



Office de la Propriété

Intellectuelle
du Canada

Un organisme
d'Industrie Canada

Canadian
Intellectual Property
Office

An agency of
Industry Canada

CA 2727549 C 2014/08/26

(11)(21) **2 727 549**

(12) **BREVET CANADIEN**
CANADIAN PATENT

(13) **C**

(86) Date de dépôt PCT/PCT Filing Date: 2009/06/05
(87) Date publication PCT/PCT Publication Date: 2009/12/17
(45) Date de délivrance/Issue Date: 2014/08/26
(85) Entrée phase nationale/National Entry: 2010/12/06
(86) N° demande PCT/PCT Application No.: NZ 2009/000101
(87) N° publication PCT/PCT Publication No.: 2009/151342
(30) Priorité/Priority: 2008/06/09 (US61/060,113)

(51) Cl.Int./Int.Cl. *C12P 7/18* (2006.01),
C10L 1/182 (2006.01)

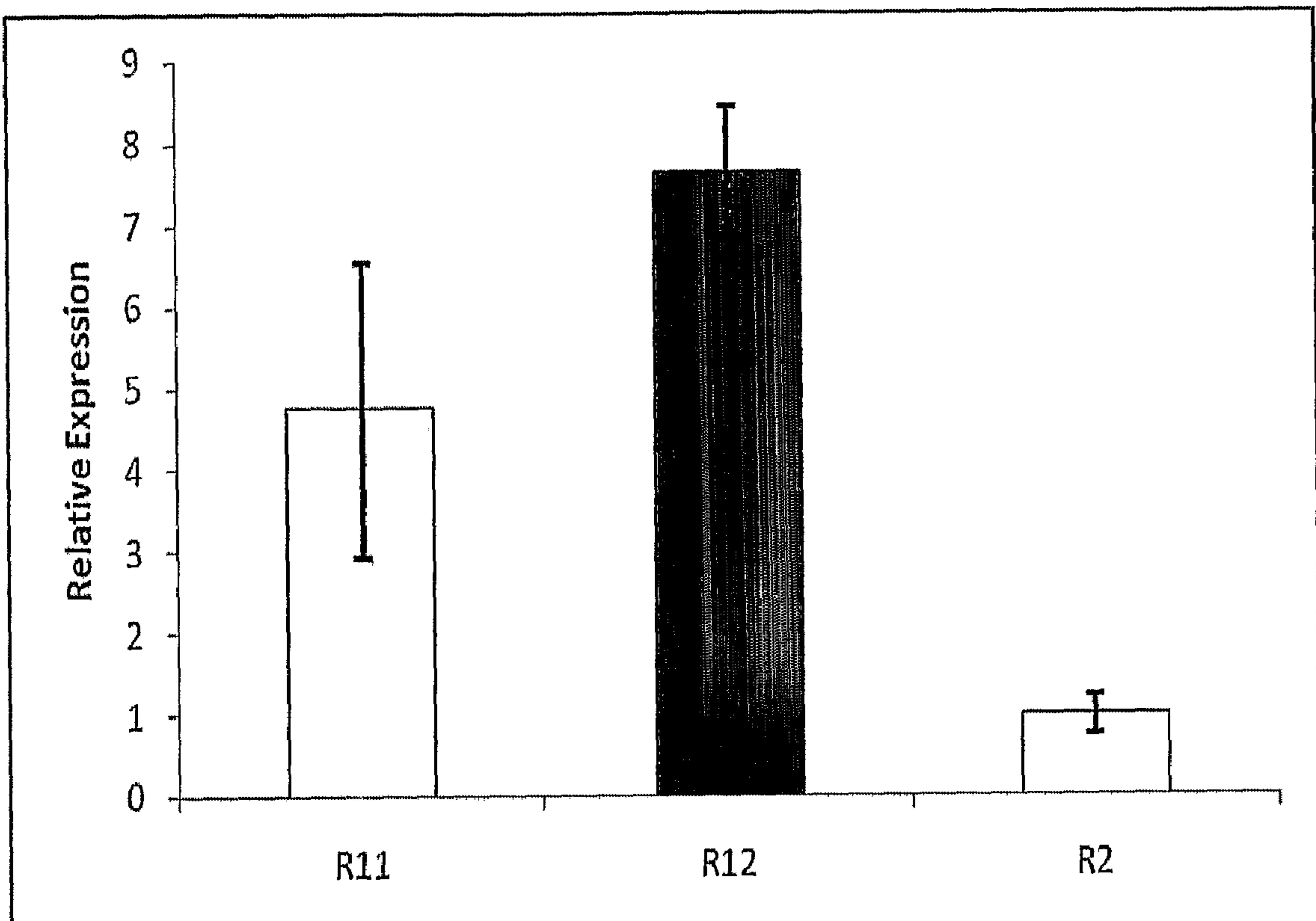
(72) Inventeurs/Inventors:
SIMPSON, SEAN DENNIS, NZ;
TRAN, PHUONG LOAN, NZ;
MIHALCEA, CHRISTOPHE DANIEL, NZ;
FUNG, JENNIFER MON YEE, US;
LIEW, FUNGMIN, NZ

(73) Propriétaire/Owner:
LANZATECH NEW ZEALAND LIMITED, NZ

(74) Agent: BERESKIN & PARR LLP/S.E.N.C.R.L.,S.R.L.

(54) Titre : PRODUCTION DE BUTANEDIOL PAR FERMENTATION MICROBIENNE ANAEROBIE

(54) Title: PRODUCTION OF BUTANEDIOL BY ANAEROBIC MICROBIAL FERMENTATION



(57) Abrégé/Abstract:

The invention provides methods for producing 2,3-butanediol by anaerobic fermentation. According to particular methods of the invention, 2,3-butanediol is produced by anaerobic fermentation of substrates including carbohydrate and carbon monoxide.

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

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
17 December 2009 (17.12.2009)(10) International Publication Number
WO 2009/151342 A1(51) International Patent Classification:
C12P 7/18 (2006.01) *C12R 1/145* (2006.01)
C10L 1/182 (2006.01)(74) Agent: BALDWINS INTELLECTUAL PROPERTY;
PO Box 5999, Wellesley Street, Auckland, 1141 (NZ).(21) International Application Number:
PCT/NZ2009/000101

(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, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(22) International Filing Date:
5 June 2009 (05.06.2009)

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (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, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(25) Filing Language: English
(26) Publication Language: English
(30) Priority Data:
61/060,113 9 June 2008 (09.06.2008) US

Published:

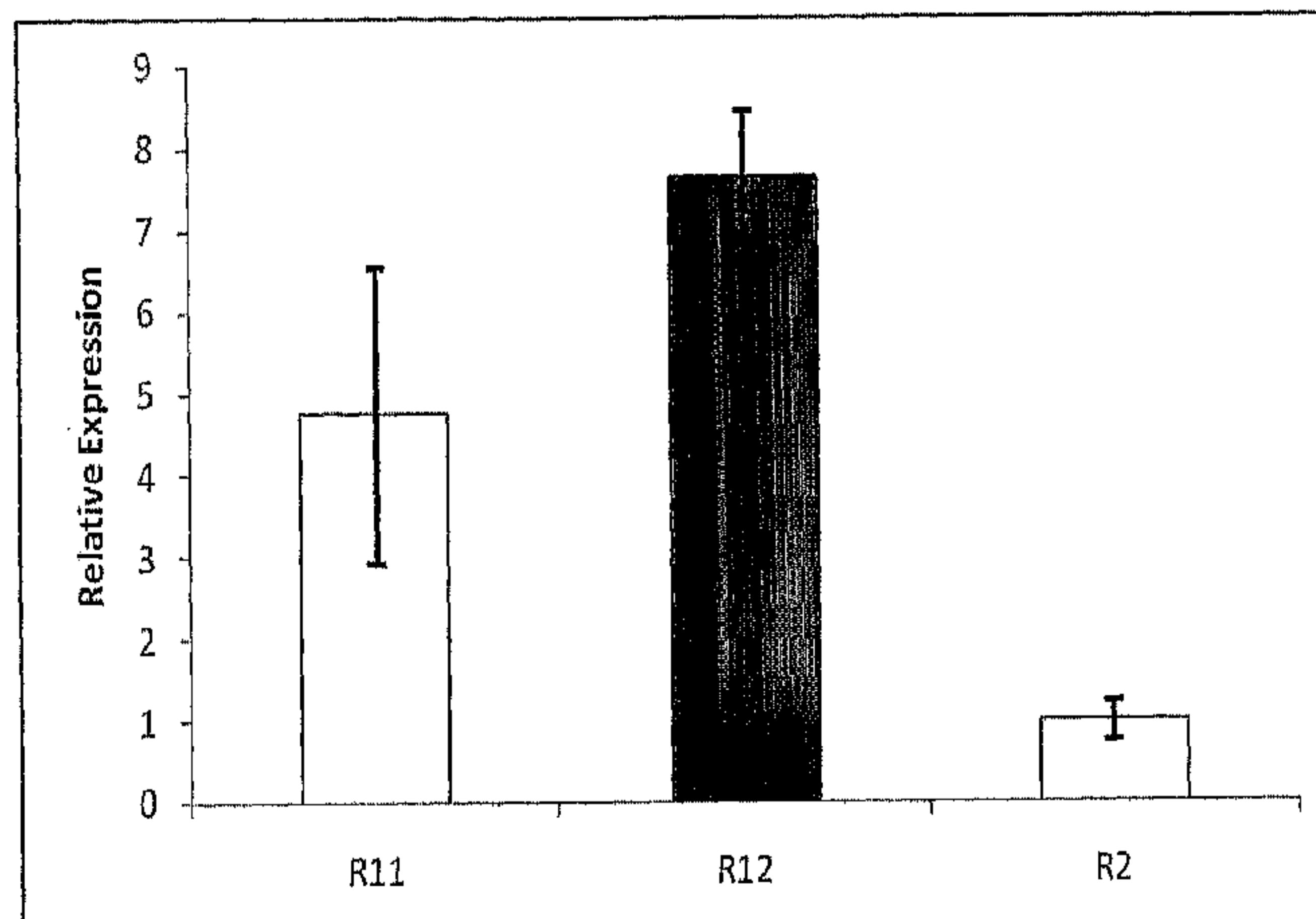
— with international search report (Art. 21(3))

(71) Applicant (for all designated States except US): LAN-ZATECH NEW ZEALAND LIMITED [NZ/NZ]; 24 Balfour Road, Parnell, Auckland, 1052 (NZ).

