclosed herein. The term "sufficiently identical" is used herein to refer to a first amino acid or nucleotide sequence that contains a sufficient or minimum number of identical or equivalent (e.g., with a similar side chain) amino acid residues or nucleotides to a second amino acid or nucleotide

sequence such that the first and second amino acid or nucleotide sequences have a common structural domain and/or common functional activity. For example, amino acid or nucleotide sequences that contain a common structural domain having at least about 45%, 55%, or 65% identity, preferably 75% identity, more preferably 85%, 90%, 95%, 96%, 97%, 98% or 99% identity are defined herein as sufficiently identical.

To determine the percent identity of two amino acid sequences or of two nucleic acids, the sequences are aligned for optimal comparison purposes. The percent identity between the two sequences is a function of the number of identical positions shared by the sequences (*i.e.*, percent identity = number of identical positions/total number of positions (*e.g.*, overlapping positions) x 100). In one embodiment, the two sequences are the same length. The percent identity between two sequences can be determined using techniques similar to those described below, with or without allowing gaps. In calculating percent identity, typically exact matches are counted.

The determination of percent identity between two sequences can be accomplished using a mathematical algorithm. A preferred, nonlimiting example of a mathematical algorithm utilized for the comparison of two sequences is the algorithm of Karlin and Altschul (1990) *Proc. Natl. Acad. Sci. USA* 87:2264, modified as in Karlin and Altschul (1993) *Proc. Natl. Acad. Sci. USA* 90:5873-5877. Such an algorithm is incorporated into the BLASTn and BLASTx programs of Altschul *et al.* (1990) *J. Mol. Biol.* 215:403. BLAST nucleotide

searches can be performed with the BLASTn program, score = 100, wordlength = 12, to obtain nucleotide sequences homologous to the polynucleotide molecules of the invention. BLAST protein searches can be performed with the BLASTx program, score = 50, wordlength = 3, to obtain amino acid sequences homologous to protein molecules of the invention. To obtain gapped alignments for comparison purposes, Gapped BLAST can be utilized as described in Altschul *et al.* (1997) *Nucleic Acids Res.* 25:3389. Alternatively, PSI-Blast can be used to perform an iterated search that detects distant relationships between molecules. *See* Altschul *et al.* (1997) *supra.* When utilizing BLAST, Gapped BLAST, and PSI-Blast programs, the default parameters of the respective programs (e.g., BLASTx and BLASTn) can be used. *See* http://www.ncbi.nlm.nih.gov. Another preferred, non-limiting example of a mathematical algorithm utilized for the comparison of sequences is the algorithm of Myers and Miller (1988) *CABIOS* 4:11-17. Such an algorithm is incorporated into the ALIGN program (version 2.0), which is part of the GCG sequence alignment software package. When utilizing the ALIGN program for comparing amino acid sequences, a PAM120 weight residue table, a gap length penalty of 12, and a gap penalty of 4 can be used. Alignment may also be performed manually by inspection.

Unless otherwise stated, sequence identity/similarity values provided herein refer to the value obtained using the full-length sequences of the invention and using multiple alignment by mean of the algorithm Clustal W (Nucleic Acid Research, 22(22):4673-4680, 1994) using the program AlignX

included in the software package Vector NTI Suite Version 7 (InforMax, Inc., Bethesda, MD, USA) using the default parameters; or any equivalent program thereof. By "equivalent program" is intended any sequence comparison program that, for any two sequences in question, generates an alignment having identical nucleotide or amino acid residue matches and an identical percent sequence identity when compared to the corresponding alignment generated by CLUSTALW (Version 1.83) using default parameters (available at the European Bioinformatics Institute website: http://www.ebi.ac.uk/Tools/clustalw/index.html).In certain embodiments, any genes encoding for enzymes with one or more of the aldehyde dehydrogenase and alcohol dehydrogenase activities may be used. These enzymes may be wild-type enzymes from a different organism, or may be artificial, recombinant or engineered enzymes.

In certain embodiments, the metabolic reactions described within this invention may be catalyzed by one or more enzymes regardless of the number of steps catalyzed by each enzyme which may be single or multi-functional and still be included within the scope of this invention.

In certain embodiments, any genes encoding for enzymes with the same activity as any of the enzymes described within this invention may be used. These enzymes may be wild-type enzymes from a different organism, or may be artificial, recombinant or engineered enzymes.

Due to the inherent degeneracy of the genetic code, other nucleic acid sequences which encode substantially the same or a functionally equivalent amino acid sequence can also be used to express such enzymes. As will be understood by those of skill in the art, it can be advantageous to modify a coding sequence to enhance its expression in a particular host. The codons that are utilized most often in a species are called "optimal codons", and those not utilized very often are classified as "rare or low-usage codons". Codons can be substituted to reflect the preferred codon usage of the host, a process sometimes called "codon optimization" or "controlling for species codon bias".

Expression of genes is a complex mechanism that may be modified by molecular biology techniques. For example, expression of heterologous genes may be controlled by an inducible promoter or a constitutive promoter. The heterologous genes may either be integrated into a chromosome of the host or present as extra-chromosomal genetic elements (such as plasmids, BAC, YAC, etc.) that can be inherited by daughter cells. Such extra-chromosomal genetic elements may contain selection markers.

Methods for expressing polypeptide from an exogenous nucleic acid molecule include constructing a nucleic acid such that a regulatory element (promoter, enhancers and the like) promotes the expression of a nucleic acid sequence that encodes the desired polypeptide at a desired condition.

In another embodiment, heterologous control elements can be used to activate or repress expression of endogenous or heterologous genes. Moreover, when expression is to be repressed or eliminated, the gene for the relevant enzyme, protein or RNA can be eliminated, for example, by knock-out mutation obtained through homologous recombination or other known deletion techniques. The use of the technique of interference RNA (iRNA) for gene post-trascriptional silencing could also be used.

Methods that modify the expression of genes in microorganisms are contemplated for use in the construction of the microbial cells of the present invention.

Any method capable of introducing an exogenous nucleic acid molecule into microorganisms can be used. For example, electroporation, conjugation, heat shock, *Agrobacterium tumefaciens* mediated transformation, protoplasts fusion, etc.

The exogenous nucleic acid molecule contained within a microorganism described herein may be maintained within that cell in any form, i.e., these molecules can be integrated into the any chromosome or maintained in an extra-chromosomal state that can be passed on to daughter cells. Additionally, these microorganisms can be stably or transiently transformed. Moreover, exogenous nucleic acid molecule may be present as single or multiple copies into the host microorganism.

The reducing equivalents needed for the conversion of the propionate/propionyl-CoA intermediate into n-propanol may be supplied to the microorganism *in vivo* through the use of a recombinant NAD(P)H recycling system and the external supply of a formate salt.

According to the present invention, it is possible to drive redox balance artificially in three main ways. As example, one way is the introduction of a recombinant NAD(P)H and/or recycling system based on a the introduction of a gene coding for an enzyme that catalyzes the conversion of formate salt into CO_2 with the concomitant regeneration of the reduced form NAD(P)H and the external supply of formate to the growth medium. See, U.S. Patent Application Publication No. 2003/0175903 A1, herein incorporated by reference.

The reducing equivalents needed for the conversion of the propionate/propionyl-CoA intermediate into n-propanol may also be supplied by the addition of an overpressure of H_2 to the bioreactor (at low or high pressures, but preferentially at 1-2 atmospheres) as described in U.S. Pat. No. 4,732,855, herein incorporated by reference. This overpressure can be used in microorganism that express a hydrogenase enzyme, native or heterologous.

Another alternative is to supply the reducing equivalents needed for the conversion of the propionate/propionyl-CoA intermediate into n-propanol through the use of cathodes and a mediator molecule. This reaction occurs simultaneous to the fermentation process in a bioelectric reactor, where the

mediator is a external molecule that has a function of transferring the electrons from a cathode to the electron carriers of the living cell (NAD(P)) as described by Thrash & Coates 2008, Environ. Sci. Technol. 42:3921-3931, herein incorporated by reference.

The working cathode can be poised at several potentials against the reference electrode, such as 10 mV, 100 mV, 200 mV, 400 mV, 600 mV and 800 mV or any potential value necessary to transfer electrons from the electrode to the growing cells. The cathodes can be constructed in different materials, shapes, sizes and superficial areas, such as single wires, nets or solid shape configurations. However, other shapes or configurations may be considered within the scope of the present invention.

The mediator molecule can be any molecule externally supplied or internally secreted and can be present at several concentrations, such as 0.2 mM, 0.4 mM, 0.6 mM, 0.8 mM, 1.0 mM, or any concentration necessary to transfer the electrons from the electrode to the cell with high performance and with the object of maximizing the concentration of interesting end-products and minimizing the electrical current generated during this process. Examples of suitable mediators for this process are benzyl viologen, methyl viologen, anthraquinone 2,6-disulfonic acid, neutral red and cobalt sepulchrate. Other suitable mediator molecules for the process of the present invention are compounds present in yeast extract and endogenous mediator present in

Propionibacterium spp. extract. Another embodiment of the invention is the use of endogenous mediator by recirculation of the cells to the bioreactor.

In the present invention, the preferred form for externally supplying reducing equivalents to the culture medium is through the use of electrodes and a mediator molecule.

The electrical current used to supply the electrodes can be originated by renewable or non-renewable energy sources. However, the preferred source is a renewable source, such as hydroelectrical plants or, more preferentially according to the biorefinery concepts, such as through the burning of sugarcane bagasse.

The bioelectrical reactor uses a two or three electrode system for precise measurement and control of the potential at the working electrode (cathode) and the auxiliary counter electrode (anode). If necessary by the reactor configuration an electron shuttle may be used. Any kind of reference electrode system known at the state of the art as adequate for aqueous media, as the hydrogen electrode or the silver chloride electrode, can be used by the present invention as a reference electrode when necessary.

The cathodic voltage should be maintained below 3.0 V, preferentially below to 1.5 V, to prevent the electrolysis of water what would undesirably increase the pH of the media and release gaseous hydrogen.

In addition, high concentrations of chloride ions must be avoided in the anodic compartment to prevent its oxidation that would undesirably form chlorine that would react with water to form hypochlorous acid, which would be very prejudicial to the growth and integrity of the microorganisms.

The anode and cathode were separated by a separator element selected among the ones known by the state of the art. The purpose of this separator is to permit only the passage of ions and electrical current and avoid, or at least reduce, the transfer of chemicals, as sugars, and metabolites across it. As examples of the separators adequate for the present invention are ceramics porous septums, fibery diaphragms and, preferably, solid permeable electrolytes as the cation-selective membranes known as permselective membrane, commercially designed as Nafion or similar.

The cathode compartment is the place where the culture medium is fed and the fermentation is conducted. Its composition, made mainly by water and soluble nutrients, substrates and metabolites, permits its use as a catholyte in addition to its ability to promote the cells growth and the fermentation development.

The anode compartment must be filled with an aqueous solution, stable to the anode potential and able to conduct electricity. It can be usually constituted by an aqueous buffer as a 100 mM sodium phosphate solution.

The electrodes could be assembled in many different configurations as single wires, bars, rods, nets, porous agglomerates, woven structures or solid or perforated foils or plates, with a smooth or a rough surface. In the case of the cathodes they are preferably used as the baffles to prevent the vortex in stirred bioelectrical reactors. In the case of the anodes they are preferably assembled in the wall of the bioelectrical reactors, separated by a permselective membrane.

Electrodes must be made of a material stable to the corrosion in the bioelectrical reactor operational conditions and that is a good electricity conductor. The anode must be preferably made of carbon, graphite, or metals or alloys as nickel, platinum, stainless steel or titanium. The cathode must be made of any material adequate for use as cathodes, such as graphite, glassy carbon, stainless steel, carbon steel or metals or alloys as nickel, iron, lead, titanium, commercially designed as monel, sanicro, 2RK65 or similar. Preferably the cathode material will be constituted by a metal or alloy of high hydrogen overpotencial as titanium, monel, sanicro, or 2RK65.

Fermentation media in the present invention contain suitable carbon sources to yield a high productivity of propionic acid by native or engineered microorganisms hosting the dicarboxylic acid pathway and the n-propanol producing pathway by native or engineered microorganims. This carbon sources can include monosaccharides such as glucose, fructose and xylose; oligosaccharides such as sucrose and lactose; polysaccharides such as starch, pectin, cellulose and hemicellulose, and lignocellulosic materials; fatty acids;

succinate; lactate; acetate; glycerol and mixtures thereof. Also, it can include other carbon sources from renewable feedstocks of complex composition such as sugarcane juice, sugarcane molasses or acid or enzymatic hydrolysates of lignocellulosic materials. Waste materials such as whey or industrial glycerol waste waters can also be used.

In certain embodiments of the present invention glycerol, sucrose and the complex multi-component sugarcane juice or sugarcane molasses are preferentially used.

In addition to the appropriate carbon sources, the culture media may be provided by other macronutrients such as nitrogen, and micronutrients such as phosphorous, potassium, sodium, calcium, vitamins and essentials metallic cofactors, known to those skilled in the art, according to the requirements of the producing microorganism.

In certain embodiments, the carbon source can be preferentially supplied with at least one nitrogen source.

In certain embodiments, the preferred nitrogen source is yeast extract.

In certain embodiments, the preferred nitrogen source is N_2.

In certain embodiments vitamin B5 (pantothenic acid) is supplied to the culture medium with the object of increasing productivity. This panthotenic acid may be provided in pure form or as a crude extract by-product of fermentation by another organism.

The microorganisms, native or engineered, must be grown in conditions for high yield production of the compounds of interest. Suitable culture conditions will be considered. The microorganisms, native or engineered for propionic acid and subsequent n-propanol production, grow at temperatures ranging from 25°C to 60°C, where temperatures 30°C to 32°C are preferred. Suitable pH ranges for the fermentation high production, are between pH 5 to pH 7.5, where pH 6.5 to 6.8 are preferred. Reaction may be performed under anaerobic, microaerobic, or aerobic conditions.

In certain embodiments, fermentation under anaerobic condition is preferred.

The fermentative process in the present invention can employ various fermentation operations modes. Batch mode fermentation is a close system where culture media and producer microorganism, set at the beginning of fermentation, don't have any more inputs except for the reagents for pH control, foam control and others required for process sustenance. The process described in the present invention can also be employed in Fed-batch or continuous mode.

The fermentative process can be performed in free cell culture and in immobilized cell culture. For immobilized cell cultures is contemplated the use of different material supports such as alginates, fibrous bed, argyle materials such as chrysotile, montmorillonite KSF and montmorillonite K-10. However, other methods of immobilization are considered here within the scope of the present invention.

In certain embodiments, the preferred condition is the use of immobilized cells.

The present invention may be practiced in several bioreactor configurations, such as stirred tank, bubble column, airlift reactor and other known to those skilled in the art.

The products, n-propanol and, eventually, iso-propanol and/or ethanol, can be extracted from the fermentation broth using processes well-known in the state-of-the-art, such as for the separation of ethanol from broth. These processes include distillation, reactive distillation, azeotropic distillation and extractive distillation. There is no need to remove the total amount of water in the media.

In addition, the alcohols n-propanol and iso-propanol and/or ethanol, obtained according to the present invention can be dehydrated together in the same reactor using operating conditions to yield high amounts of propylene and

an amount of ethylene. In certain embodiment of the invention, reactor feed stream can be a mixture of n-propanol and iso-propanol and/or ethanol or a mixture of these alcohols with water. Ethylene can be purified to used as a copolymer with propylene.

The dehydration reaction occurs in the presence of catalyst such as alumina, silica-alumina, zeolites and other metallic oxides using temperatures ranging from 180°C to 600°C, preferentially from 300°C to 500°C. The reaction is conduced in an adiabatic or isothermal reactor, which can also be a fixed or a fluidized bed reactor.

The dehydration reaction of n-propanol and, eventually, iso-propanol and/or ethanol, can be optimized using residence time ranging from 0,1 to 60 seconds, preferentially from 1 to 30 seconds. Non converted alcohol can be recycled to the dehydration reactor.