(72) Inventors; and
(75) Inventors/Applicants (for US only): SIMPSON, Sean, Dennis [GB/NZ]; 3/19 Richardson Road, Mount Albert, Auckland, 1025 (NZ). TRAN, Phuong, Loan [VN/NZ]; 31-39 Pitt Street, Newton, Auckland, 1010 (NZ). MHALCEA, Christophe, Daniel [DE/NZ]; 1A Picton Street, Apt. 2, Freemans Bay, Auckland, 1011 (NZ). FUNG, Jennifer, Mon, Yee [US/US]; 2340 Wexford Avenue, South San Francisco, California 94080 (US). LIEW, FungMin [MY/NZ]; 22 Madison Place, Forrest Hill, Auckland, 0620 (NZ).

(54) Title: PRODUCTION OF BUTANEDIOL BY ANAEROBIC MICROBIAL FERMENTATION

Figure 1



(57) Abstract: The invention provides methods for producing 2,3-butanediol by anaerobic fermentation. According to particular methods of the invention, 2,3-butanediol is produced by anaerobic fermentation of substrates including carbohydrate and carbon monoxide.

- 1 -

Production of Butanediol by Anaerobic Microbial Fermentation

FIELD

The present invention relates to the production of butanediol by microbial fermentation, particularly to production of 2,3-butanediol by microbial fermentation of substrates 5 comprising CO.

BACKGROUND

Biofuels for transportation are attractive replacements for gasoline and are rapidly penetrating fuel markets as low concentration blends. Biofuels, derived from natural plant sources, are more environmentally sustainable than those derived from fossil 10 resources (such as gasoline), their use allowing a reduction in the levels of so-called fossil carbon dioxide (CO₂) gas that is released into the atmosphere as a result of fuel combustion. In addition, biofuels can be produced locally in many geographies, and can act to reduce dependence on imported fossil energy resources. Alcohols suitable for use as biofuels include ethanol, butanol and 2,3-butanediol.

15 Ethanol is rapidly becoming a major hydrogen-rich liquid transport fuel around the world. Worldwide consumption of ethanol in 2002 was an estimated 10.8 billion gallons. The global market for the fuel ethanol industry is also predicted to grow sharply in future, due to an increased interest in ethanol in Europe, Japan, the USA and several developing nations.

20 Butanediols including 1,2-butanediol, 1,3-butanediol, 1,4-butanediol and 2,3-butanediol may be considered to have a variety of advantages over ethanol. Like ethanol, butanediols may be used directly as an automotive fuel additive. They may also be relatively easily transformed into a number of other potentially higher value and/or higher energy products. For example, 2,3-butanediol may be readily converted in a two 25 step process into an eight-carbon dimer which can be used as aviation fuel.

2,3-Butanediol derives its versatility from its di-functional backbone, i.e., 2 hydroxyl groups are located at vicinal C-atoms allowing the molecule to be transformed quite

- 2 -

easily into substances such as butadiene, butadione, acetoin, methylethyl ketone etc. These chemical compounds are used as base molecules to manufacture a vast range of industrially produced chemicals.

In addition, 2,3-butanediol may be used as a fuel in an internal combustion engine. It is in 5 several ways more similar to gasoline than it is to ethanol. As the interest in the production and application of environmentally sustainable fuels has strengthened, interest in biological processes to produce 2,3-butanediol (often referred to as bio-butanol) has increased.

2,3-Butanediol can be produced by microbial fermentation of carbohydrate containing 10 feedstock (Syu MJ, *Appl Microbial Biotechnol* 55:10-18 (2001), Qin et al., *Chinese J Chem Eng* 14(1):132-136 (2006)). 2,3-Butanediol may also be produced by microbial fermentation of biomass from crops such as sugar beet, corn, wheat and sugarcane. However, the cost of these carbohydrate feed stocks is influenced by their value as 15 human food or animal feed and the cultivation of starch or sucrose-producing crops for 2,3-butanediol production is not economically sustainable in all geographies. Therefore, it is of interest to develop technologies to convert lower cost and/or more abundant carbon resources into 2,3-butanediol.

Carbon Monoxide (CO) is a major by-product of the incomplete combustion of organic materials such as coal or oil and oil derived products. Although the complete combustion 20 of carbon containing precursors yields CO₂ and water as the only end products, some industrial processes need elevated temperatures favouring the build up of carbon monoxide over CO₂. One example is the steel industry, where high temperatures are needed to generate desired steel qualities. For example, the steel industry in Australia is reported to produce and release into the atmosphere over 500,000 tonnes of CO 25 annually.

Furthermore, CO is also a major component of syngas, where varying amounts of CO and H₂ are generated by gasification of a carbon-containing fuel. For example, syngas may be

- 3 -

produced by cracking the organic biomass of waste woods and timber to generate precursors for the production of fuels and more complex chemicals.

The release of CO into the atmosphere may have significant environmental impact. In addition, emissions taxes may be required to be paid, increasing costs to industrial plants.

5 Since CO is a reactive energy rich molecule, it can be used as a precursor compound for the production of a variety of chemicals. However, this valuable feedstock has not been utilised to produce 2,3-butanediol.

It is an object of the present invention to provide a process that goes at least some way towards overcoming the above disadvantages or at least to provide the public with a 10 useful choice.

STATEMENT OF INVENTION

In one aspect, the invention provides a method of producing butanediol by microbial fermentation of a substrate comprising carbon monoxide. In particular embodiments, the invention provides a method of producing butanediol by microbial fermentation, the 15 method including:

- a. providing a substrate comprising CO;
- b. in a bioreactor containing a culture of one or more micro-organisms, anaerobically fermenting the substrate to produce butanediol.

In certain embodiments, the butanediol is 2,3-butanediol.

20 In another aspect, the invention provides a method of increasing efficiency of 2,3-butanediol production by fermentation, the method including:

- a. providing a substrate comprising CO;
- b. in a bioreactor containing a culture of one or more micro-organisms, anaerobically fermenting the substrate to produce 2,3-butanediol.

25 In another aspect of the invention, there is provided a method of producing 2,3-butanediol by microbial fermentation, the method including:

- a. providing a substrate

- 4 -

- b. in a bioreactor containing a culture of one or more micro-organisms, anaerobically fermenting the substrate, wherein one or more micro-organisms includes one or more 2,3-butanediol dehydrogenase genes;
- c. upregulating the 2,3-butanediol dehydrogenase gene(s), such that 2,3-butanediol is produced by the micro-organism(s).

5 In particular embodiments, the substrate comprises CO.

In particular embodiments of the various aspects, the substrate comprising carbon monoxide is a gaseous substrate comprising carbon monoxide. The gaseous substrate comprising carbon monoxide can be obtained as a by-product of an industrial process. In 10 certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, non-ferrous products manufacturing, petroleum refining processes, gasification of biomass, gasification of coal, electric power production, carbon black production, ammonia production, methanol production and coke manufacturing. In one embodiment the gaseous substrate comprises a gas obtained from 15 a steel mill. In another embodiment the gaseous substrate comprises automobile exhaust fumes.

In particular embodiments, the CO-containing substrate typically contains a major proportion of CO, such as at least about 20% to about 100% CO by volume, from 40% to 95% CO by volume, from 40% to 60% CO by volume, and from 45% to 55% CO by volume.

20 In particular embodiments, the substrate comprises about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO by volume. Substrates having lower concentrations of CO, such as 6%, may also be appropriate, particularly when H₂ and CO₂ are also present.

25 In particular embodiments of the various aspects, the substrate comprising CO is provided at a sufficient level, such that 2,3-butanediol is produced. In particular embodiments, CO is provided such that a specific uptake rate of at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least 0.6mmol/g/min; or at least 0.7mmol/g/min; or at

- 5 -

least 0.8mmol/g/min; or at least 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min is maintained.

In certain embodiments of the various aspects, the method comprises microbial fermentation using *Clostridium autoethanogenum*.

- 5 In another aspect, the invention provides a method of producing 2,3-butanediol by microbial fermentation, the method including:
 - a. providing a substrate
 - b. in a bioreactor including a culture of *Clostridium autoethanogenum*, anaerobically fermenting the substrate to produce 2,3-butanediol.
- 10 In particular embodiments, the substrate is one or more carbohydrates such as fructose.. Alternatively the substrate is a substrate comprising carbon monoxide, typically a gaseous substrate comprising carbon monoxide, as herein before described
- 15 In a further aspect, the invention provides a method for producing butanediol by microbial fermentation of a first substrate and a second substrate comprising CO.

15 Preferably, the butanediol is 2,3-butanediol.

In particular embodiments, the first substrate is a carbohydrate. In certain embodiments, the first substrate is fructose. In certain embodiments, the second substrate is a gaseous substrate comprising carbon monoxide, as herein before described.

In particular embodiments, the method includes the steps of:

- 20 (a) microbial fermentation of the first substrate to produce 2,3-butanediol
- (b) microbial fermentation of the second substrate comprising CO to produce 2,3-butanediol.

In certain embodiments, steps (a) and (b) may be conducted at the same time. Alternatively, step (a) may substantially precede or follow step (b). In particular 25 embodiments, the method may alternate between step (a) and step (b).

In a further aspect of the invention, there is provided a method according to any of the previous aspects, wherein the fermentation is carried out in a bioreactor.

- 6 -

In a further aspect of the invention, there is provided a method according to any of the previous aspects, wherein the method further includes the step of capturing or recovering the butanediol.

In a further aspect, there is provided butanediol, preferably 2,3-butanediol, produced by 5 the methods of any of the previous aspects.

The invention may also be said broadly to consist in the parts, elements and features referred to or indicated in the specification of the application, individually or collectively, in any or all combinations of two or more of said parts, elements or features, and where specific integers are mentioned herein which have known equivalents in the art to which 10 the invention relates, such known equivalents are deemed to be incorporated herein as if individually set forth.