The contaminants that are generated in the process are removed through a purification section that is traditionally used in this type of reaction. Propylene can be washed with pure water or caustic solution to remove acids compounds like carbon dioxide and/or can be fed into beds to absorb polar compounds like water and also to remove carbon monoxide. Alternatively, a distillation column can be used to separate higher hydrocarbons such as propane, butane, butylene and higher compounds. The separation of propylene and ethylene is made by the methods know in the state-of-the-art as cryogenic

distillation. Polymer grade propylene is provided by the process of the present invention and has 100% of renewable carbon content.

Polypropylene and their copolymers of the present invention are produced by polymerization processes well-known in the state of art, which can be conduced via bulk polymerization process with temperatures ranging from 105°C to 300°C, or via polymerization in suspension with temperatures ranging from 50°C to 100°C. Alternatively polypropylene can be produced in a gas phase reactor in the presence of a polymerization catalyst such as Ziegler-Natta or metalocene catalysts with temperatures ranging from 60°C -80°C.

The product obtained by the processes described in the present invention has 100% of biobased content contributing to reduce greenhouse gas emission, since at the end of its life there would no fossil carbon emissions if it is incinerated.

EXAMPLE 1

Fermentation of Sugarcane Juice by *Propionibacterium acidipropionici*

A native strain of *Propionibacterium acidipropionici* (ATCC No. 4875) was used to study propionic acid and n-propanol production using sugarcane juice as a carbon source. The bacterium was cultured in a medium containing 30% sugar cane juice diluted in water and supplemented with 1 g/L of yeast extract. At this dilution, the starting concentrations of sugars in diluted

sugarcane juice medium were measured at 53 g/L of sucrose, 10.9 g/L of glucose and 7.4 g/L of fructose. The medium was sterilized at 121°C and 1 kgf/cm^2 for 20 min prior to use.

Free-cell batch fermentation was conducted in a 2.5 L bioreactor (BioFlo 3000 – New Brunswick) containing 2.0 L of the sterile medium inoculated with 20 g/l (wet weight) of the adapted cells of *P. acidipropionici*. The bioreactor temperature was maintained at 30°C and the agitation speed at 100 rpm. Constant pH of 6.5 was automatically controlled by adding a 4M NaOH solution. Anaerobic conditions were maintained through the use of a N_2 atmosphere.

Batch fermentation was stopped after 114 h and the products were quantified through High Performance Liquid Chromatography coupled to a Refraction Index detector and using standards for the desired metabolites (Varian Chromatographer using a Aminex HPX-87H Organic Acid Column from Transgenomic, operating at room temperature and using 0.002 M H_2SO_4 as the eluent at a flux of 0.6 mL/min). Table 5 shows the final concentration of the products. As can be observed, no n-propanol is detected at the growth conditions used.

Table 5. Final product concentrations after 114 h of fermentation by *Propionibacterium acidipropionici* (ATCC No. 4875) of sugarcane juice media (see composition in text) under controlled conditions of temperature, pH and agitation.

Component	Concentration (g/L)
Propionic acid	28.0
Acetic acid	9.6
Succinic acid	8.1
n-Propanol	ND

ND: Not detected

EXAMPLE 2

Engineering *Propionibacterium acidipropionici* for *In Vivo* n-propanol Production Through the Heterologous Expression of a Propionyl-CoA Reducing Pathway

Constructs:

pBK1T. A shuttle plasmid, pBK1T, is constructed in two steps. First step consists of fusing a portion of the native pRGO1 plasmid of *P. acidipropionici* with a portion of a commercial pUC18 plasmid, as described by Kiatpapan et al. 2000 (Appl. Env. Microbiol. 66:4688-4695). As a result of this fusion, the plasmid has both origins of replication in *E. coli* and *P. acidipropionici* and the

marker gene conferring resistance to ampicilin for *E. coli*; however, this resistance gene is not expressed in *P. acidipropionici* due to the differences in G+C content and codon usage. As an appropriate selection marker for *P. acidipropionici*, a synthetic construct was designed comprising a gene conferring resistance to the antibiotic thiostrepton, isolated from *Streptomyces laurentii* (GenBank Accession Number L39157.1), controlled by the promoter and terminator regions of the *pa-mmc* gene gene coding for the Methyl-malonyl CoA transcarboxilase (E.C. 2.1.3.1) of *P. acidipropionici*. This synthetic construct is built by amplifying the thiostrepton resistance gene from plasmid pIJ680 (Hopwood et al., 1985, "Genetic manipulation of Streptomyces – A Laboratory Manual", John Innes Foundation, Norwich) using adapter-primers PMMC_TSR-F (5'-CCGGGTTGCAATCAGGCTCTGATGCGCATGACTGAGTTGGACACCATCG-3') and TAPH_TSR-R (5'-TCAGGCTGAGAACGACCTGATCCGCCATTATCGGTTGGCCGCGAGAT-3'), in which the Forward primer contains a hybridization tail for fusing with the promoter region (underlined) and the Reverse primer contains a hybridization tailfor fusing with the terminator region (underlined). The promoter and terminator regions of the *pa-mmc* gene of *P. acidipropionici* are PCR amplified from genomic DNA using the primers NcoI_PMMC-F (5'-GATGACATCCATGGGTGTGCCATTTCTCACAATCC -3'), PMMC-R (5'-CCGGGTTGCAATCAGGCT CTGATGCGC-3'), TMMC-F (5'-TCAGGCTGAGAACGACCTGAT-3') and PsiI_TMMC-R (5'-GATCGTTTATAAGTAGGAGGCCTGCCTTGC-3'). Both amplicons are

joined together by single-joint PCR according to Yu et al., 2004 (Fungal Genetics and Biology **41**:973-981). The sequence of the resulting synthetic construct is provided in Figure 7.This is digested with *Nco*I and *PsiI* and inserted at the *Psi*I (blunt) and *Nco*I sites of the fusion vector in order to create our shuttle vector pBK1T.

pBK1T1. Expression plasmid pBK1T1 is constructed by inserting into pBK1T a gene coding for the bifunctional aldehyde/alcohol dehydrogenase of *Clostridium carboxidivorans* (ATCC No. BAA-624T) (Uniprot Accesion No. C6PZV5), controlled by the promoter and terminator regions of the gene coding for the Methyl-malonyl CoA transcarboxilase (E.C. 2.1.3.1) of *P. acidipropionici.* Due to differences in the G+C content and codon usage between *P. acidipropionici* and *C. carboxidivorans*, said gene was designed by reverse translation of the primary amino acid sequence. For this, a codon table is generated from host ribosomal protein genes, which are highly expressed. The codons are selected to resemble this table and the overall host G+C content, avoiding recognition sites of host restriction enzymes. Inverted repeats were also avoided to disrupt mRNA secondary structures. Finally, adaptors for digestion with the restriction enzymes *Xba*I and *Hind*III are added to the 5' and 3' ends of this sequence, respectively. The sequence of this synthetic construct is provided in Figure 8. The designed 2950 bp construct, containing the gene, its controlling regions and cloning adaptors is synthesized by Epoch Life Science (http://epochlifescience.com/Service/Gene_Synthesis.aspx). The construct is then digested and cloned into the *Xba*I and *Hind*III sites of pBK1T to generate

the expression shuttle plasmid pBK1T1. A schematic view of this plasmid is provided in Figure 5 and its sequence in Figure 10.

pBK1T2. Expression plasmid pBK1T2 is constructed by inserting into pBK1T a gene coding for the bifunctional aldehyde/alcohol dehydrogenase of *Clostridium acetobutylicum* (ATCC No. 824) (Uniprot Accesion No. P33744), controlled by the promoter and terminator regions of the gene coding for the Methyl-malonyl CoA transcarboxilase (E.C. 2.1.3.1) of *P. acidipropionici*. Due to differences in the G+C content and codon usage between *P. acidipropionici* and *C. acetobutylicum*, said gene was designed by reverse translation of the primary amino acid sequence. For this, a codon table is generated from host ribosomal protein genes, which are highly expressed. The codons are selected to resemble this table and the overall host G+C content, avoiding recognition sites of host restriction enzymes. Inverted repeats were also avoided to disrupt mRNA secondary structures. Finally, adaptors for digestion with the restriction enzymes *Xba*I and *Hind*III are added to the 5' and 3' ends of this sequence, respectively. The sequence of this synthetic construct is provided in Figure 6. The designed 2959 bp construct, containing the gene, its controlling regions and cloning adaptors is synthesized by Epoch Life Science (http://epochlifescience.com/Service/Gene_Synthesis.aspx). The construct is then digested and cloned into the *Xba*I and *Hind*III sites of pBK1T to generate the expression shuttle plasmid pBK1T2. A schematic view of this plasmid is provided in Figure 6 and its sequence in Figure 11.

Transformation:

pBK1T1 and pBK1T2 plasmids are first multiplied in *E. coli* GM2929 (*dam-*, *dcm-*) and are then recovered with high yield using standard procedures. Afterwards, these plasmids are transformed into electrocompetent cells of *Propionibacterium freudenreichii* (ATCC No. 6207) according to Kiatpapan and Murooka, 2001 (Appl. Microbiol. Biotechnol. 56:144-149) in order to obtain the appropriate methylation pattern to avoid digestion in the final host *P. acidipropionici*. Finally, the plasmids are recovered from *P. freudenreichii* and used to transform electrocompetent cells of *P. acidipropionici* (ATCC No. 4875). Transformants containing the expression plasmid pBK1T1 or pBK1T2 are selected in media containing 50 μg/mL thiostrepton and allowed to grow for 4-7 days.

Growth:

Recovered colonies of *P. acidipropionici* containing the expression plasmid pBK1T1 or pBKT2 are used to inoculate Erlenmayer flasks containing 125 mL of culture media (0.5% yeast extract, 0.5% peptone, 0.1% KH_2PO_4, 0.2% $(NH_4)_2HPO_4$, 0.1% of saline solutions 1 and 2 - solution 1: 1% $MgSO_4.7H_2O$ and 0.25% $MnSO_4.H_2O$; solution 2: 1% $CaCl_2.2H_2O$ and 1% de $CoCl_2.6H_2O$; pH 6,8) with 50 μg/mL thiostrepton and 5% glycerol as a reduced carbon source. The culture is grown in anaerobiosis until reaching OD_{600} ~2.5 and is used to seed a bioreactor culture using the same media, as explained in comparative Example 1. The production of n-propanol from this reduced carbon source is measured by High-Performance Liquid Chromatography, coupled to a

Refraction Index detector (Varian Chromatographer using a Aminex HPX-87H Organic Acid Column from Transgenomic, operating at room temperature and using 0.005 M H_2SO_4 as the eluent at a flux of 1 mL/min) and is compared to the production of this metabolite by a native *P. acidipropionici* strain (ATCC No. 4875). Native strains of *P. acidipropionici* are known to produce n-propanol from glycerol with a yield of approximately 4% (Barbirato et al., 1997, Appl. Microbiol. Biotechnol. 47: 441-446). Therefore, an increase in the production of this metabolite from glycerol can be attributed to the effect of the expression of the heterologous aldehyde/alcohol dehydrogenase gene.

EXAMPLE 3

Fermentation of sucrose by *Propionibacterium acidipropionici* using a bioelectrical reactor and a mediator molecule

A native strain of *Propionibacterium acidipropionici* (ATCC No. 4875) was used to study n-propanol production using sucrose as a carbon source. The bioelectrical reactor and different concentrations of mediator (cobalt sepulchrate) were utilized to drive the redox balance in order to obtain n-propanol.

P. acidipropionici was grown in a synthetic medium containing (per liter): 1 g KH2PO4, 2 g (NH4)2HPO4, 5 mg FeSO4·7H2O, 10 mg MgSO4·7H2O, 2.5 mg MnSO4·H2O, 10 mg CaCl2·6H2O, 10 mg CoCl2·6H2O, 10 g yeast extract (Oxoid), and the 9 g sucrose as a carbon source. The medium was autoclaved at 121 °C and 15 psig for 20 min. The cobalt sepulchrate (mediator) was added

separately to the autoclaved media in order to avoid thermal molecular instability.

Batch fermentation in a bioelectrical reactor was performed in a 2.0 L fermentor APPLIKON containing 700 ml of culture medium. The temperature was set at 30 °C and the pH was maintained at 6.5 by automatic addition of 4 M NaOH, with 50 rpm agitation. Anaerobiosis was maintained by nitrogen sparing through the culture medium before fermentation began and after each sampling. The redox potential system consists of a working electrode (WE) (a graphite bar, area 4.9 cm^2 or 10.5 cm^2 and thickness of 3.0 mm) and a counter anode (a graphite bar, area 30 cm^2 and thickness of 3.0 mm in the counter electrode compartment filled with 40 ml 3 M KCl). The working electrode (WE) was poised at 150 mV more negative than the redox potential of the mediator (around -350 mV) using a DC voltage source (2.3 - 3.1 Volts). The current between working electrode and counter electrode was recorded using a computer interface. In order to define the correct voltage to be applied into the system, a cyclic voltametry experiment was performed using a potentiostat (PGSTAT 302N model from AUTOLAB) connected to the system. The bioreactor was inoculated with 70 ml of cells in exponential phase (OD~ 3 to 5), which were grown in polypropylene test tubes at 30 °C. Samples were collected every 2 hours. After measuring the optical density (OD_{600}), the remaining volume of the sample was centrifuged at 10,000 g for 6 min. The supernatant was stored at −20 °C until HPLC and SPME-GC/MS analysis.

Cell biomass was calculated by measuring the absorbance at 600 nm in a ULTROSPEC 2000 spectrophotometer UV/visible (Pharmacia Biotech) after appropriate dilution in water. For HPLC-RI analysis, the samples were filtered through a 0.2 μm filter (Millipore). Propionic, succinic and acetic acids, n-propanol and sugars were separated and quantified by high-performance liquid chromatography (Waters 600 Chromatograph), using an ion exclusion column Aminex HPX-87H (Bio-Rad). Operating conditions were: 0.04 mol L^{-1} H_2SO_4 degassed eluent, flow rate 0.4 mL min^{-1}, column temperature 35 °C and refractometer temperature 35 °C.

The volatile products were confirmed by using the HS-SPME and gas chromatography mass spectrometry (GC-MS). The technique (SPME – Solid-phase microextraction) makes use of a fused silica optical fiber coated with a thin polymer layer to extract the analytes from a liquid (solution), from the headspace (HS) above a liquid or solid, or from a gaseous phase. All assays were carried out using 6 mL of fermented broth in pH 2-3 acidified in hydrochloric acid solution 3 mol L^{-1}. The experimental conditions of the assays were those indicated by the experimental design. Experimental conditions in SPME: Bath temperature (T: 30–35 °C), pre-equilibrium time (PET: 5 min), extraction time (Ext: 3 min). GC/MS analyses were obtained on an Agilent GC 6890/Hewlett-Packard 5973 gas chromatograph equipped with Stabilwax-DA capillary column (30 m × 0.25 mm × 0.25 μm) with helium (1 mL min^{-1}) as carrier gas. The oven temperature was programmed as follows: 40 °C for 3 min, then increased 5 °C/min up to 130 °C e then increased 40 °C/ min to 210 °C.

The injection port was equipped with a 0.75 mm i.d. liner and the injector was maintained at 210 °C in the splitless mode. Under these conditions, no sample carry-over was observed on blank runs conducted between extractions. The volatile products were identified by comparing their experimental spectra with those of WILEY Mass Spectra Library and injection of standards.

Table 6 summarizes the final concentration of n-propanol obtained after several fermentations of varying mediator concentration and working cathode area, after 36 hrs of fermentation. In the control fermentation the voltage applied and mediator concentration were zero. As can be observed, n-propanol was detected in fermentations with mediator and their final concentration increase as a function of the mediator concentration, in the concentration range used, and working cathode area.

Using the native strain, n-propanol was formed with yields ranging from 1.0-9.6% depending on the conditions, with the best results corresponding to condition 0.8 mM cobalt sepulchrate (WE area 4.9 cm^2). These results suggest that the native gene *adh* of *P. acidipropionici* is not efficient in the conversion of propionate to propanol. The next step consist of conducting fermentation with genetically modified strain expressing the gene from *C. carboxidivorans* as described in Example 2.

Figure 12(a) and (b) shows HPLC and Figure 13 shows GC-MS spectra after 36 hrs of control and 1.0 mM cobalt sepulchrate supplemented fermentations. The n-propanol peak appears only in the fermentation using bioelectrical reactor and the mediator molecule. Figure 12 shows a GC-MS chromatogram obtained in the fermentation broth using 1.0 mM cobalt sepulchrate. The products propionic and acetic acids and *n*-propanol were confirmed by GC-MS in all fermentation experiments.

A time course for cell growth of the control and the 1.0 mM cobalt sepulchrate fermentation is shown in Figure 14. In both fermentations it is possible to observe a similar behavior considering OD and formation of the common end-products, however in the fermentation using the mediator molecule n-propanol is produced at the beginning of the fermentation and its concentration increases following the cell growth.

Table 6. Final concentration of n-propanol obtained in five different fermentations (duration of 36 hrs) by *Propionibacterium acidipropionici* (ATCC No. 4875): control (no voltage applied and the mediator concentration was zero), 0.5 (WE area 4.9 cm^2), 0.8 (WE area 4.9 cm^2), 1.0 (WE area 4.9 cm^2), 0.8 (WE area 10.5 cm^2), and 1.0 (WE area 10.5 cm^2) mM mediator concentration.

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Fermentation	n-Propanol concentration (mg/L)
Control	ND
0.5 mM Cobalt Sepulchrate (WE area 4.9 cm^2)	25
0.8 mM Cobalt Sepulchrate (WE area 4.9 cm^2)	65
1.0 mM Cobalt Sepulchrate (WE area 4.9 cm^2)	81
0.8 mM Cobalt Sepulchrate (WE area 10.5 cm^2)	97
1.0 mM Cobalt Sepulchrate (WE area 10.5 cm^2)	180

ND: Not detected

In the claims which follow and in the preceding description of the invention, except where the context requires otherwise due to express language or necessary implication, the word "comprise" or variations such as "comprises" or "comprising" is used in an inclusive sense, i.e. to specify the presence of the stated features but not to preclude the presence or addition of further features in various embodiments of the invention.

It is to be understood that, if any prior art publication is referred to herein, such reference does not constitute an admission that the publication forms a part of the common general knowledge in the art, in Australia or any other country.

The claims defining the invention are as follows:

1. A method for producing n-propanol comprising:

(a) providing a suitable carbon source for fermentation by a microorganism expressing the dicarboxylic acid pathway, reducing equivalents in the form of NAD(P)H, and at least one gene coding for an enzyme that catalyzes the conversion of propionate/propionyl-CoA into n-propanol;

(b) contacting the carbon source and reducing equivalents in the form of NAD(P)H with the microorganism under conditions favorable for the production of n-propanol by the microorganism; whereby a fermentation broth is produced; and

(c) recovering n-propanol from the fermentation broth.

2. The method of claim 1, wherein the microorganism has been genetically engineered to express one or more enzymes, whereby the microorganism is capable of converting propionate/propionyl-CoA to n-propanol.

3. The method of claim 2, wherein the microorganism is selected from the group consisting of: *Propionigenium* spp., *Propionispira arboris*, *Propionibacterium* spp., and *Selenomonas*.

4. The method of claim 2, wherein the enzyme is selected from the group consisting of:

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aldehyde dehydrogenases that are capable of using propionic acid as a substrate;

aldehyde dehydrogenases that are capable of using an acyl-CoA intermediateas a substrate;

alcohol dehydrogenases that catalyze the conversion of an aldehyde to its corresponding primary alcohol; and

multifunctional enzymes that posses both aldehyde/alcohol dehydrogenase domains.

5. The method of claim 4, wherein the enzyme has alcohol dehydrogenase protein domain with e-value threshold below 1e-2.

6. The method of claim 4, wherein the enzyme has aldehyde dehydrogenase protein domain with e-value threshold below 1e-2.

7. The method of claim 4, wherein the aldehyde dehydrogenases are capable of using propionic acid as a substrate are selected from the group consisting of: *Mus musculus* (GenBank Accession No. AC162458.4); *Clostridium botulinum* A str. American Type Culture Collection (ATCC) No. 3502 (GenBank Accession No. AM412317.1); and *Saccharomyces cerevisiae* (GenBank Accession No. EU255273.1).

8. The method of claim 4, wherein the aldehyde dehydrogenases that are capable of using acyl-CoA intermediate as a substrate are selected from the

group consisting of: *Rhodococcus opacus* (GenBank Accession No. AP011115.1); *Entamoeba dispar* (GenBank Accession No. DS548207.1); and *Lactobacillus reuteri* (GenBank Accession No. ACHG01000187.1).

9. The method of claim 4, wherein the alcohol dehydrogenases that catalyze the conversion of an aldehyde to its corresponding primary alcohol are selected from the group consisting of: *Aspergillus niger* (GenBank Accession No. AM270229.1); *Streptococcus pneumoniae* Taiwan19F-14 (GenBank Accession No. CP000921.1); and *Salmonella enterica* (GenBank Accession No. CP001127.1).

10. The method of claim 4, wherein the multifunctional enzymes that posses both aldehyde/alcohol dehydrogenase domains are selected from the group consisting of: *Lactobacillus sakei* (GenBank Accession No. CR936503.1); *Giardia intestinalis* (GenBank Accession No. U93353.1); *Shewanella amazonensis* (GenBank Accession No. CP000507.1); *Thermosynechococcus elongatus* (GenBank Accession No. BA000039.2); *Clostridium acetobutylicum* (GenBank Accession No. AE001438.3); and *Clostridium carboxidivorans* ATCC No. BAA-624T (GenBank Accesion No. ACVI01000101.1).

11. The method of claim 1, wherein the fermentation broth further comprises ethanol and/or isopropanol.

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12. The method of claim 11, wherein ethanol and/or isopropanol are recovered from fermentation broth.

13. The method of claim 1, wherein the microorganism has the expression of its gene encoding for an enzyme acetate kinase (E.C. 2.7.2.1) altered so as to diminish its activity.

14. The method of any one of claims 1-13, wherein the reducing equivalents comprise NAD(P)H.

15. The method of claim 14, wherein the NAD(P)+ is reduced to NAD(P)H comprising the use of electrodes and a mediator molecule, an overpressure of H_2, or a microorganism expressing a NAD^+-dependent formate dehydrogenase in the presence of formate.

16. The method of claim 14, further comprising contacting the fermentation broth with electrodes and a mediator molecule.

17. The method of claim 16, wherein mediator molecules are benzyl viologen, methyl viologen, anthraquinone 2,6-disulfonic acid, neutral red, cobalt sepulchrate, 1,4 dihydroxy-2-naphthoic acid (DHNA) and flavins.

18. The method of claim 16, wherein mediator molecules are compounds present in yeast extract and *Propionibacterium* spp. extract.

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19. The method of any one of claims 1-18, wherein the carbon source is sugarcane juice, sugarcane molasses, hydrolyzed starch, hydrolyzed ligno-cellulosic materials, glucose, sucrose, fructose, lactate, lactose, xylose or glycerol in any form or a mixture thereof.

20. A microorganism for using in the method as defined in any one of claims 1 to 19.

21. A method of claim 1 further comprising:

dehydrating the n-propanol produced by the method as defined in any one of claims 1 to 19 to produce propylene.

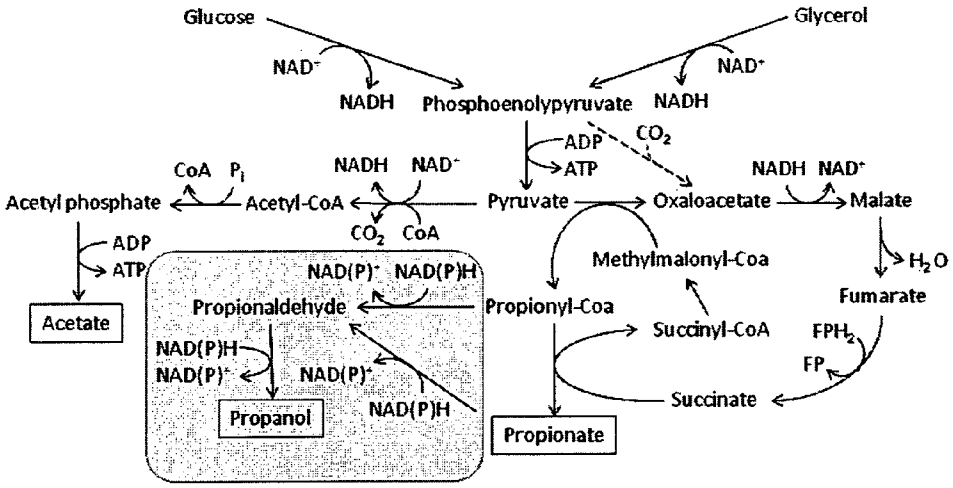
22. A method of claim 1 further comprising:

dehydrating in the same reactor n-propanol and isopropanol and/or ethanol produced by the method as defined in any of claims 1 to 19 to produce propylene.

23. A method of claim 1 further comprising:

polymerizing the propylene produced by the method as defined in any one of claims 21 and 22 to produce polypropylene.

24. n-propanol produced by the method of any one of claims 1 to 23.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NADH
Phosphoenolypyruvate
NADH
NAD+
ADP
ATP
CO2
CoA
Pi
NADH
NAD+
NADH
NAD+
Acetyl phosphate
Acetyl-CoA
Pyruvate
Oxaloacetate
Malate
CO2
CoA
ADP
ATP
Methylmalonyl-Coa
H2O
NAD(P)+
NAD(P)H
Propionaldehyde
Propionyl-Coa
Fumarate
Acetate
Succinyl-CoA
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FP
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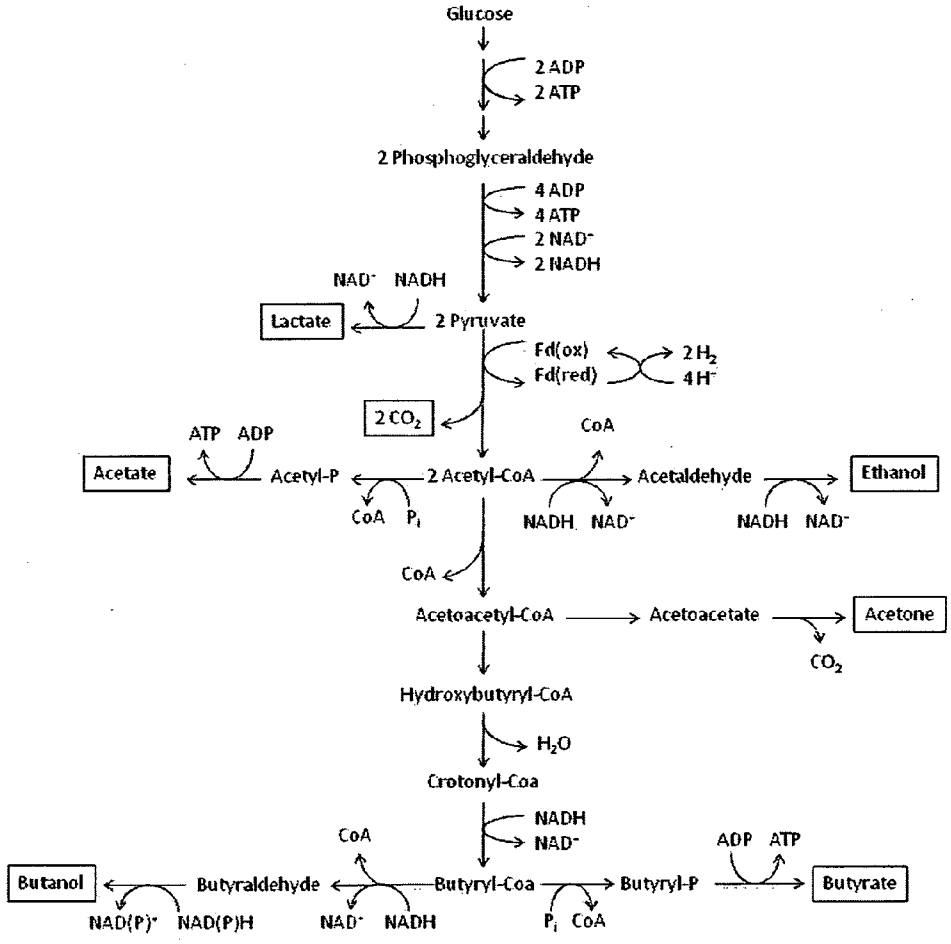
NAD(P)+
NAD(P)+
Propanol
NAD(P)H
Succinate
Propionate

Figure 1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2 ADP
2 ATP
2 Phosphoglyceraldehyde
4 ADP
4 ATP
2 NAD+
2 NADH
NAD+ NADH
Lactate
2 Pyruvate
Fd(ox)
Fd(red)
2 H2
4 H+
2 CO2
ATP ADP
Acetate
Acetyl-P
2 Acetyl-CoA
CoA
Acetaldehyde
Ethanol
CoA Pi
NADH NAD+
NADH NAD+
CoA
Acetoacetyl-CoA
Acetoacetate
Acetone
CO2
Hydroxybutyryl-CoA
H2O
Crotonyl-Coa
NADH
NAD+
CoA
ADP ATP
Butanol
Butyraldehyde
Butyryl-Coa
Butyryl-P
Butyrate
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NAD+ NADH
Pi CoA