DETAILED DESCRIPTION OF THE INVENTION

The following is a description of the present invention, including preferred embodiments thereof, given in general terms. The invention is further exemplified in the disclosure 15 given under the heading "Examples" herein below, which provides experimental data supporting the invention, specific examples of aspects of the invention, and means of performing the invention.

As used herein "butanediol" refers to all structural isomers of the diol including 1,2-butanediol, 1,3-butanediol, 1,4-butanediol and 2,3-butanediol and stereoisomers thereof. 20 The term "2,3-butanediol" should be interpreted to include all enantiomeric and diastereomeric forms of the compound, including (R,R), (S,S) and meso forms, in racemic, partially stereoisomerically pure and/or substantially stereoisomerically pure forms.

The term "bioreactor" includes a fermentation device consisting of one or more vessels and/or towers or piping arrangement, which includes the Continuous Stirred Tank 25 Reactor (CSTR), Immobilized Cell Reactor (ICR), Trickle Bed Reactor (TBR), Bubble Column, Gas Lift Fermenter, Static Mixer, or other vessel or other device suitable for gas-liquid contact. As is described herein after, in some embodiments the bioreactor may comprise

- 7 -

a first growth reactor and a second fermentation reactor. As such, when referring to the addition of a substrate, for example a substrate comprising carbon monoxide, to the bioreactor or fermentation reaction it should be understood to include addition to either or both of these reactors where appropriate.

5 The term “substrate comprising carbon monoxide” and like terms should be understood to include any substrate in which carbon monoxide is available to one or more strains of bacteria for growth and/or fermentation, for example.

“Gaseous substrates comprising carbon monoxide” include any gas which contains a level of carbon monoxide. The gaseous substrate will typically contain a major proportion of

10 CO, preferably at least about 15% to about 95% CO by volume.

Unless the context requires otherwise, the phrases “fermenting”, “fermentation process” or “fermentation reaction” and the like, as used herein, are intended to encompass both the growth phase and product biosynthesis phase of the process.

The inventors have surprisingly shown that 2,3-butanediol can be produced by microbial

15 fermentation using *Clostridium autoethanogenum*. They have found that fermentation products include a variety of alcohols, whereby ethanol and 2,3-butanediol are significant substituents. 2,3-Butanediol has not been previously identified as a fermentation product using *Clostridium autoethanogenum*. In particular, the inventors have determined that *Clostridium autoethanogenum* can be used to produce 2,3-butanediol
20 and other products from a substrate comprising carbohydrate. In particular, fructose can be converted into products including acetate, ethanol and 2,3-butanediol. It has also been surprisingly demonstrated that 2,3-butanediol can be produced by *Clostridium autoethanogenum* from substrates comprising CO, particularly gaseous substrates comprising CO. The use of a gaseous carbon source, particularly a source including CO, in
25 fermentation processes has not previously resulted in the production of 2,3-butanediol.

In particular embodiments of the invention, the efficiency of 2,3-butanediol production can be increased by providing the substrate at a sufficient level such that 2,3-butanediol

- 8 -

is produced. It has been recognised that increasing the amount of substrate provided to a microbial culture, increases the amount of 2,3-butanediol produced by the culture.

In particular embodiments of the invention, the substrate comprising CO is provided at a sufficient level such that 2,3-butanediol is produced. It has been shown that a microbial culture comprising *C.autoethanogenum* can uptake CO at a rate up to approximately 1.5 to 2mmol/gram dry weight microbial cells/minute (specific CO uptake). In particular embodiments of the invention, a substrate comprising CO is provided to the microbial culture comprising *C.autoethanogenum* such that a specific uptake is maintained substantially at or at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least 5 0.6mmol/g/min; or at least 0.7mmol/g/min; or at least 0.8mmol/g/min; or at least 10 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min. In such embodiments, 2,3-butanediol is a significant fermentation product of at least 0.5g/L; or at least 1g/L; or at least 2g/L; or at least 5g/L. In particular embodiments, 2,3-butanediol is produced at a rate of at least 0.5g/L/day; or at least 15 1g/L/day.

In particular embodiments of the invention, apparatus used for conducting methods of the invention enable measurement and/or control of parameters such as CO supply, CO uptake, biomass level, 2,3-butanediol production. For example, samples can be taken from a bioreactor to determine one or more of the above parameters and the bioreactor 20 conditions optionally adjusted to improve 2,3-butanediol production. For example, in a bioreactor, wherein the microbial culture is producing no or insignificant amounts of 2,3-butanediol, the CO supply can be increased such that 2,3-butanediol is produced.

It is accepted that products such as acetate and ethanol are produced from CO via a combination of the acetyl-CoA cycle and the THF cycle as described in Phillips, J.R, *et al*, 25 1994, *Applied Biochemistry and Biotechnology*, 45/46: 145. However, in accordance with the methods of the invention, it has been surprisingly shown that 2,3-butanediol can be produced, particularly where CO is provided such that specific CO uptake rates of at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least 0.6mmol/g/min; or at least

- 9 -

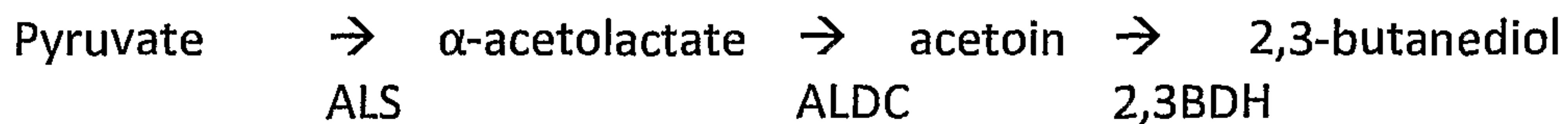
0.7mmol/g/min; or at least 0.8mmol/g/min; or at least 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min are maintained. Without wishing to be bound by theory, it is considered that by providing sufficient or elevated levels of CO, higher energy products, such as 2,3-butanediol can be produced

5 during fermentation. It is considered precursors of products, such as 2,3-butanediol act as electron acceptors to relieve the microbial cell of excess reducing power, in the form of NAD(P)H, thus restoring a favourable NAD(P):NAD(P)H equilibrium. It is further considered that carbohydrates fermented by the culture can also be converted into 2,3-butanediol in a similar manner.

10 The following genes have been putatively identified in *C.autoethanogenum*: α -acetolactate synthase (ALS), α -acetolactate decarboxylase (ALDC) and 2,3-butanediol dehydrogenase (2,3BDH). The putative 2,3-butanediol dehydrogenase gene (ORF 1283) of *C.autoethanogenum* (strain deposited at DSMZ under the accession number 19630) shows strong homology to the 2,3BDH of *Clostridium novyi* (NT01CX_0344) with amino acid identities of 73% (262/357) and positives of 84% (300/357). ORF 1283 also shows significant homology to the gene YdjL (bdhA) of *Bacillus subtilis* (47% amino acid identities, 63% positives and E-value of 3e-89. Further evidence that ORF 1283 of LZ1560 is 2,3BDH comes from homology to 2,3BDH (YAL060W) of *Saccharomyces cerevisiae* (E=2e-53).

15

20 Without wishing to be bound by theory, it is considered 2,3-butanediol is produced from pyruvate (an intermediate in anabolism produced from acetyl CoA) as follows:



Real-Time PCR studies of 2,3-butanediol dehydrogenase in *C.autoethanogenum*, indicate

25 that it is substantially upregulated in cultures where significant amounts of 2,3-butanediol are produced. Thus, 2,3-butanediol dehydrogenase can be upregulated in accordance with the methods of the invention. For example, where CO is supplied at sufficient levels, 2,3-butanediol dehydrogenase is upregulated. In particular, where CO is supplied such

- 10 -

that the specific CO uptake by the microbial culture is at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least 0.6mmol/g/min; or at least 0.7mmol/g/min; or at least 0.8mmol/g/min; or at least 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min; 2,3-butanediol dehydrogenase is 5 upregulated. As such, the invention provides a method of producing 2,3-butanediol by microbial fermentation of a substrate by upregulation of 2,3-butanediol dehydrogenase.

The inventors have further demonstrated that different substrates, such as a carbohydrate substrate and a gaseous substrate comprising CO, can be switched during microbial production of 2,3-butanediol, without deleterious effect. In addition, they 10 contemplate that substrates could be alternated, for example when one substrate is unavailable, and would continue to produce 2,3-butanediol.

In accordance with the results obtained, in one embodiment of the invention, 2,3-butanediol is produced by microbial fermentation of a substrate comprising carbohydrate. In another embodiment of the invention, a substrate comprising carbon 15 monoxide, preferably a gaseous substrate comprising CO, is converted into various products including 2,3-butanediol, by *Clostridium autoethanogenum*.

In a further embodiment of the invention, a first substrate comprising carbohydrate (preferably fructose) may be used in initial stages of the fermentation reaction and following complete consumption of the substrate, the substrate can be switched to a 20 second substrate comprising CO. Again, the inventors have surprisingly determined that 2,3-butanediol is produced in the initial stages where the first substrate comprising carbohydrate is the sole carbon source and is also produced in the latter stages where the substrate comprising CO is the sole carbon source.

The inventors have shown that 2,3-butanediol is produced under a variety of conditions, 25 including media containing alternative buffer solutions such as acetate buffer and citrate buffer. The inventors also submit that in embodiments where the pH is uncontrolled and may be variable, 2,3-butanediol is still produced. Examples of media suitable for carrying out the desired fermentation are described in the examples section hereinafter.

- 11 -

The inventors contemplate that the 2,3-butanediol produced in such processes may be readily recovered using separation techniques known in the art. Furthermore, the 2,3-butanediol may be readily converted into substances such as butadiene, butadione, acetoin, methylethyl ketone and the like. Such chemical compounds are valuable base 5 molecules used to manufacture a significant percentage of all chemical industry products. Therefore, the inventors contemplate that the 2,3-butanediol produced in the processes disclosed herein may be used in the manufacture of a wide range of well known industrial products.

The invention is generally described herein in relation to preferred embodiments of the 10 invention which utilise *Clostridium autoethanogenum* and/or produce 2,3-butanediol. However, it should be appreciated that alternative micro-organisms may be substituted for *C. autoethanogenum*. Similarly, the methods may be used to produce and recover butanediols other than 2,3-butanediol. Accordingly, unless the context requires otherwise, reference to "2,3-butanediol" may be substituted by the general term 15 "butanediol".