Figure 2

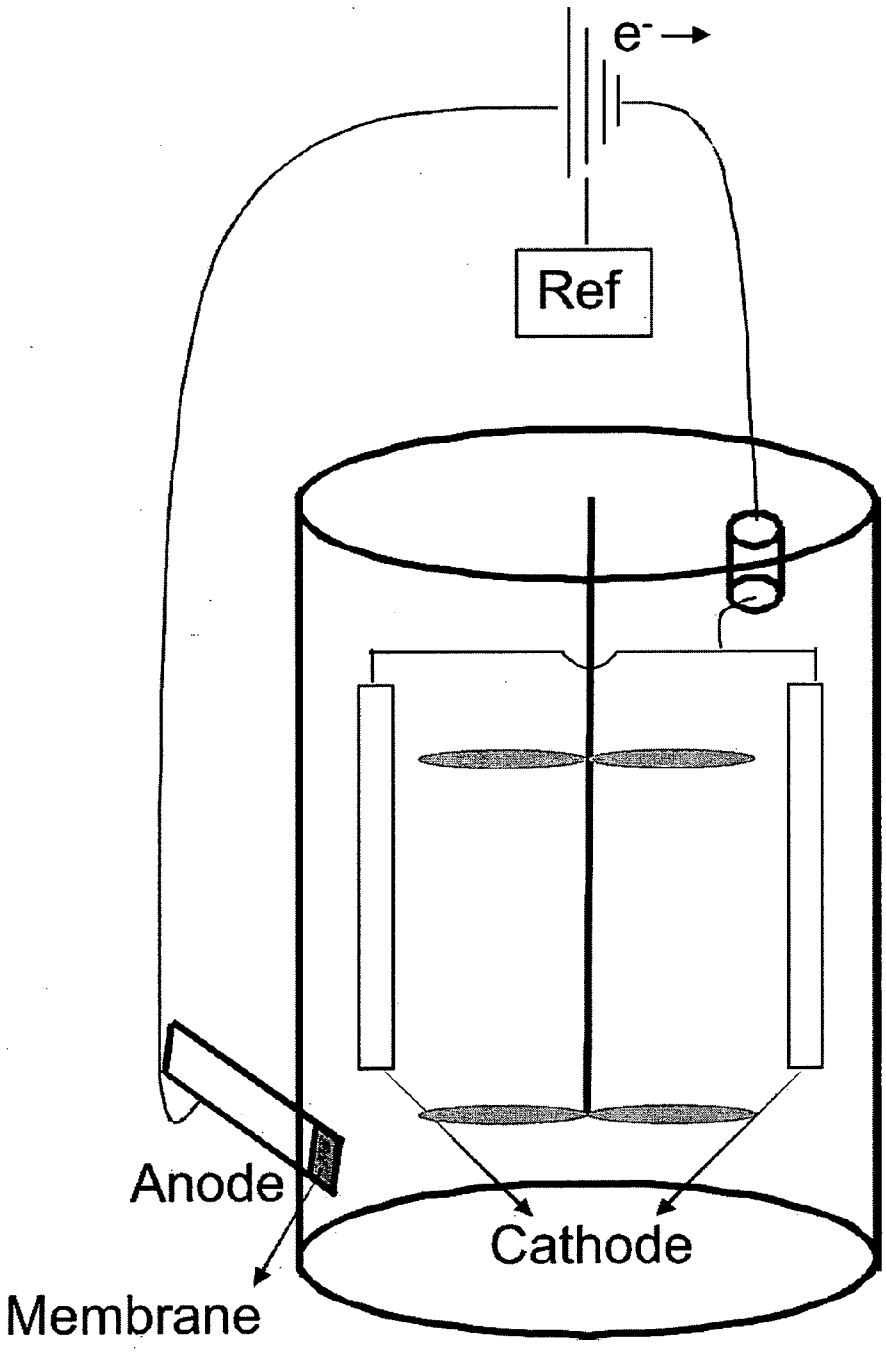

Figure 3

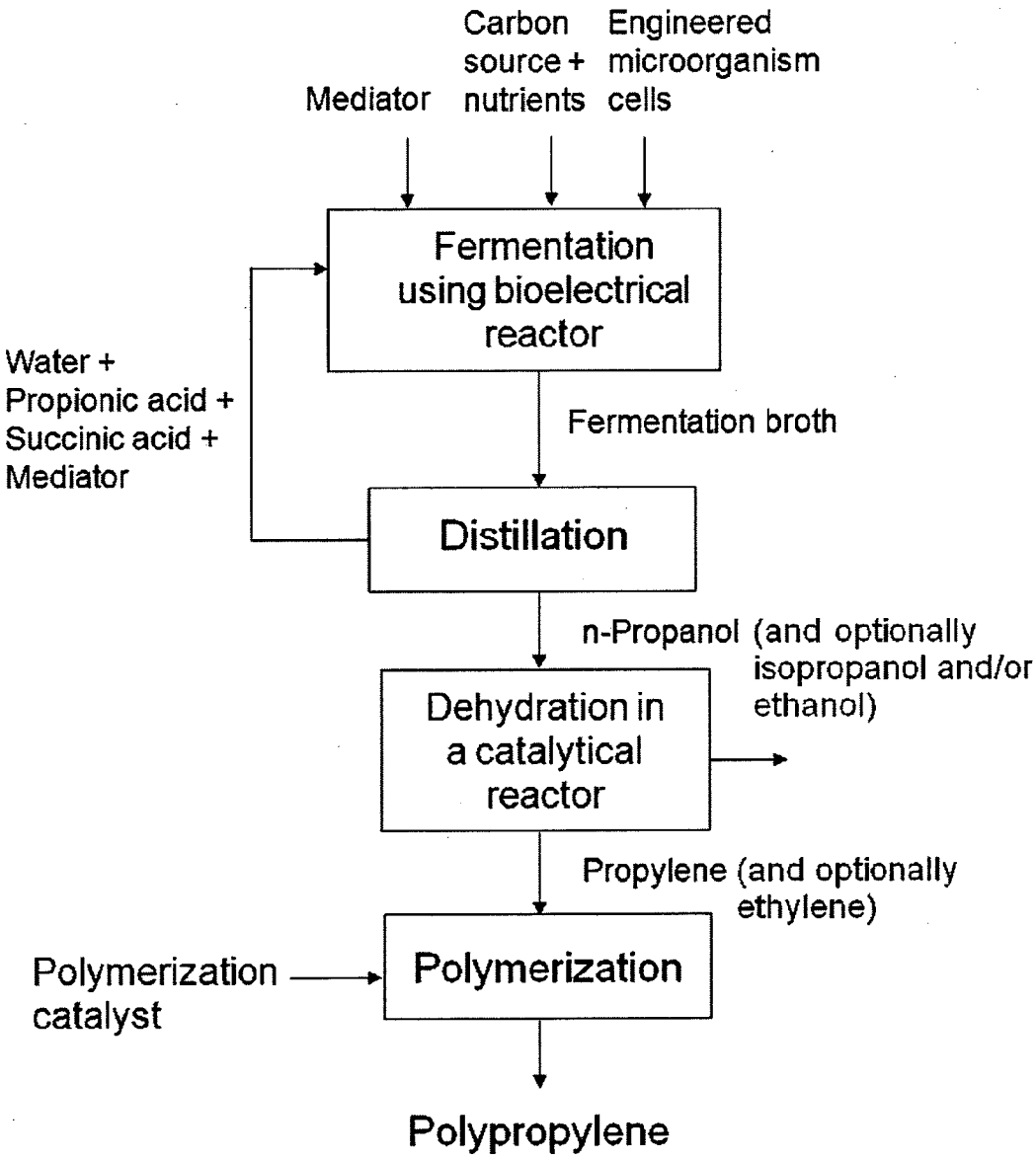
Mediator
Carbon source + nutrients
Engineered microorganism cells
Fermentation using bioelectrical reactor
Water + Propionic acid + Succinic acid + Mediator
Fermentation broth
Distillation
n-Propanol (and optionally isopropanol and/or ethanol)
Dehydration in a catalytical reactor
Propylene (and optionally ethylene)
Polymerization catalyst
Polymerization
Polypropylene

Figure 4

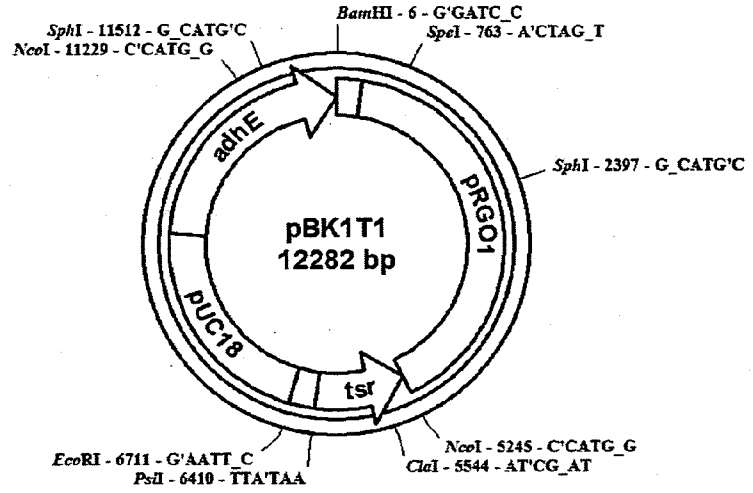

Figure 5

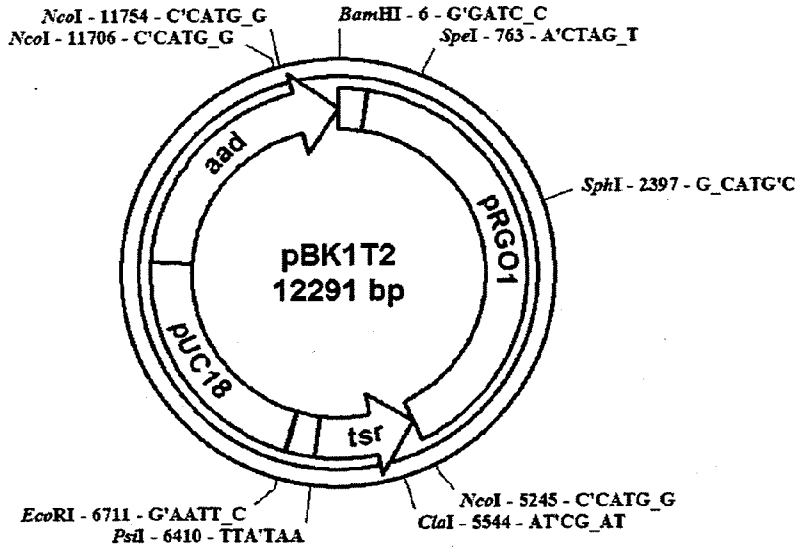

Figure 6

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 151 TTGTCCGTCG CGGGTGCAAG GATTGGACCC ATGAGTCCGC GAAAGATTGG
 201 CGTTACCGAG CTCGCGCTCC GCGACGCGCA TCAGAGCCTG ATTGCAACCC
 251 GG(ATG)ACTGA GTTGGACACC ATCGCAAATC CGTCCGATCC CGCGGTGCAG
 301 CGGATCATCG ATGTCACCAA GCCGTCGCGA TCCAACATAA AGACAACGTT
 351 GATCGAGGAC GTCGAGCCCC TCATGCACAG CATCGCGGCC GGGGTGGAGT
 401 TCATCGAGGT CTACGGCAGC GACAGCAGTC CTTTTCCATC TGAGTTGCTG
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1151 GGGGCAAGGC AGGCCTCCTA CTTATAAACG ATC
```

Figure 7

```
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 501 ACCTGCGGCA TCCTGGAGGA GGACGAGGGC TTCGGCATGG TCAAGATCGC
 551 CGAGCCGGTC GGCGTCATCG CCGCGGTCAT CCCGACCACC AACCCCACCT
 601 CCACCGCCAT CTTCAAGGCC CTCCTGGCCC TCAAGACCCG CAACGGCATC
 651 ATCTTCTCCC CGCACCCGCG CGCCAAGAAG TGCACCATCG CCGCGGCCAA
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1051 GCGCCTACAT CCTGTCCAAG GACGAGACCA CCAAGGTCGG CAAGATCCTC
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1451 GGGCGCCATC GGCGACATCT ACAACTTCAA GCTCGCCCCC TCCCTGACCC
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```

Figure 8 (Sheet 1 of 2)

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2751 GACGACCAGT GCACCGGCGC CAACCCGCGC TACCCGCTCA TCTCCGAGAT
2801 CAAGCAGATG TACGTGAACG CCTTC(TGA)TG ATCAGGCTGA GAACGACCTG
2851 ATCCGCCACT CGCGGAACTC CGGACGCCGC GTCCCCTCGG GGGCGCGGCG
2901 TCCTGCATGT CCGGGCCGCAG GGGCAAGGCA GGCCTCCTAC AAGCTTGAGT

Figure 8 (Sheet 2 of 2)

```
   1 GAGTTCTAGA GTGTGCCATT TCTCACAATC CCGGGGTGCG ATTGTCGCGT
  51 TTCCCACAGG AATCGGCGCG GGGATCTGGA GGGTGCTGCG ACACGCCCAT
 101 ATTTTGAACG ATGTTCAGTG CGTCAACCTC GACCCCAGTG CTGAACTTGT
 151 CCGTCGCGGG TGCAAGGATT GGACCCATGA GTCCGCGAAA GATTGGCGTT
 201 ACCGAGCTCG CGCTCCGCGA CGCGCATCAG AGCCTGATTG CAACCCGG(AT
 251 G)AAGGTCACC ACCGTCAAGG AGCTGGACGA GAAGCTCAAG GTCATCAAGG
 301 AGGCCCAGAA GAAGTTCTCG TGCTACTCGC AGGAGATGGT GGACGAGATC
 351 TTCCGCAACG CCGCGATGGC CGCGATCGAC GCCCGCATCG AGCTCGCCAA
 401 GGCCGCGGTC CTGGAGACCG GCATGGGCCT CGTCGAGGAC AAGGTGATCA
 451 AGAACCACTT CGCCGGCGAG TACATCTACA ACAAGTACAA GGACGAGAAG
 501 ACCTGCGGCA TCATCGAGCG CAACGAGCCG TACGGCATCA CCAAGATCGC
 551 CGAGCCCATC GGCGTCGTCG CCGCGATCAT CCCCGTCACC AACCCGACCT
 601 CCACCACGAT CTTCAAGTCG CTGATCTCGC TCAAGACCCG CAACGGCATC
 651 TTCTTCTCGC CGCACCCGCG CGCCAAGAAG TCGACCATCC TGGCCGCGAA
 701 GACCATCCTG GACGCCGCGG TCAAGTCGGG CGCCCCCGAG AACATCATCG
 751 GCTGGATCGA CGAGCCCTCG ATCGAGCTGA CCCAGTACCT GATGCAGAAG
 801 GCCGACATCA CCCTCGCCAC CGGCGGGCCC TCGCTCGTCA AGTCGGCCTA
 851 CTCGTCCGGC AAGCCCGCCA TCGGCGTGGG GCCGGGCAAC ACCCCCGTCA
 901 TCATCGACGA GTCCGCCCAC ATCAAGATGG CCGTCTCCTC CATCATCCTC
 951 TCCAAGACCT ACGACAACGG CGTCATCTGC GCCTCGGAGC AGTCCGTGAT
1001 CGTCCTCAAG TCGATCTACA ACAAGGTCAA GGACGAGTTC CAGGAGCGCG
1051 GCGCCTACAT CATCAAGAAG AACGAGCTGG ACAAGGTGCG CGAGGTCATC
1101 TTCAAGGACG GCTCGGTGAA CCCCAAGATC GTCGGCCAGT CGGCCTACAC
1151 CATCGCCGCG ATGGCCGGCA TCAAGGTCCC GAAGACCACG CGCATCCTCA
1201 TCGGCGAGGT CACCTCCCTG GGCGAGGAGG AGCCCTTCGC CCACGAGAAG
1251 CTCTCGCCCG TCCTGGCCAT GTACGAGGCC GACAACTTCG ACGACGCCCT
1301 CAAGAAGGCC GTCACCCTGA TCAACCTCGG CGGGCTGGGC CACACCTCCG
1351 GCATCTACGC CGACGAGATC AAGGCCCGCG ACAAGATCGA CCGCTTCTCC
1401 TCGGCCATGA AGACCGTCCG CACCTTCGTC AACATCCCCA CCTCGCAGGG
1451 CGCCTCCGGC GACCTGTACA ACTTCCGCAT CCCGCCCTCC TTCACCCTCG
1501 GCTGCGGCTT CTGGGGGGGC AACTCCGTCT CGGAGAACGT GGGCCCGAAG
1551 CACCTGCTGA ACATCAAGAC CGTGGCCGAG CGCCGCGAGA ACATGCTGTG
1601 GTTCCGCGTC CCCCACAAGG TCTACTTCAA GTTCGGCTGC CTCCAGTTCG
1651 CCCTCAAGGA CCTCAAGGAC CTCAAGAAGA AGCGCGCCTT CATCGTCACC
```

Figure 9 (Sheet 1 of 2)

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1701 GACTCGGACC CCTACAACCT GAACTACGTC GACTCCATCA TCAAGATCCT
1751 CGAGCACCTC GACATCGACT TCAAGGTCTT CAACAAGGTG GGCCGCGAGG
1801 CCGACCTCAA GACCATCAAG AAGGCCACCG AGGAGATGTC GTCCTTCATG
1851 CCCGACACCA TCATCGCCCT GGGCGGGACC CCGGAGATGT CCTCCGCCAA
1901 GCTGATGTGG GTCCTCTACG AGCACCCCGA GGTCAAGTTC GAGGACCTGG
1951 CCATCAAGTT CATGGACATC CGCAAGCGCA TCTACACCTT CCCCAAGCTG
2001 GGCAAGAAGG CCATGCTCGT GGCCATCACC ACGTCCGCCG GCTCCGGCTC
2051 CGAGGTCACC CCCTTCGCCC TCGTGACCGA CAACAACACC GGCAACAAGT
2101 ACATGCTCGC CGACTACGAG ATGACCCCCA ACATGGCCAT CGTGGACGCC
2151 GAGCTCATGA TGAAGATGCC GAAGGGCCTC ACCGCCTACT CGGGCATCGA
2201 CGCCCTGGTC AACTCGATCG AGGCCTACAC CTCCGTCTAC GCCTCCGAGT
2251 ACACCAACGG CCTCGCCCTC GAGGCCATCC GCCTGATCTT CAAGTACCTC
2301 CCGGAGGCCT ACAAGAACGG CCGCACCAAC GAGAAGGCCC GCGAGAAGAT
2351 GGCCCACGCG TCCACCATGG CCGGCATGGC GTCCGCCAAC GCCTTCCTCG
2401 GCCTCTGCCA CTCCATGGCC ATCAAGCTGT CCTCGGAGCA CAACATCCCC
2451 TCCGGCATCG CCAACGCCCT CCTCATCGAG GAGGTCATCA AGTTCAACGC
2501 CGTGGACAAC CCGGTGAAGC AGGCCCCCTG CCCGCAGTAC AAGTACCCCA
2551 ACACCATCTT CCGCTACGCC CGCATCGCCG ACTACATCAA GCTGGGCGGG
2601 AACACCGACG AGGAGAAGGT CGACCTCCTC ATCAACAAGA TCCACGAGCT
2651 CAAGAAGGCC CTCAACATCC CGACCTCCAT CAAGGACGCC GGCGTGCTGG
2701 AGGAGAACTT CTACTCCTCC CTGGACCGCA TCTCGGAGCT CGCCCTGGAC
2751 GACCAGTGCA CCGGCGCCAA CCCGCGCTTC CCGCTCACCT CCGAGATCAA
2801 GGAGATGTAC ATCAACTGCT TCAAGAAGCA GCCC(TGA)TGA TCAGGCTGAG
2851 AACGACCTGA TCCGCCACTC GCGGAACTCC GGACGCCGCG TCCCCTCGGG
2901 GGCGCGGCGT CCTGCATGTC CGGGCGCAGG GGCAAGGCAG GCCTCCTACA
2951 AGCTTGAGT
```

Figure 9 (Sheet 2 of 2)

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   1 CTAGAGGATC CGGCGGAACT TCACGTCCTG GCGGTGGAGT TGGCGGGCGC
  51 GTTCCAGCCG TTCCTCCAGC ACGGTGATCC GGGCCTCCAG ACGCTCACGC
 101 TCACCCTGCT CCAGGTGCCG GGTCACCGTC ACCGTCCGCA CCGGCCGGGC
 151 CTCGGCCTGG GCGGCCCGGC GTTCCTCACT GGCCCGCTTC CGGCAATCGT
 201 CGGAACACCA CACCCGGGGC CGACCCCGCC CACCGTGGGC CTCCACCGGC
 251 GCCCCGCAGT GGGGACACGC CCGCAGCGCC GACGCATCCT CATCCAAGGC
 301 CATCACCGGG TCGGAATCCA TACCCGAAAC CATATCGTCC GGACGATGAA
 351 CTGCGCCAGA CAGCTAAGAA TGCACGAGGT GTGTCTCCGA TTCTCAGGAA
 401 ACGCTCAGCA TTTTCCGAGA CGTTCGGCGC ACGCACACAC CCCCACAAGA
 451 ACCGACCCGC CCAGCATCCG CCGACACGTC GATCCGCACC CGCGATGGGC
 501 TGGCCGAGGC CGACTACGAC CGCTAGTCAG CACCTGCGCT GATCTACCGT
 551 CGCCCTGACC GACTCTCCCG TCGGGATTGT CGCCGGCCGC TGCCAGCATG
 601 GACCTGCGGC CCCGCCCCCT CGCCCTGCAA CTCGAGGGAG GCGGGGCCGT
 651 CCACCCCCCA CACCACCCCG ACACCGTGAT GCGCCCATGT CGCCTAACGG
 701 GTTGCCCGAC CTCCCCGACA TCAAGAAAAC CTGACACCGT CGCCGCAAGC
 751 GCTACACTGA CTACTAGTAG TCAGGAGGTG CGTGATGACC ATCGCCACAT
 801 CGGTGAAACT CTCCGAAGAG ACCGGCCGCA AACTCGATGA ACTAGCCCGG
 851 GCCACCGGGC GATCCAAGTC CTACTACCTG CGCGAGGCCA TCGAGGACCA
 901 CATCGACCAG ATGGTCCACG ACTACGCCAT CGCCCGACTC GCCGACGACG
 951 TGCGAGCCGG CCGGGCCGCC ACCTACAGCG CCGACGAAGT GGACCAGATC
1001 CTTGGCCTGG ACGATTGAGT ACACCGACCC CGCCGTCAAA GCACTGCGCA
1051 AACTCGACCG AGCCCAGGCC CGCCGCATCA CCGCCTACAT ACGTGAGCTC
1101 ACCGGCCTGG ACGATCCCCA CCAACGCGGG AAAGGCCTCA CCGGGCCCCT
1151 GGCCGGACTC TGGCGCTACC GCGTCGGGGA CTACCGGATC ATCTGCGACC
1201 TGAACGCCGA CCGCCTGGCC ATCATCGCCC TGACCATCGA GCACCGATCC
1251 CAGGCCTACC GCTGACACGC AACCCCGCAC CCTCGGCCAA GACGTCACAC
1301 ACCACCCGCC CCACCGAGCA CTGAGGATGT CAACTCGCCC GAGCCGGCCT
1351 GCCGGCCGTC TTACGGGTTG TCTTGGCGGG CGGGGTGTCT TTGCCCTGGC
1401 CCAGCAGCCC CACGATCTCC CGCAGCGTGT CGGCGGTGGC GGCGTCCCGG
1451 GCCGCCTGAC GCTCCGCCTC CGCCCTGGCC TGCTCGGCTG CCTGCGCCCG
1501 ATCCTCCGCG GCGGCGGCCT GCTCCCTCGC CTCGGCCAGC TCGCCGGTCA
1551 GGGCCTCGAC CCGGGCCTGC ACCTGCCCCA GGCGCGCCTC CGCCTCCTGC
1601 TGCACCTGCT CGGCCCGGGC CTCCGCCTGG TCCCGGGCCG CCTCGGCCTC
1651 GGCCCGGTGC TGATCCGCCA GGGCCGCCTC GGCCACCGCT TCGGCCTGCC
```

Figure 10 (Sheet 1 of 7)

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1701 CATCCACCGC CTGCTCGGCC CGAGCCCCGA ACTCCTCGCG GGCCGCATCA
1751 CTCGCCTGAC GCCACGCCGC CGCCCACACC AGACCCAACG GCTCCGACAG
1801 ATCCGGCGGG GCCGGCGTCT GGACCGACGC CGAGACGTCG CGCAGGAACC
1851 CCGCCGCAGC GTCGGTGGAG CACCCCGCCT CCGCCTTCAA CGACCGCACC
1901 GTCACCCGCC GACCCGCACC GCTCAACCGC GCATAGGCCG CCGCCAACCT
1951 TGACCCATTC GACTCCATGA CCCACCCTCC CATTCTGTAC CCTGTACCTG
2001 TTCCTAGGTA CGTTCCTAAT GTACCTCACC GGATGCAGAA CCCGCAACCC
2051 CCCTCACACT CCCCCTGCAC GGGGCCCGCC CCCTGCACCC CCGCTGCCGC
2101 GCCCGCTCCT GCGTCGCGGC CTTGCCCCTG CCCAACGCCG GGCCGGCGGG
2151 CAGCCCACCA GAGGCTCTGT GAGACGTCGG CGCCCCCGTC CACCTACCCT
2201 AAAGACCAAC CGGCCGTGGA AACGTCTGTG AGGAGCCTTG TAGGAGTTCC
2251 CAGGACAAGC CAGCAAGGCC GGGCCTGACG GCCCGGAAAG GAAGTCGCTG
2301 CGCTCCTACG AAGAAGCCCC TCTGGGGACC CCCAGACCCC GGAACTATCT
2351 GATTTGGTTT AGCGGCGTAC TTCCGTCATA CCGGAATTTA TGGCATGCTG
2401 TGGTCATGGC GACGACGACG GTCGATGAGC AGTGGGAGCA GGTGTGGCTG
2451 CCCCGCTGGC CCCTGGCCTC CGACGACCTG GCAGCGGGCA TCTACCGGAT
2501 GGCCCGCCCC TCGGCGCTGG GGGTCCGATA CATCGAGGTC AACCCCCAAG
2551 CCATCAGCAA CCTCCTCGTG GTCGACTGCG ACCACCCCGA CGCTGCCATG
2601 CGCGCCGTCT GGGACCGCCA CGACTGGCTG CCCAACGCCA TCGTCGAGAA
2651 CCCCGACAAC GGCCACGCCC ACGCCGTGTG GGCCCTGGAA GCAGCCATCC
2701 CGCGCACCGA GTACGCCCAC CGCAAGCCCA TCGCCTACGC CGCCGCCGTC
2751 ACCGAGGGCC TGCGCCGATC CGTCGACGGA GACGCCTCCT ACGCCGGCCT
2801 GATCACCAAG AACCCCGAAC ACCCCGCCTG GAACACCACC TGGTGCACCG
2851 ACCACCTCTA CCGGCTGGCC GAGCTCGACA CCCACCTGGA TGCCGCCGGC
2901 CTCATGCCCG CCCCCTCCTG GCGACGCACC CGCCGGCGCA ACCCCGTCGG
2951 CCTGGGCCGC AACTGCGCCA TCTTCGAGAC CGCCCGCACC TGGGCCTACC
3001 GCGACGCCCG CCGCATCCGA CAACGCCACG AATACCCGAC CGCCGAGGAC
3051 TCGGCCGACC TGCACGCCGT CATCGCCTCC ACCGTCGAGG CGCTCAACGC
3101 CGGCTACAGC GAACCCCTGC CGGCCCGCGA GGCCGCCGGC ATCGCCGCCA
3151 GCATCCACCG ATGGATCACC CACCGTTTCT ACGGCTGGAT CGACTCCCAC
3201 ACCGTCAACG AGGCCACTTT CTCCACCATC CAGAGCTACA GAGGACACAA
3251 GGGAGCCGGC AAGGCTCGTC CTCGTGCCCG CCGTGCTGCT TCTATCACCG
3301 ATTGGGAGGC ATGATGGCTG ACGTCCAGCA CCGCGTGAAG CGTCGGGGCA
3351 CGGCCCGCGA GGCCGCAGAA CGTGTAGGGG CCTCCATCCG AACCGCCCAG
```

Figure 10 (Sheet 2 of 7)

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3401 CGGTGGACCT CCATCCCCCG TGAGGAATGG ATCACTCAGA AGGCCGTCGA
3451 GCGTGAGGAG ATCCGGGCCT ACAAGTACGA CGAGGGGCAC ACGTGGGGCG
3501 AGACCTCGCG CCACTTCGGG ATCGCGAAGA CCACCGCCCA GGAGCGGGCC
3551 CGGCGGGCTC GAAGGGAGCG GGCGGCCGAA GCGGAGAAGG CTGCCGAGGA
3601 GGCCGAGGCC GCGCTGCGTC CGACACTCTT CGAGGGCCAG GAGCAAGGTT
3651 CTGCATGAGC AACCCCGAGT CCTCGGGTAG ACCGTCTGGC CCGACGTTAA
3701 GCATGGCTGA AGCGGCCCGT GCCTGTGGGG TTTCAGTGTC CACGGTGAGG
3751 CGTCACCGTG ATGCCCTGGT GGCCCACGGT GCTACCCGTC ATGACGCGTC
3801 ATGGGTGATA CCCCTATCAG CGTTGATTTC ATGCGGTTTG ATGCCCCGGG
3851 TGACACCCCC TGATGCCCCG TCACCCAATA ACGTGGCGCC TGCCATGACG
3901 TCCCACGGTG ACGCCCCCCT GACGGGGGAA GTCCAAGAGC TGCGCGAGCG
3951 ACTGGCCAAC GCTGAGCATC GAGCCGAGCT AGCCGAAGCC ATCGCGGCCG
4001 AGCGACAACA CACGATCGAC GCCCAGCGCA TCGCCTTACG GGCCTTAGAA
4051 CCCGGCTCGA CCCATAACAG CCCGGCAACC GATGAGCCGG CTACCGCTCG
4101 CGAGCAACCT CCCGGTCCAG AACCCAGCGA CTCCAGGCCA CACCGCCGGA
4151 GTTGGTGGCG TCGGCTGACT GGTGGCGCCT GACCGGCCCC GGTCGTCTTC
4201 GAGGGGAACC TCTCGCCTGC GAGAGGACAC AGCAGCCGGC TGTGCTGGTA
4251 GGGCATCCCA GCACGACACC CCTCTGACGC GAGAAGTTCA AGGACTACGC
4301 GAATTGCTGA CTACCGCCGA GCGGCAGCAC ACGATCGAGA TGCTCAACGA
4351 ACCGCACTAC GCGGCCTTAG AAGGCCCCAA GGCACGCTCA CCTACCACGT
4401 GGATCACCAC CGATCGGCGC CGACAGCTAT GGACCCCATC GCAAGATCAA
4451 AACCCCTGAG CAGCCATCGC ACCGAGCGCC CGGCACGCCG GAAGAAGCTC
4501 CGACGCCCCT GCTGTCCGGA CACGGCCTAA CGCGTCCAGA CCAGAACCAG
4551 TGCTCCGATC TAAACCGAAG GCCCTTCATG TGAGAGCATA GTCGTGACGT
4601 CGGCACAGTA GTCGTGCCCG GCGGGGGTAA CGCTACACAA CGCTTAAAAA
4651 GCATCGGAGC AAGCTAACAC AGGGGGACTG ATGAACAAAA CACACAAAAT
4701 GGCGACGCTG GTAATTGCCG CGATCTTGGC CGCCGGAATG ACCGCACCAA
4751 CTGCCTATGC AGATTCTCCT GGAAACACCA GAATTACAGC CAGCGAGCAA
4801 AGCGTCCTTA CCCAGATACT CGGCCACAAA CCTACACAAA CTGAATATAA
4851 CCGATACGTT GAGACTTACG GAAGCGTACC GACCGAAGCA GACATCAACG
4901 CATATATAGA AGCGTCTGAA TCTGAGGGAT CATCAAGTCA AACGGCTGCT
4951 CACGATGACT CGACATCACC CGGCACGAGT ACCGAAATCT ACACGCAGGC
5001 AGCCCCTGCC AGGTTCTCAA TGTTTTTCCT GTCCGGAACT TGGATCACTA
5051 GGAGTGGTGT AGTATCGCTC TCCTTGAAGC CAAGGAAGGG TGGTATTGGC
```

Figure 10 (Sheet 3 of 7)