Method

In one embodiment, the invention provides a method for the production of butanediol by microbial fermentation. In a preferred embodiment the method comprises at least the step of anaerobically fermenting a substrate comprising CO, preferably a gaseous 20 substrate comprising CO, to obtain 2,3-butanediol.

In a particular embodiment of the invention, the method includes the steps of:

- (a) providing a substrate comprising CO, preferably a gaseous substrate comprising CO;
- (b) in a bioreactor containing a culture of one or more micro-organisms 25 anaerobically fermenting the substrate to produce 2,3-butanediol.

In another embodiment, the invention provides a method of increasing efficiency of 2,3-butanediol production by fermentation, the method including:

- (a) providing a substrate comprising CO;

- 12 -

(b) in a bioreactor containing a culture of one or more micro-organisms, anaerobically fermenting the substrate to produce 2,3-butanediol.

In particular embodiments, the substrate comprising CO is provided at a level sufficient to produce significant amounts of 2,3-butanediol, such as at least 0.5g/L of fermentation media, or at least 1g/L, or at least 2g/L, or at least 5g/L. In certain embodiments, CO is provided at a level sufficient to produce 2,3-butanediol at a rate of at least 0.5g/L/day; or at least 1g/L/day. In particular embodiments, CO is provided such that a specific uptake rate of at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least 0.6mmol/g/min; or at least 0.7mmol/g/min; or at least 0.8mmol/g/min; or at least 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min is maintained. Those skilled in the art will appreciate methods of supplying CO, particularly gaseous CO, such that the required uptake rate is achieved. However, by way of example, factors such as increasing gas hold-up in a fermentation media will increase the amount of CO available for conversion to products by the microbial culture. Gas hold-up can typically be increased by mechanical means, such as increasing agitation in a CSTR. Furthermore, supplying CO at a faster rate or a higher partial pressure will also increase the CO availability in a fermentation broth.

In another embodiment, the method involves fermentation of a substrate comprising carbohydrate by *Clostridium autoethanogenum* to produce butanediol, preferably, 2,3-butanediol.

In another embodiment, the method includes the steps of:

- (a) microbial fermentation of the first substrate to produce 2,3-butanediol
- (b) microbial fermentation of the second substrate comprising CO to produce 2,3-butanediol.

25 In certain embodiments, the first substrate is carbohydrate and in some embodiments, the substrate is fructose. Preferably, the second substrate is a gaseous substrate comprising CO. In particular embodiments, steps (a) and (b) may be conducted at the

- 13 -

same time. Alternatively, step (a) may substantially precede or follow step (b). Preferably, the method may alternate between step (a) and step (b).

In certain embodiments of the invention, the method further includes the step of capturing or recovering the 2,3-butanediol produced.

5 Micro-organisms

In embodiments of the invention the one or more micro-organisms used in the fermentation is *Clostridium autoethanogenum*. In a preferred embodiment the *Clostridium autoethanogenum* is a *Clostridium autoethanogenum* having the identifying characteristics of the strain deposited at the German Resource Centre for Biological

10 Material (DSMZ) under the identifying deposit number 19630. In another embodiment the *Clostridium autoethanogenum* is a *Clostridium autoethanogenum* having the identifying characteristics of DSMZ deposit number DSMZ 10061.

Culturing of the bacteria used in a method of the invention may be conducted using any number of processes known in the art for culturing and fermenting substrates using

15 anaerobic bacteria. Exemplary techniques are provided in the "Examples" section of this document. By way of further example, those processes generally described in the following articles using gaseous substrates for fermentation may be utilised: K. T. Klasson, M. D. Ackerson, E. C. Clausen and J. L. Gaddy (1991). Bioreactors for synthesis gas fermentations resources. Conservation and Recycling, 5; 145-165; K. T. Klasson, M. D.

20 Ackerson, E. C. Clausen and J. L. Gaddy (1991). Bioreactor design for synthesis gas fermentations. Fuel. 70. 605-614; K. T. Klasson, M. D. Ackerson, E. C. Clausen and J. L. Gaddy (1992). Bioconversion of synthesis gas into liquid or gaseous fuels. Enzyme and Microbial Technology. 14; 602-608; J. L. Vega, G. M. Antorrena, E. C. Clausen and J. L.

25 Gaddy (1989). Study of Gaseous Substrate Fermentation: Carbon Monoxide Conversion to Acetate. 2. Continuous Culture. Biotech. Bioeng. 34. 6. 785-793; J. L. Vega, E. C. Clausen and J. L. Gaddy (1989). Study of gaseous substrate fermentations: Carbon monoxide conversion to acetate. 1. Batch culture. Biotechnology and Bioengineering. 34. 6. 774-

784; and, J. L. Vega, E. C. Clausen and J. L. Gaddy (1990). Design of Bioreactors for Coal

Synthesis Gas Fermentations. Resources, Conservation and Recycling. 3. 149-160. Methods for culturing bacteria on substrates comprising carbohydrates are also well known in the art.

Substrates

- 5 In one embodiment of the invention, 2,3-butanediol is produced by microbial fermentation of a substrate comprising carbohydrate using *Clostridium autoethanogenum*. It will be appreciated there are many examples of carbohydrates suitable for fermentation known in the art and many examples of the types of processes used to ferment the carbohydrate substrate. By way of example, suitable substrates may 10 include, but are not limited to, monosaccharides such as glucose and fructose, oligosaccharides such as sucrose or lactose, polysaccharides, such as cellulose or starch. Although it is contemplated that any of these carbohydrate substrates (and mixtures thereof) are suitable in the present invention, preferred carbohydrate substrates are fructose and sucrose (and mixtures thereof).
- 15 Those skilled in the art will appreciate fermentable sugars may be obtained from cellulosic and lignocellulosic biomass through processes of pre-treatment and saccharification, as described, for example, in US20070031918. Biomass refers to any cellulose or lignocellulosic material and includes materials comprising cellulose, and optionally further comprising hemicellulose, lignin, starch, oligosaccharides and/or 20 monosaccharides. Biomass includes, but is not limited to bioenergy crops, agricultural residues, municipal solid waste, industrial solid waste, sludge from paper manufacture, yard waste, wood and forestry waste. However, in exemplary embodiments of the invention commercially available fructose is used as the carbon and energy source for the fermentation.
- 25 In a particular embodiment, a substrate comprising carbon monoxide, preferably a gaseous substrate comprising carbon monoxide is used in the methods of the invention. The gaseous substrate may be a waste gas obtained as a by-product of an industrial process, or from some other source such as from combustion engine (for example

- 15 -

automobile) exhaust fumes. In certain embodiments, the industrial process is selected from the group consisting of ferrous metal products manufacturing, such as a steel mill, non-ferrous products manufacturing, petroleum refining processes, gasification of coal, electric power production, carbon black production, ammonia production, methanol 5 production and coke manufacturing. In these embodiments, the CO-containing gas may be captured from the industrial process before it is emitted into the atmosphere, using any convenient method. Depending on the composition of the gaseous substrate comprising carbon monoxide, it may also be desirable to treat it to remove any undesired impurities, such as dust particles before introducing it to the fermentation. For example, 10 the gaseous substrate may be filtered or scrubbed using known methods.

In other embodiments of the invention, the gaseous substrate comprising carbon monoxide may be sourced from the gasification of biomass. The process of gasification involves partial combustion of biomass in a restricted supply of air or oxygen. The resultant gas typically comprises mainly CO and H₂, with minimal volumes of CO₂, 15 methane, ethylene and ethane. For example, biomass by-products obtained during the extraction and processing of foodstuffs such as sugar from sugarcane, or starch from maize or grains, or non-food biomass waste generated by the forestry industry may be gasified to produce a CO-containing gas suitable for use in the present invention.

The CO-containing substrate will typically contain a major proportion of CO, such as at 20 least about 20% to about 100% CO by volume, from 40% to 95% CO by volume, from 40% to 60% CO by volume, and from 45% to 55% CO by volume. In particular embodiments, the substrate comprises about 25%, or about 30%, or about 35%, or about 40%, or about 45%, or about 50% CO, or about 55% CO, or about 60% CO by volume. Substrates having lower concentrations of CO, such as 6%, may also be appropriate, particularly when H₂ 25 and CO₂ are also present.

In particular embodiments, CO is supplied at a level sufficient for 2,3-butanediol production to occur. In particular embodiments, CO is provided such that a specific uptake rate of at least 0.4mmol/g/min; or at least 0.5mmol/g/min; or at least

- 16 -

0.6mmol/g/min; or at least 0.7mmol/g/min; or at least 0.8mmol/g/min; or at least 0.9mmol/g/min; or at least 1.0mmol/g/min; or at least 1.2mmol/g/min; or at least 1.5mmol/g/min is maintained. Those skilled in the art will appreciate methods of supplying CO, particularly gaseous CO, such that the required uptake rate is achieved.

5 However, by way of example, factors such as increasing gas hold-up in a fermentation media will increase the amount of CO available for conversion to products by the microbial culture. Those skilled in the art will appreciate methods of increasing gas hold-up. However, by way of non-limiting example, gas hold-up is typically increased by mechanical means such as increasing agitation in a CSTR. Furthermore, supplying CO at a
10 faster rate or a higher partial pressure will also increase the CO availability in a fermentation broth.

It is not necessary for the gaseous substrate to contain any hydrogen, however this is not considered detrimental to 2,3-butanediol production. The gaseous substrate may also contain some CO₂ for example, such as about 1% to about 80% by volume, or 1% to about
15 30% by volume. In one embodiment it contains about 5% to about 10% by volume. In another embodiment the gaseous substrate contains approximately 20% CO₂ by volume.