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5101 AACGAGGGGG ACGAGCGTAC CTGGAAGACT GTATACGACA AATTCCATAA
5151 CGCTGGGCAA TGGACACGAT ACAAGAACAA CGGCGTAGAC GCCAGCATGA
5201 AAAAGCAGTA CATGTGCCAC TTCAAGTACG GGATGGTGAA GACGCCATGG
5251 GTGTGCCATT TCTCACAATC CCGGGGTGCG ATTGTCGCGT TTCCCACAGG
5301 AATCGGCGCG GGGATCTGGA GGGTGCTGCG ACACGCCCAT ATTTTGAACG
5351 ATGTTCAGTG CGTCAACCTC GACCCCAGTG CTGAACTTGT CCGTCGCGGG
5401 TGCAAGGATT GGACCCATGA GTCCGCGAAA GATTGGCGTT ACCGAGCTCG
5451 CGCTCCGCGA CGCGCATCAG AGCCTGATTG CAACCCGGAT GACTGAGTTG
5501 GACACCATCG CAAATCCGTC CGATCCCGCG GTGCAGCGGA TCATCGATGT
5551 CACCAAGCCG TCGCGATCCA ACATAAAGAC AACGTTGATC GAGGACGTCG
5601 AGCCCCTCAT GCACAGCATC GCGGCCGGGG TGGAGTTCAT CGAGGTCTAC
5651 GGCAGCGACA GCAGTCCTTT TCCATCTGAG TTGCTGGATC TGTGCGGGCG
5701 GCAGAACATA CCGGTCCGCC TCATCGACTC CTCGATCGTC AACCAGTTGT
5751 TCAAGGGGGA GCGGAAGGCC AAGACATTCG GCATCGCCCG CGTCCCTCGC
5801 CCGGCCAGGT TCGGCGATAT CGCGAGCCGG CGTGGGGACG TCGTCGTTCT
5851 CGACGGGGTG AAGATCGTCG GGAACATCGG CGCGATAGTA CGCACGTCGC
5901 TCGCGCTCGG AGCGTCGGGG ATCATCCTGG TCGACAGTGA CATCACCAGC
5951 ATCGCGGACC GGCGTCTCCA AAGGGCCAGC CGAGGTTACG TCTTCTCCCT
6001 TCCCGTCGTT CTCTCCGGTC GCGAGGAGGC CATCGCCTTC ATTCGGGACA
6051 GCGGTATGCA GCTGATGACG CTCAAGGCGG ATGGCGACAT TTCCGTGAAG
6101 GAACTCGGGG ACAATCCGGA TCGGCTGGCC TTGCTGTTCG GCAGCGAAAA
6151 GGGTGGGCCT TCCGACCTGT TCGAGGAGGC GTCTTCCGCC TCGGTTTCCA
6201 TCCCCATGAT GAGCCAGACC GAGTCTCTCA ACGTTTCCGT TTCCCTCGGA
6251 ATCGCGCTGC ACGAGAGGAT CGACAGGAAT CTCGCGGCCA ACCGATAATC
6301 AGGCTGAGAA CGACCTGATC CGCCACTCGC GGAACTCCGG ACGCCGCGTC
6351 CCCTCGGGGG CGCGGCGTCC TGCATGTCCG GGCGCAGGGG CAAGGCAGGC
6401 CTCCTACTTA TAATTGTCCC ATACGCGTCA TACTGGTTAG TCGCTGGAGA
6451 TCCAGACGTT TGGGACTTCT ATCGTTCTTT ATGGTGGATT CCAGTGGCTT
6501 TTCTAGGAAT AGTTTCAATA GTACTGATGG CTAGCAGTAG AGGTTGGGGA
6551 CGACGTCTCG GCGACTCCGG AGAACACCAA GTCAGGGTCT CATGAGTGTG
6601 CGATAGCTTG AGCTGTCTAC CAATCTGGAT ATAGCTATAT CGGTCGTTTG
6651 TGTCTGATTC GCCAGTGAGC CAACGGCGGG GGCGACACGC GGTGGCGAAA
6701 CCCCCTGGCA GAATTCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT
6751 TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG
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6801 TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC
6851 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA
6901 TGAATCGGCC AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC
6951 CGCTTCCTCG CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG
7001 CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG
7051 GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA
7101 CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG
7151 ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA
7201 GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC
7251 TCCTGTTCCG ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT
7301 CGGGAAGCGT GGCGCTTTCT CAAAGCTCAC GCTGTAGGTA TCTCAGTTCG
7351 GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA
7401 GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG
7451 TAAGACACGA CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC
7501 AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA
7551 CTACGGCTAC ACTAGAAGAA CAGTATTTGG TATCTGCGCT CTGCTGAAGC
7601 CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC
7651 ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG
7701 AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG
7751 CTCAGTGGAA CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA
7801 AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC
7851 AATCTAAAGT ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA
7901 TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT
7951 GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC
8001 TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG
8051 ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT
8101 CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC
8151 TAGAGTAAGT AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG
8201 CTACAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC
8251 TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA
8301 AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG
8351 CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT
8401 GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA
8451 GTCATTCTGA GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCGGCGT

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 8501 CAATACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC
 8551 ATTGGAAAAC GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
 8601 GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT
 8651 CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT
 8701 GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT
 8751 CTTCCTTTTT CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA
 8801 GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG
 8851 CGCACATTTC CCCGAAAAGT GCCACCTGAC GTCTAAGAAA CCATTATTAT
 8901 CATGACATTA ACCTATAAAA ATAGGCGTAT CACGAGGCCC TTTCGTCTCG
 8951 CGCGTTTCGG TGATGACGGT GAAAACCTCT GACACATGCA GCTCCCGGAG
 9001 ACGGTCACAG CTTGTCTGTA AGCGGATGCC GGGAGCAGAC AAGCCCGTCA
 9051 GGGCGCGTCA GCGGGTGTTG GCGGGTGTCG GGGCTGGCTT AACTATGCGG
 9101 CATCAGAGCA GATTGTACTG AGAGTGCACC ATATGCGGTG TGAAATACCG
 9151 CACAGATGCG TAAGGAGAAA ATACCGCATC AGGCGCCATT CGCCATTCAG
 9201 GCTGCGCAAC TGTTGGGAAG GGCGATCGGT GCGGGCCTCT TCGCTATTAC
 9251 GCCAGCTGGC GAAAGGGGGA TGTGCTGCAA GGCGATTAAG TTGGGTAACG
 9301 CCAGGGTTTT CCCAGTCACG ACGTTGTAAA ACGACGGCCA GTGCCACTAG
 9351 AGTGTGCCAT TTCTCACAAT CCCGGGGTGC GATTGTCGCG TTTCCCACAG
 9401 GAATCGGCGC GGGGATCTGG AGGGTGCTGC GACACGCCCA TATTTTGAAC
 9451 GATGTTCAGT GCGTCAACCT CGACCCCAGT GCTGAACTTG TCCGTCGCGG
 9501 GTGCAAGGAT TGGACCCATG AGTCCGCGAA AGATTGGCGT TACCGAGCTC
 9551 GCGCTCCGCG ACGCGCATCA GAGCCTGATT GCAACCCGGA TGAAGGTCAC
 9601 CAACGTCGAG GAGCTGATGA AGAAGATGCA GGAGGTGCAG AACGCCCAGA
 9651 AGAAGTTCGG CTCCTTCACC CAGGAGCAGG TCGACGAGAT CTTCCGCCAG
 9701 GCCGCGCTGG CCGCGAACTC GGCCCGCATC GACCTGGCCA AGATGGCCGT
 9751 CGAGGAGACC AAGATGGGCA TCGTCGAGGA CAAGGTGATC AAGAACCACT
 9801 TCGTCGCCGA GTACATCTAC AACAAGTACA AGAACGAGAA GACCTGCGGC
 9851 ATCCTGGAGG AGGACGAGGG CTTCGGCATG GTCAAGATCG CCGAGCCGGT
 9901 CGGCGTCATC GCCGCGGTCA TCCCGACCAC CAACCCCACC TCCACCGCCA
 9951 TCTTCAAGGC CCTCCTGGCC CTCAAGACCC GCAACGGCAT CATCTTCTCC
10001 CCGCACCCGC GCGCCAAGAA GTGCACCATC GCCGCGGCCA AGCTGGTGCT
10051 CGACGCCGCG GTGAAGGCCG GCGCCCCGAA GGGCATCATC GGCTGGATCG
10101 ACGAGCCCTC CATCGAGCTG TCGCAGATCG TCATGAAGGA GGCCGACATC
10151 ATCCTGGCCA CCGGCGGCCC CGGCATGGTG AAGGCCGCGT ACTCGTCCGG
```

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10201 CAAGCCCGCC ATCGGCGTCG GCCCCGGCAA CACCCCCGCC CTGATCGACG
10251 AGTCCGCCGA CATCAAGATG GCCGTCAACT CCATCCTGCT GTCCAAGACC
10301 TTCGACAACG GCATGATCTG CGCCTCCGAG CAGTCGGTGG TCGTCGTCGA
10351 CTCGATCTAC GAGGAGGTGA AGAAGGAGTT CGCCCACCGC GGCGCCTACA
10401 TCCTGTCCAA GGACGAGACC ACCAAGGTCG GCAAGATCCT CCTGGTCAAC
10451 GGCACCCTGA ACGCCGGCAT CGTCGGCCAG TCGGCCTACA AGATCGCCGA
10501 GATGGCCGGC GTGAAGGTCC CGGAGGACGC CAAGGTGCTC ATCGGCGAGG
10551 TCAAGTCGGT GGAGCACTCC GAGGAGCCGT TCTCCCACGA GAAGCTCTCG
10601 CCCGTCCTGG CCATGTACCG CGCCAAGAAC TTCGACGAGG CCCTGCTCAA
10651 GGCCGGCCGC CTCGTCGAGC TGGGCGGGAT GGGCCACACC TCGGTCCTGT
10701 ACGTCAACGC CATCACCGAG AAGGTGAAGG TGGAGAAGTT CCGCGAGACC
10751 ATGAAGACCG GCCGCACCCT GATCAACATG CCCTCCGCCC AGGGCGCCAT
10801 CGGCGACATC TACAACTTCA AGCTCGCCCC CTCCCTGACC CTCGGCTGCG
10851 GCTCCTGGGG CGGCAACTCC GTGTCCGAGA ACGTGGGCCC GAAGCACCTG
10901 CTGAACATCA AGTCGGTGGC CGAGCGCCGC GAGAACATGC TGTGGTTCCG
10951 CGTGCCGGAG AAGGTCTACT TCAAGTACGG CTCCCTCGGC GTCGCCCTCA
11001 AGGAGCTCGA CATCCTCGAC AAGAAGAAGG TGTTCATCGT GACCGACAAG
11051 GTGCTGTACC AGCTGGGCTA CATCGACCGC GTCACCAAGA TCCTCGAGGA
11101 GCTCAAGATC TCCTACAAGA TCTTCACCGA CGTCGAGCCC GACCCCACCC
11151 TGGCCACCGC CAAGAAGGGC GCCGAGGAGC TGCTGTCCTT CAACCCCGAC
11201 ACCATCATCG CCGTGGGCGG GGGCTCCGCC ATGGACGCCG CCAAGATCAT
11251 GTGGGTGATG TACGAGCACC CGGAGGTGCG CTTCGAGGAC CTCGCCATGC
11301 GCTTCATGGA CATCCGCAAG CGCGTCTACA CCTTCCCGAA GATGGGCGAG
11351 AAGGCCATGA TGATCTCGGT GGCCACCTCG GCCGGCACCG GCTCGGAGGT
11401 CACCCCCTTC GCCGTCATCA CCGACGAGAA GACCGGCGCC AAGTACCCCC
11451 TGGCCGACTA CGAGCTGACC CCGAACATGG CCATCATCGA CGCCGAGCTC
11501 ATGATGGGCA TGCCGAAGGG CCTCACCGCC GCGTCCGGCA TCGACGCCCT
11551 GACCCACGCG ATCGAGGCCT ACGTGTCGAT CATGGCCTCC GAGTACACCA
11601 ACGGCCTGGC CCTGGAGGCC ATCCGCCTGA TCTTCAAGTA CCTCCCGATC
11651 GCCTACTCGG AGGGCACCAC CTCCATCAAG GCCCGCGAGA AGATGGCCCA
11701 CGCCTCGACC ATCGCCGGCA TGGCCTTCGC CAACGCCTTC CTCGGCGTCT
11751 GCCACTCGAT GGCCCACAAG CTGGGCTCGA CCCACCACGT CCCCCACGGC
11801 ATCGCCAACG CCCTGCTGAT CAACGAGGTG ATCAAGTTCA ACGCCGTCGA
11851 GAACCCCCGC AAGCAGGCCG CCTTCCCGCA GTACAAGTAC CCGAACATCA

11901 AGAAGCGCTA CGCCCGCATC GCCGACTACC TCAACCTCGG CGGCTCGACC
11951 GACGACGAGA AGGTCCAGCT CCTGATCAAC GCCATCGACG AGCTCAAGGC
12001 CAAGATCAAC ATCCCGGAGT CCATCAAGGA GGCCGGCGTC ACCGAGGAGA
12051 AGTTCTACGC CACCCTCGAC AAGATGTCGG AGCTCGCCTT CGACGACCAG
12101 TGCACCGGCG CCAACCCGCG CTACCCGCTC ATCTCCGAGA TCAAGCAGAT
12151 GTACGTGAAC GCCTTCTGAT GATCAGGCTG AGAACGACCT GATCCGCCAC
12201 TCGCGGAACT CCGGACGCCG CGTCCCCTCG GGGGCGCGGC GTCCTGCATG
12251 TCCGGGCGCA GGGGCAAGGC AGGCCTCCTA CA
```

Figure 10 (Sheet 7 of 7)

```
   1 CTAGAGGATC CGGCGGAACT TCACGTCCTG GCGGTGGAGT TGGCGGGCGC
  51 GTTCCAGCCG TTCCTCCAGC ACGGTGATCC GGGCCTCCAG ACGCTCACGC
 101 TCACCCTGCT CCAGGTGCCG GGTCACCGTC ACCGTCCGCA CCGGCCGGGC
 151 CTCGGCCTGG GCGGCCCGGC GTTCCTCACT GGCCCGCTTC CGGCAATCGT
 201 CGGAACACCA CACCCGGGGC CGACCCCGCC CACCGTGGGC CTCCACCGGC
 251 GCCCCGCAGT GGGGACACGC CCGCAGCGCC GACGCATCCT CATCCAAGGC
 301 CATCACCGGG TCGGAATCCA TACCCGAAAC CATATCGTCC GGACGATGAA
 351 CTGCGCCAGA CAGCTAAGAA TGCACGAGGT GTGTCTCCGA TTCTCAGGAA
 401 ACGCTCAGCA TTTTCCGAGA CGTTCGGCGC ACGCACACAC CCCCACAAGA
 451 ACCGACCCGC CCAGCATCCG CCGACACGTC GATCCGCACC CGCGATGGGC
 501 TGGCCGAGGC CGACTACGAC CGCTAGTCAG CACCTGCGCT GATCTACCGT
 551 CGCCCTGACC GACTCTCCCG TCGGGATTGT CGCCGGCCGC TGCCAGCATG
 601 GACCTGCGGC CCCGCCCCCT CGCCCTGCAA CTCGAGGGAG GCGGGGCCGT
 651 CCACCCCCCA CACCACCCCG ACACCGTGAT GCGCCCATGT CGCCTAACGG
 701 GTTGCCCCGAC CTCCCCGACA TCAAGAAAAC CTGACACCGT CGCCGCAAGC
 751 GCTACACTGA CTACTAGTAG TCAGGAGGTG CGTGATGACC ATCGCCACAT
 801 CGGTGAAACT CTCCGAAGAG ACCGGCCGCA AACTCGATGA ACTAGCCCGG
 851 GCCACCGGGC GATCCAAGTC CTACTACCTG CGCGAGGCCA TCGAGGACCA
 901 CATCGACCAG ATGGTCCACG ACTACGCCAT CGCCCGACTC GCCGACGACG
 951 TGCGAGCCGG CCGGGCCGCC ACCTACAGCG CCGACGAAGT GGACCAGATC
1001 CTTGGCCTGG ACGATTGAGT ACACCGACCC CGCCGTCAAA GCACTGCGCA
1051 AACTCGACCG AGCCCAGGCC CGCCGCATCA CCGCCTACAT ACGTGAGCTC
1101 ACCGGCCTGG ACGATCCCCA CCAACGCGGG AAAGGCCTCA CCGGGCCCCT
1151 GGCCGGACTC TGGCGCTACC GCGTCGGGGA CTACCGGATC ATCTGCGACC
1201 TGAACGCCGA CCGCCTGGCC ATCATCGCCC TGACCATCGA GCACCGATCC
1251 CAGGCCTACC GCTGACACGC AACCCCGCAC CCTCGGCCAA GACGTCACAC
1301 ACCACCCGCC CCACCGAGCA CTGAGGATGT CAACTCGCCC GAGCCGGCCT
1351 GCCGGCCGTC TTACGGGTTG TCTTGGCGGG CGGGGTGTCT TTGCCCTGGC
1401 CCAGCAGCCC CACGATCTCC CGCAGCGTGT CGGCGGTGGC GGCGTCCCGG
1451 GCCGCCTGAC GCTCCGCCTC CGCCCTGGCC TGCTCGGCTG CCTGCGCCCG
1501 ATCCTCCGCG GCGGCGGCCT GCTCCCTCGC CTCGGCCAGC TCGCCGGTCA
1551 GGGCCTCGAC CCGGGCCTGC ACCTGCCCCA GGCGCGCCTC CGCCTCCTGC
1601 TGCACCTGCT CGGCCCGGGC CTCCGCCTGG TCCCGGGCCG CCTCGGCCTC
1651 GGCCCGGTGC TGATCCGCCA GGGCCGCCTC GGCCACCGCT TCGGCCTGCC
```

Figure 11 (Sheet 1 of 7)

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1701 CATCCACCGC CTGCTCGGCC CGAGCCCCGA ACTCCTCGCG GGCCGCATCA
1751 CTCGCCTGAC GCCACGCCGC CGCCCACACC AGACCCAACG GCTCCGACAG
1801 ATCCGGCGGG GCCGGCGTCT GGACCGACGC CGAGACGTCG CGCAGGAACC
1851 CCGCCGCAGC GTCGGTGGAG CACCCCGCCT CCGCCTTCAA CGACCGCACC
1901 GTCACCCGCC GACCCGCACC GCTCAACCGC GCATAGGCCG CCGCCAACCT
1951 TGACCCATTC GACTCCATGA CCCACCCTCC CATTCTGTAC CCTGTACCTG
2001 TTCCTAGGTA CGTTCCTAAT GTACCTCACC GGATGCAGAA CCCGCAACCC
2051 CCCTCACACT CCCCCTGCAC GGGGCCCGCC CCCTGCACCC CCGCTGCCGC
2101 GCCCGCTCCT GCGTCGCGGC CTTGCCCCTG CCCAACGCCG GGCCGGCGGG
2151 CAGCCCACCA GAGGCTCTGT GAGACGTCGG CGCCCCCGTC CACCTACCCT
2201 AAAGACCAAC CGGCCGTGGA AACGTCTGTG AGGAGCCTTG TAGGAGTTCC
2251 CAGGACAAGC CAGCAAGGCC GGGCCTGACG GCCCGGAAAG GAAGTCGCTG
2301 CGCTCCTACG AAGAAGCCCC TCTGGGGACC CCCAGACCCC GGAACTATCT
2351 GATTTGGTTT AGCGGCGTAC TTCCGTCATA CCGGAATTTA TGGCATGCTG
2401 TGGTCATGGC GACGACGACG GTCGATGAGC AGTGGGAGCA GGTGTGGCTG
2451 CCCCGCTGGC CCCTGGCCTC CGACGACCTG GCAGCGGGCA TCTACCGGAT
2501 GGCCCCGCCCC TCGGCGCTGG GGGTCCGATA CATCGAGGTC AACCCCCAAG
2551 CCATCAGCAA CCTCCTCGTG GTCGACTGCG ACCACCCCGA CGCTGCCATG
2601 CGCGCCGTCT GGGACCGCCA CGACTGGCTG CCCAACGCCA TCGTCGAGAA
2651 CCCCGACAAC GGCCACGCCC ACGCCGTGTG GGCCCTGGAA GCAGCCATCC
2701 CGCGCACCGA GTACGCCCAC CGCAAGCCCA TCGCCTACGC CGCCGCCGTC
2751 ACCGAGGGCC TGCGCCGATC CGTCGACGGA GACGCCTCCT ACGCCGGCCT
2801 GATCACCAAG AACCCCGAAC ACCCCGCCTG GAACACCACC TGGTGCACCG
2851 ACCACCTCTA CCGGCTGGCC GAGCTCGACA CCCACCTGGA TGCCGCCGGC
2901 CTCATGCCCG CCCCCTCCTG GCGACGCACC CGCCGGCGCA ACCCCGTCGG
2951 CCTGGGCCGC AACTGCGCCA TCTTCGAGAC CGCCCGCACC TGGGCCTACC
3001 GCGACGCCCG CCGCATCCGA CAACGCCACG AATACCCGAC CGCCGAGGAC
3051 TCGGCCGACC TGCACGCCGT CATCGCCTCC ACCGTCGAGG CGCTCAACGC
3101 CGGCTACAGC GAACCCCTGC CGGCCCGCGA GGCCGCCGGC ATCGCCGCCA
3151 GCATCCACCG ATGGATCACC CACCGTTTCT ACGGCTGGAT CGACTCCCAC
3201 ACCGTCAACG AGGCCACTTT CTCCACCATC CAGAGCTACA GAGGACACAA
3251 GGGAGCCGGC AAGGCTCGTC CTCGTGCCCG CCGTGCTGCT TCTATCACCG
3301 ATTGGGAGGC ATGATGGCTG ACGTCCAGCA CCGCGTGAAG CGTCGGGGCA
3351 CGGCCCGCGA GGCCGCAGAA CGTGTAGGGG CCTCCATCCG AACCGCCCAG
```

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```
3401 CGGTGGACCT CCATCCCCCG TGAGGAATGG ATCACTCAGA AGGCCGTCGA
3451 GCGTGAGGAG ATCCGGGCCT ACAAGTACGA CGAGGGGCAC ACGTGGGGCG
3501 AGACCTCGCG CCACTTCGGG ATCGCGAAGA CCACCGCCCA GGAGCGGGCC
3551 CGGCGGGCTC GAAGGGAGCG GGCGGCCGAA GCGGAGAAGG CTGCCGAGGA
3601 GGCCGAGGCC GCGCTGCGTC CGACACTCTT CGAGGGCCAG GAGCAAGGTT
3651 CTGCATGAGC AACCCCGAGT CCTCGGGTAG ACCGTCTGGC CCGACGTTAA
3701 GCATGGCTGA AGCGGCCCGT GCCTGTGGGG TTTCAGTGTC CACGGTGAGG
3751 CGTCACCGTG ATGCCCTGGT GGCCCACGGT GCTACCCGTC ATGACGCGTC
3801 ATGGGTGATA CCCCTATCAG CGTTGATTTC ATGCGGTTTG ATGCCCCGGG
3851 TGACACCCCC TGATGCCCCG TCACCCAATA ACGTGGCGCC TGCCATGACG
3901 TCCCACGGTG ACGCCCCCCT GACGGGGGAA GTCCAAGAGC TGCGCGAGCG
3951 ACTGGCCAAC GCTGAGCATC GAGCCGAGCT AGCCGAAGCC ATCGCGGCCG
4001 AGCGACAACA CACGATCGAC GCCCAGCGCA TCGCCTTACG GGCCTTAGAA
4051 CCCGGCTCGA CCCATAACAG CCCGGCAACC GATGAGCCGG CTACCGCTCG
4101 CGAGCAACCT CCCGGTCCAG AACCCAGCGA CTCCAGGCCA CACCGCCGGA
4151 GTTGGTGGCG TCGGCTGACT GGTGGCGCCT GACCGGCCCC GGTCGTCTTC
4201 GAGGGGAACC TCTCGCCTGC GAGAGGACAC AGCAGCCGGC TGTGCTGGTA
4251 GGGCATCCCA GCACGACACC CCTCTGACGC GAGAAGTTCA AGGACTACGC
4301 GAATTGCTGA CTACCGCCGA GCGGCAGCAC ACGATCGAGA TGCTCAACGA
4351 ACCGCACTAC GCGGCCTTAG AAGGCCCCAA GGCACGCTCA CCTACCACGT
4401 GGATCACCAC CGATCGGCGC CGACAGCTAT GGACCCCATC GCAAGATCAA
4451 AACCCCTGAG CAGCCATCGC ACCGAGCGCC CGGCACGCCG GAAGAAGCTC
4501 CGACGCCCCT GCTGTCCGGA CACGGCCTAA CGCGTCCAGA CCAGAACCAG
4551 TGCTCCGATC TAAACCGAAG GCCCTTCATG TGAGAGCATA GTCGTGACGT
4601 CGGCACAGTA GTCGTGCCCG GCGGGGGTAA CGCTACACAA CGCTTAAAAA
4651 GCATCGGAGC AAGCTAACAC AGGGGGACTG ATGAACAAAA CACACAAAAT
4701 GGCGACGCTG GTAATTGCCG CGATCTTGGC CGCCGGAATG ACCGCACCAA
4751 CTGCCTATGC AGATTCTCCT GGAAACACCA GAATTACAGC CAGCGAGCAA
4801 AGCGTCCTTA CCCAGATACT CGGCCACAAA CCTACACAAA CTGAATATAA
4851 CCGATACGTT GAGACTTACG GAAGCGTACC GACCGAAGCA GACATCAACG
4901 CATATATAGA AGCGTCTGAA TCTGAGGGAT CATCAAGTCA AACGGCTGCT
4951 CACGATGACT CGACATCACC CGGCACGAGT ACCGAAATCT ACACGCAGGC
5001 AGCCCCTGCC AGGTTCTCAA TGTTTTTCCT GTCCGGAACT TGGATCACTA
5051 GGAGTGGTGT AGTATCGCTC TCCTTGAAGC CAAGGAAGGG TGGTATTGGC
```

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```
5101 AACGAGGGGG ACGAGCGTAC CTGGAAGACT GTATACGACA AATTCCATAA
5151 CGCTGGGCAA TGGACACGAT ACAAGAACAA CGGCGTAGAC GCCAGCATGA
5201 AAAAGCAGTA CATGTGCCAC TTCAAGTACG GGATGGTGAA GACGCCATGG
5251 GTGTGCCATT TCTCACAATC CCGGGGTGCG ATTGTCGCGT TTCCCACAGG
5301 AATCGGCGCG GGGATCTGGA GGGTGCTGCG ACACGCCCAT ATTTTGAACG
5351 ATGTTCAGTG CGTCAACCTC GACCCCAGTG CTGAACTTGT CCGTCGCGGG
5401 TGCAAGGATT GGACCCATGA GTCCGCGAAA GATTGGCGTT ACCGAGCTCG
5451 CGCTCCGCGA CGCGCATCAG AGCCTGATTG CAACCCGGAT GACTGAGTTG
5501 GACACCATCG CAAATCCGTC CGATCCCGCG GTGCAGCGGA TCATCGATGT
5551 CACCAAGCCG TCGCGATCCA ACATAAAGAC AACGTTGATC GAGGACGTCG
5601 AGCCCCTCAT GCACAGCATC GCGGCCGGGG TGGAGTTCAT CGAGGTCTAC
5651 GGCAGCGACA GCAGTCCTTT TCCATCTGAG TTGCTGGATC TGTGCGGGCG
5701 GCAGAACATA CCGGTCCGCC TCATCGACTC CTCGATCGTC AACCAGTTGT
5751 TCAAGGGGGA GCGGAAGGCC AAGACATTCG GCATCGCCCG CGTCCCTCGC
5801 CCGGCCAGGT TCGGCGATAT CGCGAGCCGG CGTGGGGACG TCGTCGTTCT
5851 CGACGGGGTG AAGATCGTCG GGAACATCGG CGCGATAGTA CGCACGTCGC
5901 TCGCGCTCGG AGCGTCGGGG ATCATCCTGG TCGACAGTGA CATCACCAGC
5951 ATCGCGGACC GGCGTCTCCA AAGGGCCAGC CGAGGTTACG TCTTCTCCCT
6001 TCCCGTCGTT CTCTCCGGTC GCGAGGAGGC CATCGCCTTC ATTCGGGACA
6051 GCGGTATGCA GCTGATGACG CTCAAGGCGG ATGGCGACAT TTCCGTGAAG
6101 GAACTCGGGG ACAATCCGGA TCGGCTGGCC TTGCTGTTCG GCAGCGAAAA
6151 GGGTGGGCCT TCCGACCTGT TCGAGGAGGC GTCTTCCGCC TCGGTTTCCA
6201 TCCCCATGAT GAGCCAGACC GAGTCTCTCA ACGTTTCCGT TTCCCTCGGA
6251 ATCGCGCTGC ACGAGAGGAT CGACAGGAAT CTCGCGGCCA ACCGATAATC
6301 AGGCTGAGAA CGACCTGATC CGCCACTCGC GGAACTCCGG ACGCCGCGTC
6351 CCCTCGGGGG CGCGGCGTCC TGCATGTCCG GGCGCAGGGG CAAGGCAGGC
6401 CTCCTACTTA TAATTGTCCC ATACGCGTCA TACTGGTTAG TCGCTGGAGA
6451 TCCAGACGTT TGGGACTTCT ATCGTTCTTT ATGGTGGATT CCAGTGGCTT
6501 TTCTAGGAAT AGTTTCAATA GTACTGATGG CTAGCAGTAG AGGTTGGGGA
6551 CGACGTCTCG GCGACTCCGG AGAACACCAA GTCAGGGTCT CATGAGTGTG
6601 CGATAGCTTG AGCTGTCTAC CAATCTGGAT ATAGCTATAT CGGTCGTTTG
6651 TGTCTGATTC GCCAGTGAGC CAACGGCGGG GGCGACACGC GGTGGCGAAA
6701 CCCCCTGGCA GAATTCGTAA TCATGGTCAT AGCTGTTTCC TGTGTGAAAT
6751 TGTTATCCGC TCACAATTCC ACACAACATA CGAGCCGGAA GCATAAAGTG
```

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6801 TAAAGCCTGG GGTGCCTAAT GAGTGAGCTA ACTCACATTA ATTGCGTTGC
6851 GCTCACTGCC CGCTTTCCAG TCGGGAAACC TGTCGTGCCA GCTGCATTAA
6901 TGAATCGGCC AACGCGCGGG GAGAGGCGGT TTGCGTATTG GGCGCTCTTC
6951 CGCTTCCTCG CTCACTGACT CGCTGCGCTC GGTCGTTCGG CTGCGGCGAG
7001 CGGTATCAGC TCACTCAAAG GCGGTAATAC GGTTATCCAC AGAATCAGGG
7051 GATAACGCAG GAAAGAACAT GTGAGCAAAA GGCCAGCAAA AGGCCAGGAA
7101 CCGTAAAAAG GCCGCGTTGC TGGCGTTTTT CCATAGGCTC CGCCCCCCTG
7151 ACGAGCATCA CAAAAATCGA CGCTCAAGTC AGAGGTGGCG AAACCCGACA
7201 GGACTATAAA GATACCAGGC GTTTCCCCCT GGAAGCTCCC TCGTGCGCTC
7251 TCCTGTTCCG ACCCTGCCGC TTACCGGATA CCTGTCCGCC TTTCTCCCTT
7301 CGGGAAGCGT GGCGCTTTCT CAAAGCTCAC GCTGTAGGTA TCTCAGTTCG
7351 GTGTAGGTCG TTCGCTCCAA GCTGGGCTGT GTGCACGAAC CCCCCGTTCA
7401 GCCCGACCGC TGCGCCTTAT CCGGTAACTA TCGTCTTGAG TCCAACCCGG
7451 TAAGACACGA CTTATCGCCA CTGGCAGCAG CCACTGGTAA CAGGATTAGC
7501 AGAGCGAGGT ATGTAGGCGG TGCTACAGAG TTCTTGAAGT GGTGGCCTAA
7551 CTACGGCTAC ACTAGAAGAA CAGTATTTGG TATCTGCGCT CTGCTGAAGC
7601 CAGTTACCTT CGGAAAAAGA GTTGGTAGCT CTTGATCCGG CAAACAAACC
7651 ACCGCTGGTA GCGGTGGTTT TTTTGTTTGC AAGCAGCAGA TTACGCGCAG
7701 AAAAAAAGGA TCTCAAGAAG ATCCTTTGAT CTTTTCTACG GGGTCTGACG
7751 CTCAGTGGAA CGAAAACTCA CGTTAAGGGA TTTTGGTCAT GAGATTATCA
7801 AAAAGGATCT TCACCTAGAT CCTTTTAAAT TAAAAATGAA GTTTTAAATC
7851 AATCTAAAGT ATATATGAGT AAACTTGGTC TGACAGTTAC CAATGCTTAA
7901 TCAGTGAGGC ACCTATCTCA GCGATCTGTC TATTTCGTTC ATCCATAGTT
7951 GCCTGACTCC CCGTCGTGTA GATAACTACG ATACGGGAGG GCTTACCATC
8001 TGGCCCCAGT GCTGCAATGA TACCGCGAGA CCCACGCTCA CCGGCTCCAG
8051 ATTTATCAGC AATAAACCAG CCAGCCGGAA GGGCCGAGCG CAGAAGTGGT
8101 CCTGCAACTT TATCCGCCTC CATCCAGTCT ATTAATTGTT GCCGGGAAGC
8151 TAGAGTAAGT AGTTCGCCAG TTAATAGTTT GCGCAACGTT GTTGCCATTG
8201 CTACAGGCAT CGTGGTGTCA CGCTCGTCGT TTGGTATGGC TTCATTCAGC
8251 TCCGGTTCCC AACGATCAAG GCGAGTTACA TGATCCCCCA TGTTGTGCAA
8301 AAAAGCGGTT AGCTCCTTCG GTCCTCCGAT CGTTGTCAGA AGTAAGTTGG
8351 CCGCAGTGTT ATCACTCATG GTTATGGCAG CACTGCATAA TTCTCTTACT
8401 GTCATGCCAT CCGTAAGATG CTTTTCTGTG ACTGGTGAGT ACTCAACCAA
8451 GTCATTCTGA GAATAGTGTA TGCGGCGACC GAGTTGCTCT TGCCCGGCGT
```