Typically, the carbon monoxide will be added to the fermentation reaction in a gaseous state. However, the invention should not be considered to be limited to addition of the substrate in this state. For example, the carbon monoxide could be provided in a liquid.
20 For example, a liquid may be saturated with a carbon monoxide containing gas and then that liquid added to a bioreactor. This may be achieved using standard methodology. By way of example a microbubble dispersion generator (Hensirisak et. al. Scale-up of microbubble dispersion generator for aerobic fermentation; Applied Biochemistry and Biotechnology Volume 101, Number 3 / October, 2002) could be used.
25 In one embodiment of the invention, the inventors have determined that 2,3-butanediol can be produced by fermentation of a first substrate and a second substrate. In one particular embodiment of the invention, 2,3-butanediol will be produced when a first

- 17 -

substrate, for example a carbohydrate such as fructose and a second substrate, preferably a substrate comprising CO, are provided.

In a further embodiment, the inventors have determined that 2,3-butanediol will be produced by a first substrate and on complete consumption, the first substrate may be replaced with a second substrate and the 2,3-butanediol continues to be produced. In a particular embodiment, the first substrate is fructose and on complete consumption of the fructose, a substrate comprising CO can be provided. The inventors have surprisingly found that 2,3-butanediol continues to be produced. The inventors further contemplate that the first substrate and second substrate may be alternated if needed. For example if a first substrate is unavailable, an alternative substrate may be used until the availability of the first substrate improves.

Media

It will be appreciated that for growth of the bacteria and substrate to butanediol fermentation to occur, in addition to the substrate, a suitable nutrient medium will need to be fed to the bioreactor. A nutrient medium will contain components, such as vitamins and minerals, sufficient to permit growth of the micro-organism used. Anaerobic media suitable for the growth of *Clostridium autoethanogenum* are known in the art, as described for example by Abrini *et al* (*Clostridium autoethanogenum*, sp. Nov., An Anaerobic Bacterium That Produces Ethanol From Carbon Monoxide; Arch. Microbiol., 161: 345-351 (1994)). The "Examples" section herein after provides further examples of suitable media.

Fermentation conditions

The fermentation should desirably be carried out under appropriate conditions for the substrate to butanediol fermentation to occur. Reaction conditions that should be considered include temperature, media flow rate, pH, media redox potential, agitation rate (if using a continuous stirred tank reactor), inoculum level, maximum substrate concentrations and rates of introduction of the substrate to the bioreactor to ensure that substrate level does not become limiting, and maximum product concentrations to avoid

product inhibition. Examples of fermentation conditions suitable for anaerobic fermentation of a substrate comprising CO are detailed in WO2007/117157, WO2008/115080, WO2009/022925 and WO2009/064200, the disclosure of which are incorporated herein by reference. It is recognised the fermentation conditions reported 5 therein can be readily modified in accordance with the methods of the instant invention.

The inventors have determined that, in one embodiment where pH is not controlled, there does not appear to be a deleterious effect on 2,3-butanediol production.

Bioreactor

Fermentation reactions may be carried out in any suitable bioreactor as described 10 previously herein. In some embodiments of the invention, the bioreactor may comprise a first, growth reactor in which the micro-organisms are cultured, and a second, fermentation reactor, to which broth from the growth reactor is fed and in which most of the fermentation product (2,3-butanediol, for example) is produced.

Product Recovery

15 The fermentation will result in a fermentation broth comprising a desirable product (such as butanediol) and/or one or more by-products (such as ethanol, acetate and butyrate) as well as bacterial cells, in a nutrient medium. In a preferred embodiment, the fermentation products include 2,3-butanediol.

2,3-butanediol, or a mixed alcohol stream containing 2,3-butanediol and one or more 20 other alcohols, may be recovered from the fermentation broth by methods known in the art, such as fractional distillation or evaporation, pervaporation, and extractive fermentation. By-products such as acids including acetate and butyrate may also be recovered from the fermentation broth using methods known in the art. For example, an adsorption system involving an activated charcoal filter or electrodialysis may be used.

25 In certain embodiments of the invention, 2,3-butanediol and by-products are recovered from the fermentation broth by continuously removing a portion of the broth from the bioreactor, separating microbial cells from the broth (conveniently by filtration, for

- 19 -

example), and recovering 2,3-butanediol and optionally other alcohols and acids from the broth. Alcohols may conveniently be recovered for example by distillation, and acids may be recovered for example by adsorption on activated charcoal. The separated microbial cells are preferably returned to the fermentation bioreactor. The cell free permeate 5 remaining after the alcohol(s) and acid(s) have been removed is also preferably returned to the fermentation bioreactor. Additional nutrients (such as B vitamins) may be added to the cell free permeate to replenish the nutrient medium before it is returned to the bioreactor.

Also, if the pH of the broth was adjusted during recovery of 2,3-butanediol and/or by-10 products, the pH should be re-adjusted to a similar pH to that of the broth in the fermentation bioreactor, before being returned to the bioreactor.

The invention will now be described in more detail with reference to the following non-limiting examples.

EXAMPLES

15 Materials and Methods

Solution A			
NH ₄ Ac	3.083g	KCl	0.15g
MgCl ₂ .6H ₂ O	0.61g	NaCl (optional)	0.12g
CaCl ₂ .2H ₂ O	0.294g	Distilled Water	Up to 1L
Solution B			
Biotin	20.0 mg	Calcium D-(*)-pantothenate	50.0 mg
Folic acid	20.0 mg	Vitamin B12	50.0 mg
Pyridoxine. HCl	10.0 mg	p-Aminobenzoic acid	50.0 mg
Thiamine. HCl	50.0 mg	Thioctic acid	50.0 mg
Riboflavin	50.0 mg	Distilled water	To 1 Litre
Nicotinic acid	50.0 mg		

- 20 -

Solution(s) C			
Component/0.1M solution (aq)	Quantity/ml into 1L media	Component/0.1M solution (aq)	Quantity/ml into 1L media
FeCl ₃	1ml	MnCl ₂	0.1ml
CoCl ₂	0.5ml	Na ₂ WO ₄	0.1ml
NiCl ₂	0.5ml	ZnCl ₂	0.1ml
H ₃ BO ₃	0.1ml	Na ₂ SeO ₃	0.1ml
Na ₂ MoO ₄	0.1ml		
Solution D		Solution E	
Media Component	Concentration per 1.0L of Media	Concentration per 1.0L of Media	
MgCl ₂ .6H ₂ O	0.5g	0.5g	
NaCl	0.2g	0.2g	
CaCl ₂	0.2g	0.2g	
100mM sodium phosphate buffer (pH 6.0)	-	160mL	
NaH ₂ PO ₄	2.04g	-	
NH ₄ Cl	2.5g	0.6g	
85% H ₃ PO ₄	-	0.5mL	
KCl	0.15g	0.15g	
Solution C	10mL	10mL	
Solution E	10mL	10mL	
Resazurin (1000 mg/L stock)	2mL	1mL	
FeCl ₃	0.01g	0.0025g	
Cysteine HCl monohydrate	0.5g	0.25g	
Agarose (optional)	15g	-	
Distilled water	To 1 Litre	To 1 Litre	

- 21 -

Solution F	
<i>Composite trace metal solution</i>	<i>per L of stock</i>
Nitrilotriacetic Acid	1.5g
MgSO ₄ .7H ₂ O	3.0g
MnSO ₄ .H ₂ O	0.5g
NaCl	1.0g
FeSO ₄ .7H ₂ O	0.1g
Fe(SO ₄) ₂ (NH ₄) ₂ . 6H ₂ O	0.8g
CoCl ₂ . 6H ₂ O	0.2g
ZnSO ₄ .7H ₂ O	0.2g
CuCl ₂ . 2H ₂ O	0.02g
AlK(SO ₄) ₂ .12H ₂ O	0.02g
H ₃ BO ₃	0.30g
NaMoO ₄ .2H ₂ O	0.03g
Na ₂ SeO ₃	0.02g
NiCl ₂ . 6H ₂ O	0.02g
Na ₂ WO ₄ .6H ₂ O	0.02g
Distilled water	To 1 Litre

Preparation of Na₂S_x

A 500ml flask was charged with Na₂S (93.7g, 0.39mol) and 200ml H₂O. The solution was 5 stirred until the salt had dissolved and sulfur (25g, 0.1mol) was added under constant N₂ flow. After 2 hours stirring at room temperature, the "Na₂S_x" solution (approx 4M with respect to [Na] and approx 5M with respect to sulfur), now a clear reddish brown liquid, was transferred into N₂ purged serum bottles, wrapped in aluminium foil.

Preparation of Cr (II) solution

10 A 1 L three necked flask was fitted with a gas tight inlet and outlet to allow working under inert gas and subsequent transfer of the desired product into a suitable storage flask. The flask was charged with CrCl₃.6H₂O (40g, 0.15 mol), zinc granules [20 mesh] (18.3g, 0.28

- 22 -

mol), mercury (13.55g, 1mL, 0.0676 mol) and 500 mL of distilled water. Following flushing with N₂ for one hour, the mixture was warmed to about 80°C to initiate the reaction. Following two hours of stirring under a constant N₂ flow, the mixture was cooled to room temperature and continuously stirred for another 48 hours by which time 5 the reaction mixture had turned to a deep blue solution. The solution was transferred into N₂ purged serum bottles and stored in the fridge for future use.

Bacteria:

The *Clostridium autoethanogenum* used is that deposited at the German Resource Centre for Biological Material (DSMZ) and allocated the accession number 19630.

10 *Sampling and analytical procedures*

Media samples were taken from the fermentation reactor (e.g. CSTR or serum bottle) at intervals over the course of the fermentation.. Each time the media was sampled care was taken to ensure that no gas was allowed to enter into or escape from the reactor.

HPLC:

15 HPLC System Agilent 1100 Series. Mobile Phase: 0.0025N Sulfuric Acid. Flow and pressure: 0.800 mL/min. Column: Alltech IOA; Catalog # 9648, 150 x 6.5 mm, particle size 5 µm. Temperature of column: 60°C. Detector: Refractive Index. Temperature of detector: 45°C.

Method for sample preparation:

20 400 µL of sample and 50 µL of 0.15M ZnSO₄ and 50 µL of 0.15M Ba(OH)₂ are loaded into an Eppendorf tube. The tubes are centrifuged for 10 min. at 12,000rpm, 4°C. 200 µL of the supernatant are transferred into an HPLC vial, and 5µL are injected into the HPLC instrument.

Headspace Analysis:

25 Measurements were carried out on a Varian CP-4900 micro GC with two installed channels. Channel 1 was a 10m Mol-sieve column running at 70°C, 200kPa argon and a backflush time of 4.2s, while channel 2 was a 10m PPQ column running at 90°C, 150kPa

- 23 -

helium and no backflush. The injector temperature for both channels was 70°C. Runtimes were set to 120s, but all peaks of interest would usually elute before 100s. Specific CO uptake was determined by calculating CO consumption per gram of cells (dry weight – see below).

5 **Cell Density:**

Cell density was determined by counting bacterial cells in a defined aliquot of fermentation broth. Alternatively, the absorbance of the samples was measured at 600nm (spectrophotometer) and the dry weight determined via calculation according to published procedures.

10 **Metal sulfide solution 1:**

Approximately 950mL of solution A was transferred into a 1L fermenter and sparged with nitrogen. H₃PO₄ (85% solution, 1.5mL) was added and the pH adjusted to 5.3 using concentrated NH₄OH(aq). Resazurin (1mL of a 2g/L solution) was added and the solution sparged with N₂. Chromium(II)chloride was added until the ORP of the solution 15 decreased to approximately -150mV. 10 x Solution(s) C were added before sodium polysulfide (1.44mL of a 4.3M or 1mL of a 6M solution) were added and the solution sparged with N₂.

Example 1A: Production of 2,3-butanediol by fermentation

Fermentative conversion of a substrate, using *Clostridium autoethanogenum* was 20 conducted in a CSTR reactor over a two week period, with periodic monitoring. The media used for the CSTR experiments were prepared in accordance with the components listed in Table E. The phosphate salt mixture consisted of 0.65mM Na₂HPO₄ and 15.3 mM NaH₂PO₄. All other components such as the phosphoric acid, the ammonium salts and the cysteine-hydrochloride were mixed into 800 ml of water before the buffer salts 25 were added to the solution. Proceeding in this manner ensured that the pH increased above about 6.5 avoiding the precipitation of media components. The solution was diluted to 1L and made anaerobic by heating to boiling and allowing it to cool to room temperature under a constant flow of N₂ gas. Once cool, the solution was adjusted to

- 24 -

the final pH of 5.3 and the B vitamins added. Anaerobicity was maintained throughout the experiment. Carbohydrate (5g/L fructose) was added to the basic media formulation. The media solutions were introduced into the fermenters and optionally sparged with the respective CO containing gases from the start of the experiment, or after a 5 predetermined interval. During these experiments, the pH was controlled to remain at 5.5 by adding an aqueous solution of NaOH. An actively growing *Clostridium autoethanogenum* culture was inoculated into the reactor at a level of 5 %(v/v). The temperature of the reactor was maintained at 37°C and agitation rate was 400 rpm.

Results:

10 Initially, the fermentation contained fructose as a substrate, which resulted in the production of acetic acid, ethanol and 2,3-butanediol. Over time, the fructose was consumed and a gas stream including CO (95% CO, 5% CO₂) was sparged through the media. The media was maintained at pH 5.5 (Table 1). It should be noted that even when the carbohydrate had been consumed, the above mentioned products increased in 15 concentration, clearly demonstrating that the CO was used to produce the products including 2,3-butanediol.

Table 1: Monitoring of production of 2,3-butanediol, ethanol and acetate (concentrations in g/L) over time in a CSTR reactor.

Time \ hours	Fructose	Acetic acid	Ethanol	2,3-Butanediol
0	5	0	0	0
23	5	0.123	0.018	0
45	3.8	0.579	0.167	0.05
110	0	4.753	2.8	1.2
185	0	7.2	3.8	1.9
324	0	6.736	4.9	1.91

- 25 -

Example 1B: Production of 2,3-butanediol by fermentation

In a further experiment, the conversion of a substrate by *Clostridium autoethanogenum* was conducted in a CSTR reactor over a 10 day period, with periodic monitoring. In this instance the fermenter and media were prepared in accordance with Example 1A, 5 however the substrate was exclusively simulated steel mill gas (70% CO, 1% H₂, 14% N₂, 15% CO₂) sparged continuously and the pH of the media was kept constant at 5.5 (Table 2). Conversion of the substrate again resulted in acetic acid, ethanol and 2,3-butanediol, demonstrating that even in the absence of a carbohydrate substrate at the commencement of the fermentation, acetic acid, ethanol and butanediol are produced.

10 *Table 2: Monitoring of production of 2,3-butanediol, ethanol and acetate (concentrations in g/L) over time in a CSTR batch reactor.*

Time \ days	Acetic acid	Ethanol	2,3- Butanediol
0	0	0	0
6	4.5	0.5	0
10	5	4	0.5

Example 2: Production of 2,3-butanediol by fermentation

In a further experiment, the conversion of a substrate by *Clostridium autoethanogenum* 15 was conducted in a CSTR reactor over a 3 day period, with periodic monitoring. In this instance, the fermenter and media were prepared in accordance with that described in Example 1A, however the substrate was simulated steel mill gas (70% CO, 1% H₂, 14% N₂, 15% CO₂), sparged continuously and fructose (10g/L) and the pH of the media was kept constant at 5.5 (Table 3). Conversion of the substrate again resulted in acetic acid, 20 ethanol and 2,3-butanediol.

- 26 -

Table 3: Monitoring of production of 2,3-butanediol, ethanol and acetate (concentrations in g/L) over time in a CSTR batch reactor.

Time \ hours	Fructose	Acetic acid	Ethanol	2,3- Butanediol
0	10	0	0	0
15	9.8	0.8	0.2	0.05
23	8.87	1.7	0.7	0.2
39	5.3	3.7	2.3	0.9
69	1.8	7.3	4	3.1

The final concentrations of acetate, ethanol and 2,3-butanediol were also compared between the fermenter experiments outlined in Examples 1A, 1B and 2, at the end of 5 each experiment (note, these results relate to the final concentrations measured in tables 1-3 and are summarised for comparison in Table 4).

Table 4: Examples of 2,3-butanediol production using alternative substrates in a CSTR reactor, measured on conclusion of each experiment. Results are given in g/L.

Substrate	Final Acetate	Final Ethanol	Final 2,3- Butanediol	Fermentation time (days)
Fructose then Mill gas (Ex 1A)	6.7	4.9	1.9	13.5
Mill gas only (Ex 1B)	5	4	0.5	10
Fructose and Mill gas (Ex 2)	7.3	4	3.1	2.9

- 27 -

Example 3: Production of 2,3-butanediol by fermentation

In order to ascertain how media conditions may affect the production of 2,3-butanediol, serum bottles containing media comprising a selection of buffers were prepared and the fermentation products analysed at the end of the experiment (Table 5). Incubation was 5 performed in 234ml sealed serum bottles each containing 50 ml of the above described media (Table E), optionally buffered with either an acetate buffer (0.02M) or a citrate buffer (0.02M) and adjusted to pH 5.3. The 184ml headspace of each serum bottle was initially N₂ and then filled to an overpressure of 30psi with either 95% CO₂, 5% CO₂, or 10 70% CO₂, 15% CO₂, 14% N₂, 1% H₂. Each bottle was inoculated with 2 ml of a *Clostridium autoethanogenum* culture. A shaking incubator was used and the reaction temperature was maintained at 37 °C.

Once again, it is clear that 2,3-butanediol is produced irrespective of the buffer used in the experiment. Furthermore, it should also be noted that since the serum bottles were not pH controlled, the product also appeared to be produced with limited (or no) control 15 of pH.

Table 5: Examples of 2,3-butanediol production in a variety of media. The media of the serum bottles were analysed after the active growth, i.e., the increase in cell mass levelled out after several days (4 to 7 days). Results are given in g/L.

Media used and system	Final Acetate	Final Ethanol	Final 2,3-Butanediol
Serum Bottle 0.02M acetate buffer	5.597	1.1	0.43
Serum Bottle 0.02M Citrate buffer	6.547	0.364	0.16

Example 4: Batch fermentation in CSTR

Approximately 1.3 L of solution A was transferred into a 2L fermenter and sparged with nitrogen. Resazurin (1.35mL of a 2g/L solution) was added. H_3PO_4 (85% solution, 2.025mL) was added and the pH adjusted to 5.3 using conc. $NH_4OH(aq)$.

5 Chromium(II)chloride was added until the ORP of the solution decreased to approximately -150mV. Sodium polysulfide (6.07ml of a 4.3M solution) was added and the pH adjusted to 5.5 using concentrated HCl. The solution was sparged with N2 for 1 hour before the addition of metal sulfide solution 1 (150ml) and Solution B (15ml). The solution was sparged with N2 then CO containing gas (3% H2; 30% N2; 47% CO; 20% 10 CO2), before inoculation with an actively growing *Clostridium autoethanogenum* culture at a level of approximately 5% (v/v). Gas flow rate was adjusted to ensure the microbial culture was not limited in CO in order to maintain a high specific CO uptake. Results of the fermentation are shown in Table 6.

Table 6: 2,3-butanediol productivity in a batch culture at varying specific CO uptakes.

Day	Average specific CO uptake (mmol/g/min)	Average 2,3-butanediol production rate
1-3	0.8	1.5g/L/day
5-6	0.1	0.25g/L/day

15 Total 2,3-butanediol accumulation over 7.5 days was approx 7.5g/L. It is recognised that 2,3-butanediol is produced at low levels at lower specific CO uptake rates. However, when the gas is supplied such that the CO uptake rate can be maintained over 0.4mmol/g/min, 2,3-butanediol productivity increase significantly. In this instance, the specific CO uptake is maintained at an average of 0.8mmol/g/min over several days and 20 1,3-butanediol is produced at a rate in excess of 1g/L.