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 8501 CAATACGGGA TAATACCGCG CCACATAGCA GAACTTTAAA AGTGCTCATC
 8551 ATTGGAAAAC GTTCTTCGGG GCGAAAACTC TCAAGGATCT TACCGCTGTT
 8601 GAGATCCAGT TCGATGTAAC CCACTCGTGC ACCCAACTGA TCTTCAGCAT
 8651 CTTTTACTTT CACCAGCGTT TCTGGGTGAG CAAAAACAGG AAGGCAAAAT
 8701 GCCGCAAAAA AGGGAATAAG GGCGACACGG AAATGTTGAA TACTCATACT
 8751 CTTCCTTTTT CAATATTATT GAAGCATTTA TCAGGGTTAT TGTCTCATGA
 8801 GCGGATACAT ATTTGAATGT ATTTAGAAAA ATAAACAAAT AGGGGTTCCG
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 9001 ACGGTCACAG CTTGTCTGTA AGCGGATGCC GGGAGCAGAC AAGCCCGTCA
 9051 GGGCGCGTCA GCGGGTGTTG GCGGGTGTCG GGGCTGGCTT AACTATGCGG
 9101 CATCAGAGCA GATTGTACTG AGAGTGCACC ATATGCGGTG TGAAATACCG
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 9201 GCTGCGCAAC TGTTGGGAAG GGCGATCGGT GCGGGCCTCT TCGCTATTAC
 9251 GCCAGCTGGC GAAAGGGGGA TGTGCTGCAA GGCGATTAAG TTGGGTAACG
 9301 CCAGGGTTTT CCCAGTCACG ACGTTGTAAA ACGACGGCCA GTGCCACTAG
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 9401 GAATCGGCGC GGGGATCTGG AGGGTGCTGC GACACGCCCA TATTTTGAAC
 9451 GATGTTCAGT GCGTCAACCT CGACCCCAGT GCTGAACTTG TCCGTCGCGG
 9501 GTGCAAGGAT TGGACCCATG AGTCCGCGAA AGATTGGCGT TACCGAGCTC
 9551 GCGCTCCGCG ACGCGCATCA GAGCCTGATT GCAACCCGGA TGAAGGTCAC
 9601 CACCGTCAAG GAGCTGGACG AGAAGCTCAA GGTCATCAAG GAGGCCCAGA
 9651 AGAAGTTCTC GTGCTACTCG CAGGAGATGG TGGACGAGAT CTTCCGCAAC
 9701 GCCGCGATGG CCGCGATCGA CGCCCGCATC GAGCTCGCCA AGGCCGCGGT
 9751 CCTGGAGACC GGCATGGGCC TCGTCGAGGA CAAGGTGATC AAGAACCACT
 9801 TCGCCGGCGA GTACATCTAC AACAAGTACA AGGACGAGAA GACCTGCGGC
 9851 ATCATCGAGC GCAACGAGCC GTACGGCATC ACCAAGATCG CCGAGCCCAT
 9901 CGGCGTCGTC GCCGCGATCA TCCCCGTCAC CAACCCGACC TCCACCACGA
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10051 GGACGCCGCG GTCAAGTCGG GCGCCCCCGA GAACATCATC GGCTGGATCG
10101 ACGAGCCCTC GATCGAGCTG ACCCAGTACC TGATGCAGAA GGCCGACATC
10151 ACCCTCGCCA CCGGCGGGCC CTCGCTCGTC AAGTCGGCCT ACTCGTCCGG
```

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10201 CAAGCCCGCC ATCGGCGTGG GGCCGGGCAA CACCCCCGTC ATCATCGACG
10251 AGTCCGCCCA CATCAAGATG GCCGTCTCCT CCATCATCCT CTCCAAGACC
10301 TACGACAACG GCGTCATCTG CGCCTCGGAG CAGTCCGTGA TCGTCCTCAA
10351 GTCGATCTAC AACAAGGTCA AGGACGAGTT CCAGGAGCGC GGCGCCTACA
10401 TCATCAAGAA GAACGAGCTG GACAAGGTGC GCGAGGTCAT CTTCAAGGAC
10451 GGCTCGGTGA ACCCCAAGAT CGTCGGCCAG TCGGCCTACA CCATCGCCGC
10501 GATGGCCGGC ATCAAGGTCC CGAAGACCAC GCGCATCCTC ATCGGCGAGG
10551 TCACCTCCCT GGGCGAGGAG GAGCCCTTCG CCCACGAGAA GCTCTCGCCC
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10701 CCGACGAGAT CAAGGCCCGC GACAAGATCG ACCGCTTCTC CTCGGCCATG
10751 AAGACCGTCC GCACCTTCGT CAACATCCCC ACCTCGCAGG GCGCCTCCGG
10801 CGACCTGTAC AACTTCCGCA TCCCGCCCTC CTTCACCCTC GGCTGCGGCT
10851 TCTGGGGGGG CAACTCCGTC TCGGAGAACG TGGGCCCGAA GCACCTGCTG
10901 AACATCAAGA CCGTGGCCGA GCGCCGCGAG AACATGCTGT GGTTCCGCGT
10951 CCCCCACAAG GTCTACTTCA AGTTCGGCTG CCTCCAGTTC GCCCTCAAGG
11001 ACCTCAAGGA CCTCAAGAAG AAGCGCGCCT TCATCGTCAC CGACTCGGAC
11051 CCCTACAACC TGAACTACGT CGACTCCATC ATCAAGATCC TCGAGCACCT
11101 CGACATCGAC TTCAAGGTCT TCAACAAGGT GGGCCGCGAG GCCGACCTCA
11151 AGACCATCAA GAAGGCCACC GAGGAGATGT CGTCCTTCAT GCCCGACACC
11201 ATCATCGCCC TGGGCGGGAC CCCGGAGATG TCCTCCGCCA AGCTGATGTG
11251 GGTCCTCTAC GAGCACCCCG AGGTCAAGTT CGAGGACCTG GCCATCAAGT
11301 TCATGGACAT CCGCAAGCGC ATCTACACCT TCCCCAAGCT GGGCAAGAAG
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11401 CCCCTTCGCC CTCGTGACCG ACAACAACAC CGGCAACAAG TACATGCTCG
11451 CCGACTACGA GATGACCCCC AACATGGCCA TCGTGGACGC CGAGCTCATG
11501 ATGAAGATGC CGAAGGGCCT CACCGCCTAC TCGGGCATCG ACGCCCTGGT
11551 CAACTCGATC GAGGCCTACA CCTCCGTCTA CGCCTCCGAG TACACCAACG
11601 GCCTCGCCCT CGAGGCCATC CGCCTGATCT TCAAGTACCT CCCGGAGGCC
11651 TACAAGAACG GCCGCACCAA CGAGAAGGCC CGCGAGAAGA TGGCCCACGC
11701 GTCCACCATG GCCGGCATGG CGTCCGCCAA CGCCTTCCTC GGCCTCTGCC
11751 ACTCCATGGC CATCAAGCTG TCCTCGGAGC ACAACATCCC CTCCGGCATC
11801 GCCAACGCCC TCCTCATCGA GGAGGTCATC AAGTTCAACG CCGTGGACAA
11851 CCCGGTGAAG CAGGCCCCCT GCCCGCAGTA CAAGTACCCC AACACCATCT

11901 TCCGCTACGC CCGCATCGCC GACTACATCA AGCTGGGCGG GAACACCGAC
11951 GAGGAGAAGG TCGACCTCCT CATCAACAAG ATCCACGAGC TCAAGAAGGC
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12051 TCTACTCCTC CCTGGACCGC ATCTCGGAGC TCGCCCTGGA CGACCAGTGC
12101 ACCGGCGCCA ACCCGCGCTT CCCGCTCACC TCCGAGATCA AGGAGATGTA
12151 CATCAACTGC TTCAAGAAGC AGCCCTGATG ATCAGGCTGA GAACGACCTG
12201 ATCCGCCACT CGCGGAACTC CGGACGCCGC GTCCCCTCGG GGGCGCGGCG
12251 TCCTGCATGT CCGGGCGCAG GGGCAAGGCA GGCCTCCTAC A

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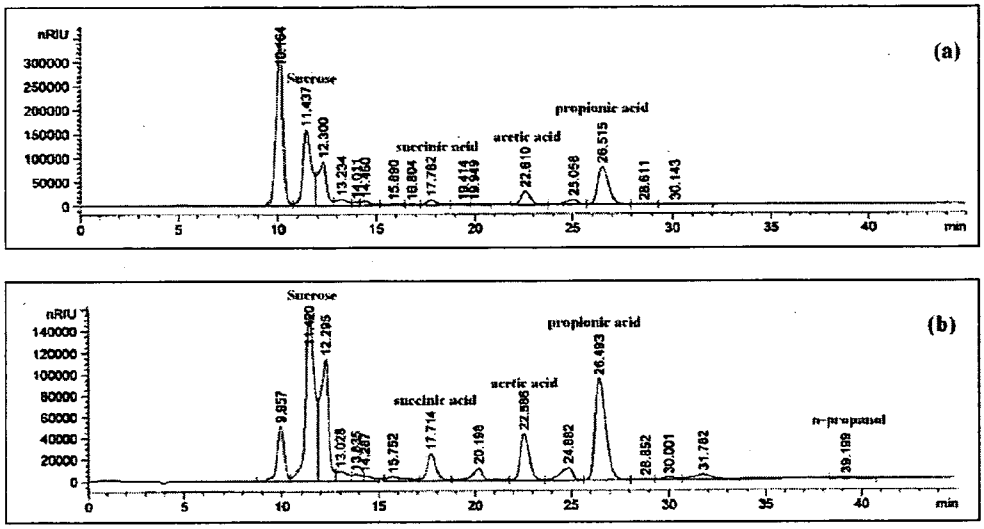
(a)
nRIU
Sucrose
propionic acid
succinic acid
acetic acid
min
(b)
nRIU
Sucrose
propionic acid
succinic acid
acetic acid
n-propanol
min

Figure 12

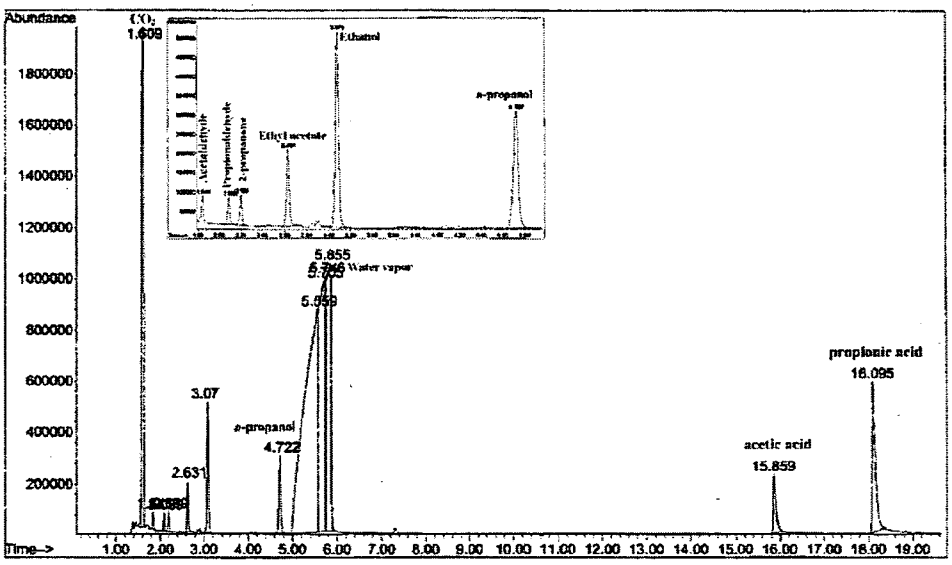
Abundance
CO2
1.609
1800000
1600000
1400000
1200000
1000000
800000
600000
400000
200000
Time-->
1.00 2.00 3.00 4.00 5.00 6.00 7.00 8.00 9.00 10.00 11.00 12.00 13.00 14.00 15.00 16.00 17.00 18.00 19.00
Acetaldehyde
Propanaldehyde
2-propanone
Ethyl acetate
Ethanol
n-propanol
5.855
Water vapor
3.07
2.631
n-propanol
4.722
acetic acid
15.859
propionic acid
16.095

Figure 13

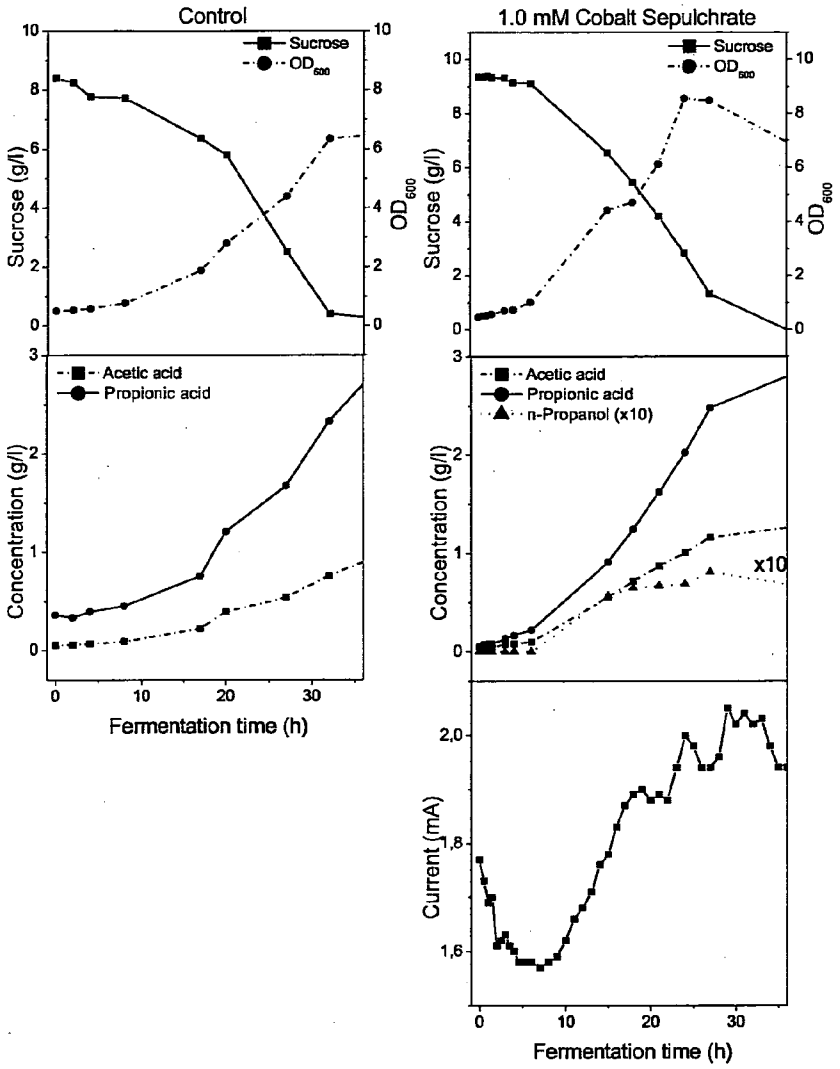
Control
1.0 mM Cobalt Sepulchrate
Sucrose
OD600
Sucrose (g/l)
Acetic acid
Propionic acid
n-Propanol (x10)
Concentration (g/l)
x10
Current (mA)
Fermentation time (h)

Figure 14