Example 5: Batch fermentation in CSTR

Approximately 1.3 L of solution A was transferred into a 2L fermenter and sparged with nitrogen. H_3PO_4 (85% solution, 2.025mL, 30mM) was added and the pH adjusted to 5.3 using concentrated $NH_4OH(aq)$. Solution B (13.5ml) was added and the solution sparged

5 with N_2 . Chromium(II)chloride was added until the ORP of the solution decreased to approximately -150mV. Resazurin (1.35mL of a 2g/L solution) was added. Sodium polysulfide (2.85ml of a 6M solution) was added and the solution sparged with N_2 for 12 hours before switching to CO containing gas (1% H_2 ; 14% N_2 ; 70% CO; 15% CO_2). The pH was adjusted to 5.5 with concentrated HCl before the addition of metal sulfide solution 10 (150ml). The solution was sparged with the CO containing gas for a further 30 minutes before inoculation with an actively growing *Clostridium autoethanogenum* culture at a level of approximately 5% (v/v). Again, gas flow rate was adjusted to ensure the microbial culture was not limited in CO in order to maintain a high specific CO uptake.

Results of the fermentation are shown in Table 7.

15 *Table 7: 2,3-butanediol productivity in a batch culture at varying specific CO uptakes.*

Day	Average CO uptake (mmol/g/min)	Average 2,3-butanediol production rate
1-4	0.85	0.8g/L/day
5-6	0.3	0.25g/L/day

Total 2,3-butanediol after approx 6 days was 5g/L. Once again, elevated specific CO uptake results in significantly higher 2,3-butanediol productivity of at least 0.5g/L/day.

Example 6: Batch fermentation in CSTR

Approximately 1.3 L of solution A was transferred into a 1.5L fermenter and sparged with

20 nitrogen. H_3PO_4 (85% solution, 2.25mL) was added and the pH adjusted to 5.3 using concentrated $NH_4OH(aq)$. Solution B (15ml) was added and the solution sparged with N_2 . Chromium(II)chloride was added until the ORP of the solution decreased to approximately -150mV. Resazurin (1.5mL of a 2g/L solution) was added. Sodium

- 30 -

polysulfide (1.5ml of a 3M solution) was added and the solution sparged with N₂ for 1 hour. 0.1M solutions of FeCl₂ (3.75mL), CoCl₂ (1.875mL), NiCl₂ (1.875mL), H₃BO₃ (0.375ml), Na₂MoO₄ (0.375ml), MnCl₂ (0.375ml), Na₂WO₄ (0.375ml) and ZnCl₂ (0.1875ml) were added and the solution sparged with CO containing gas (50% H₂; 32% CO; 4% CO₂; 5 32% CH₄). The pH was adjusted to 5.5 with concentrated HCl before the addition of Solution C (150ml). The solution was sparged with the CO containing gas for a further 30 minutes before inoculation with an actively growing *Clostridium autoethanogenum* culture at a level of approximately 5% (v/v). Gas flow rate was adjusted to ensure the microbial culture was not limited in CO in order to maintain a high specific CO uptake.

10 Results of the fermentation are shown in Table 8.

Table 8: 2,3-butanediol productivity in a batch culture at varying specific CO uptakes.

Day	Average CO uptake (mmol/g/min)	Average 2,3-butanediol production rate
0-4	0.07	0
5-14	0.15	0.2g/L/day

15 Total 2,3-butanediol concentration after 4 days was approximately 3 g/L. While the rates achieved are less than previous fermentations (examples 4 and 5), the substrate stream comprises a substantial portion of hydrogen. The results show that 2,3-butanediol is produced when using a mixed CO/H₂ substrate.

Example 7: Continuous Fermentation in Continuous Stirred Tank Reactor

Media was prepared at pH 5.5 as follows. All ingredients in Solution D, with the exception of Cysteine-HCl were mixed in 400ml distilled water. This solution was made anaerobic by heating to boiling and allowing it to cool to room temperature under a constant flow 20 of 95% CO, 5% CO₂ gas. Once cool, the Cysteine-HCl was added and the pH of the solution adjusted to 5.5 before making the volume up to 1000ml; anaerobicity was maintained throughout the experiments.

- 31 -

A five-litre bioreactor was charged with 4.9L of LM33 media prepared as described above. The gas was switched to CO containing gas (1% H₂; 14% N₂; 70% CO; 15% CO₂) at atmospheric pressure prior to inoculation with 100 ml of a *Clostridium autoethanogenum* culture. The bioreactor was maintained at 37°C stirred at 200 rpm at the start of the 5 culture. During the growth phase, the agitation was increased to 400 rpm. The pH was adjusted to 5.5 and maintained by automatic addition of 5 M NaOH. Fresh anaerobic media was continuously added into the bioreactor to maintain a defined biomass and acetate level in the bioreactor. 2,3 butanediol productivity is highlighted in Table 9.

Table 9: 2,3-butanediol productivity in a continuous culture.

Day	Average CO uptake (mmol/g/min)	Average 2,3-butanediol production rate
1-87	0.3	<0.1g/L/day
90-92	0.6	1.2g/L/day
93-95	0.4	0.87g/L/day

10 During the first 89 days of continuous operation, the fermenter was operated under CO limited conditions and minimal 2,3-butanediol was produced. However, around day 88, gas flow was increased, such that specific CO uptake increased. At this stage, 2,3-butanediol productivity increased significantly to at least 1.2 g/L/day. Around day 92, the gas flow was reduced such that the specific uptake of the culture decreased to around 15 0.4mmol/g/min and the 2,3-butanediol productivity also dropped. However, even at an average specific uptake of approximately 0.4mmol/g/min, 2,3-butanediol productivity remained at least 0.5g/L/day.

Example 8: Batch fermentation in CSTR

Approximately 1.3 L of solution A was transferred into a 2L fermenter and sparged with 20 nitrogen. H₃PO₄ (85% solution, 1.5ml) was added and the pH adjusted to 5.3 using conc. NH₄OH(aq). Solution B (15ml) was added and the solution sparged with N₂.

- 32 -

Chromium(II)chloride was added until the ORP of the solution decreased to approximately -150mV. Sodium polysulfide (1ml of a 3M solution) was added and the solution sparged with N2 for 12 hours. 0.1M solutions of FeCl₂ (3.75mL), CoCl₂ (1.875mL), NiCl₂ (1.875mL), H₃BO₃ (0.375ml), Na₂MoO₄ (0.375ml), MnCl₂ (0.375ml), Na₂WO₄ 5 (0.375ml) and ZnCl₂ (0.2ml) were added and the solution sparged with CO containing gas (1% H₂; 14% N₂; 70% CO; 15% CO₂).

Resazurin (1mL of a 2g/L solution) was added. The pH was adjusted to 5.5 with concentrated HCl and the solution was sparged with the CO containing gas for a further 30 minutes before inoculation with an actively growing *Clostridium autoethanogenum* 10 culture at a level of approximately 5% (v/v). Table 10 shows the accumulated 2,3-butanediol product in a fermenter after approximately 2 weeks operation. The specific CO uptake rates have been corrected for the culture viability. Culture viability was determined using the methods described in WO2009/022925, which is incorporated herein by reference.

15 *Table 10: 2,3-butanediol accumulation after 14 days batch fermentation.*

Day	Specific CO uptake (mmol/g/min)	2,3-butanediol (accumulated product)
13	0.6	8.67g/L
14	0.5	9.27g/L

Over the 24 hour period from day 13-14, the specific CO uptake was maintained at approximately 0.5mmol/g/min and 2,3-butanediol productivity was 0.6g/L/day.

Example 9: Gene Regulation of 2,3-Butanediol production in LZ1560

Samples were taken from three fermentations to determine gene expression during 2,3- 20 butanediol production. One sample was taken from the batch fermentation described in Example 8 on day 13, wherein products including ethanol and 2,3-butanediol were being produced. The sample is designated R12 in the results hereinafter. The second sample was taken from a batch fermentation producing both ethanol and 2,3-butanediol. The

- 33 -

sample is designated R11 in the results. The third sample (R2) was taken from the continuous fermentation operating under similar conditions as Example 7 on days 1-89. The microbial culture was CO limited and the fermentation broth had a stable acetate concentration of approximately 13g/L, ethanol concentration of less than 1g/L and 5 insignificant amounts of 2,3-butanediol. Real-Time PCR was used to determine whether genes were upregulated or downregulated relative to R2.

RNA extraction and cDNA synthesis procedure:

Total RNA was isolated from approximately 2.5×10^9 bacterial cells using Aurum Total RNA Fatty and Fibrous Tissue Kit (Biorad). On-column DNase was digested using the 10 RNase-free DNase set (Biorad). Total RNA was quantified using spectrophotometer and its purity (measured by the $A_{260/280}$ ratio) was determined before cDNA synthesis using iScript Select cDNA synthesis kit (Biorad).

Real-Time PCR procedure:

Primers for Real-Time PCR, were designed using the freeware Primer3 based on 15 LanzaTech's proprietary in-house genome sequence. Real-Time PCR reaction mixtures containing 12.5 μ L 2x SYBR Green PCR Master Mix (Biorad), 1.5 μ L of each of 1 μ M primer forward and reverse, 5 μ L of 10x diluted cDNA template, and sterile water to a total volume of 25 μ L were assembled. The mixtures were heated to 95°C for 3 minutes, followed by 40 cycles of 95°C for 15 seconds, 55°C for 15 seconds and 72°C for 30 20 seconds. For detection of primer dimerisation or other artifacts of amplification, a melting-curve analysis was performed immediately after completion of the real-time PCR (38 cycles of 58°C to 95°C at 1°C/seconds). All reactions were performed in triplicate. Quantification of gene expression was performed using a MyiQ Single Colour Real-Time 25 PCR Detection System (Biorad) and real-time data were analyzed using the iQ5 optical system software (Biorad).

Results:

The raw Ct values, together with the relative gene expression and standard errors, generated from the Real-Time PCR assay are presented in Table 11. RNA polymerase beta

- 34 -

chain (*rpoB*) was selected as reference gene for normalizing gene expression. Relative quantification using the Comparative ΔC_T method was used to calculate the relative gene expression of 2,3BDH. The acetate-producing culture (R2) was selected as calibrator (reference standard) in all analysis.

5 *Table 11: Derivation of relative gene expression values from raw Ct data. Relative expressions were normalized by *rpoB* and calibrated using Reactor 2. SE = standard error of the mean. (Any relative expression above 1 shows up regulation)*

Genes	Reactor	Raw Ct	Mean Ct	SD Ct	Relative expression	Relative expression SE
<i>rpoB</i>	11	36.36	35.4	0.846	NA	NA
	11	35.07				
	11	34.77				
	12	32.47	32.7	0.258	NA	NA
	12	32.65				
	12	32.98				
	2	31.76	31.76	0.051	NA	NA
	2	31.81				
2,3BDH	11	27.01	26.57	0.422	4.75	1.8
	11	26.53				
	11	26.17				
	12	23.23	23.18	0.038	7.64	0.8
	12	23.15				
	12	23.17				
	2	24.81	25.18	0.559	1	0.23

- 35 -

2	24.91					
2	25.82					

Real-time PCR data presented in this study shows that 2,3-butanediol gene expression is significantly higher in solventogenic cultures (R11/R12) compared to acetogenic cultures (R2). The microbial culture of R12, which was producing approximately 0.6g/L/day 2,3-butanediol at the time of cell harvest, shows the highest gene up-regulation (7.64 ± 0.8 fold), relative to R2. This is followed by R11 with 4.75 ± 1.8 fold up-regulation of the gene, which had a total 2,3-butanediol production of 1.53g/L, when the cells were harvested.

Figure 1 shows relative gene expression of 2,3-butanediol dehydrogenase (2,3BDH) in three fermenters (R11, R12 and R2). Acetate-producing R2 is selected as calibrator and gene expression was normalized using *rpoB* as reference gene. Error bar = standard error of mean. N=3. Clearly, 2,3-butanediol dehydrogenase is upregulated in microbial cultures that produce 2,3-butanediol. The microbial culture in R2 has a specific CO uptake of approximately 0.3mmol/g/min, whereas the culture in R12 has a specific uptake of approximately 0.6mmol/g/min. Increasing the amount of CO provided to the culture results in an increase in CO uptake and a subsequent increase in 2,3-butanediol dehydrogenase gene expression. The increase in 2,3-butanediol dehydrogenase gene expression results in an increase in overall 2,3-butanediol productivity.

The invention has been described herein with reference to certain preferred embodiments, in order to enable the reader to practice the invention without undue experimentation. Those skilled in the art will appreciate that the invention is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. Furthermore, titles, headings, or the like are provided to enhance the reader's comprehension of this document, and should not be read as limiting the scope of the present invention.

WO 2009/151342

PCT/NZ2009/000101

- 36 -

The reference to any prior art in this specification is not, and should not be taken as, an acknowledgment or any form of suggestion that that prior art forms part of the common general knowledge in the United States of America or any country in the world.

Throughout this specification and any claims which follow, unless the context requires otherwise, the words "comprise", "comprising" and the like, are to be construed in an inclusive sense as opposed to an exclusive sense, that is to say, in the sense of "including, but not limited to".

WHAT IS CLAIMED IS:

1. A method of producing 2,3-butanediol by microbial fermentation of a gaseous substrate comprising CO, the method comprising:
 - a. continuously providing the gaseous substrate to a bioreactor; and
 - b. anaerobically fermenting the substrate in the bioreactor, the bioreactor comprising a culture of at least one acetogenic bacterium to produce 2,3-butanediol; wherein the gaseous substrate is provided such that a specific rate of CO uptake of at least 0.4mmol CO/gram dry cells weight of bacteria/minute by the culture is maintained and 2,3-butanediol is produced at a productivity greater than 0.2g/L/per day.
2. The method of claim 1, wherein the butanediol is 2,3-butanediol.
3. The method of claim 1 further comprising converting the 2,3-butanediol to a compound selected from the group consisting of butene(s), butadiene, methyl ethyl ketone (MEK) and mixtures thereof.
4. The method of claim 3 further comprising converting the butene(s), butadiene or methyl ethyl ketone to a chemical product.
5. The method of claim 4 where the methyl ethyl ketone is converted to 2-butanol.
6. The method according to claim 1, wherein the substrate is provided such that a specific uptake rate of at least 0.6mmol CO/g/min is maintained.
7. The method according to claim 1, wherein the substrate is provided such that a specific uptake rate of at least 0.8mmol CO/g/min is maintained.
8. The method according to claim 1, wherein the substrate is provided such that a specific uptake rate of at least 1.0mmol CO/g/min is maintained.
9. The method according to claim 1, wherein the gaseous substrate comprises at least 15% to about 100% CO by volume.
10. The method according to claim 1, wherein the gaseous substrate comprises a gas obtained as a by-product from an industrial process.
11. The method according to claim 10, wherein the gaseous substrate comprises waste gas from a steel mill.
12. The method according to claim 1 wherein the acetogenic bacterium is *Clostridium autoethanogenum*.
13. The method according to claim 1 wherein ethanol is produced as a co-product of the fermentation.

- 38 -

14. The method according to claim 1 wherein the acetogenic bacterium is the *Clostridium autoethanogenum* strain deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number DSM 19630.

15. A method of producing 2,3-butanediol by microbial fermentation of a gaseous substrate comprising CO, the method comprising:

- a. continuously providing the gaseous substrate to a bioreactor;
- b. anaerobically fermenting the gaseous substrate in the bioreactor, the bioreactor comprising a culture of at least one acetogenic bacterium, wherein the at least one acetogenic bacterium comprises at least one 2,3-butanediol dehydrogenase gene;
- c. upregulating the 2,3-butanediol dehydrogenase gene by providing the gaseous substrate such that the specific uptake of CO by the at least one acetogenic bacterium is at least 0.4mmol/g/min, and such that 2,3-butanediol is produced at a productivity greater than 0.2g/L/per day; and
- d. converting the 2,3-butanediol produced in step (c) to a compound selected from the group consisting of butane, butadiene, methyl ethyl ketone and mixtures thereof.

16. The method according to claim 15 wherein the acetogenic bacterium comprises *Clostridium autoethanogenum*.

17. The method according to claim 16 wherein the acetogenic bacterium is the *Clostridium autoethanogenum* strain deposited at the German Collection of Microorganisms and Cell Cultures (DSMZ) under the accession number DSM 19630.

18. The method of claim 15 further comprising converting the butene(s), butadiene or methyl ethyl ketone to a chemical product.

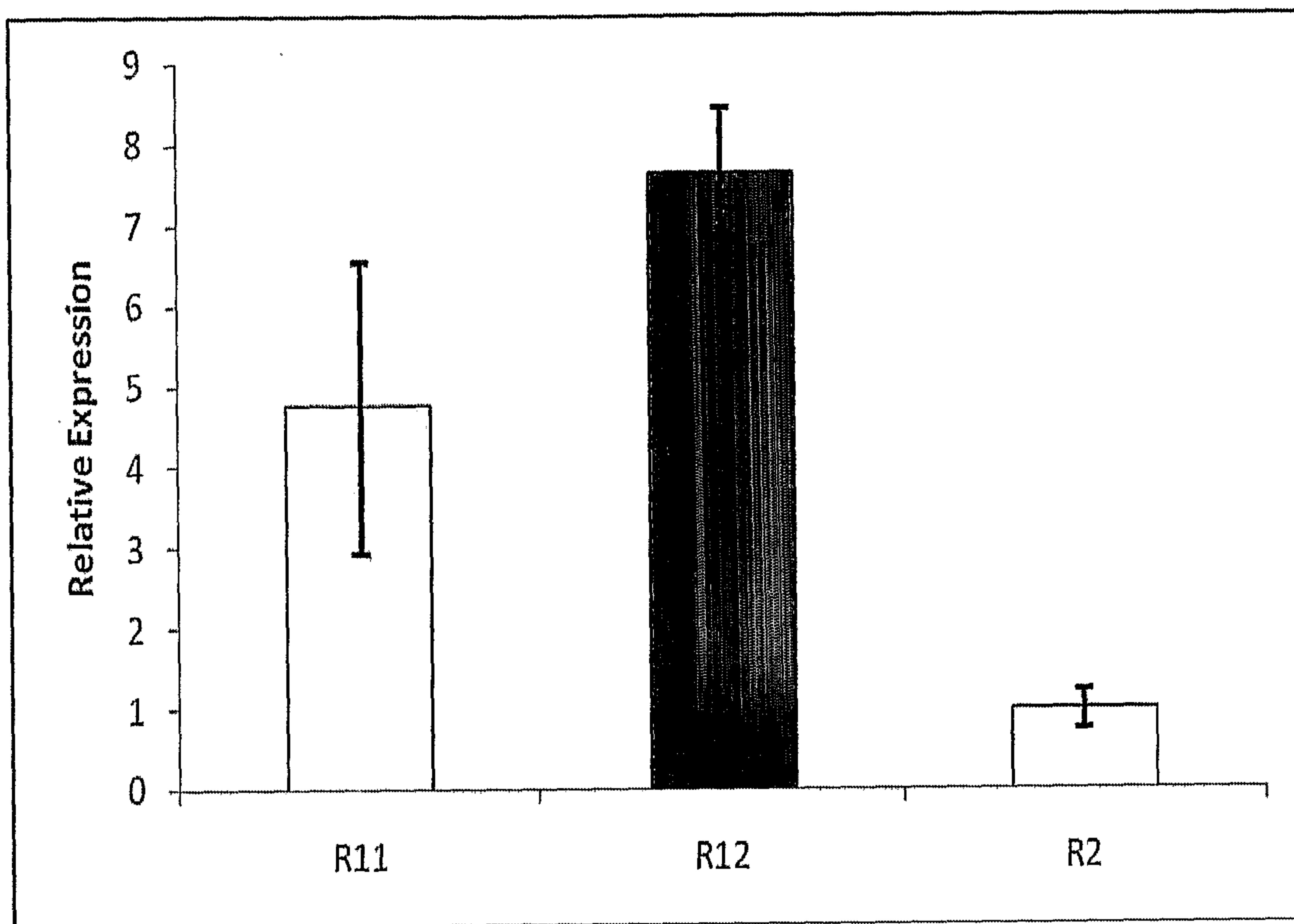
19. The method according to claim 15, wherein the substrate is provided such that a specific uptake rate of at least 0.6mmol CO/g/min is maintained.

20. The method according to claim 15, wherein the gaseous substrate comprises at least 15% to about 100% CO by volume.

21. The method according to claim 15, wherein the gaseous substrate comprises waste gas from a steel mill.

22. The method according to claim 15 wherein ethanol is produced as a co-product of the fermentation.

1 / 1

**Figure 1**

