



(43) International Publication Date
15 September 2016 (15.09.2016)

WIPO | PCT

(10) International Publication Number
WO 2016/142419 A1

(51) International Patent Classification:

C12N 15/01 (2006.01) C12N 1/20 (2006.01)
C12P 7/18 (2006.01) C12R 1/46 (2006.01)

(21) International Application Number:

PCT/EP2016/055012

(22) International Filing Date:

9 March 2016 (09.03.2016)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

15158254.1 9 March 2015 (09.03.2015) EP

(71) Applicant: FUNDACION TECNALIA RESEARCH & INNOVATION [ES/ES]; Parque Científico y Tecnológico de Bizkaia, C. Geldo, Edificio 700, 48160 Derio (ES).

(72) Inventors: RONCAL MARTÍNEZ, Tomás; Parque Tecnológico de San Sebastián, Mikeletegi Pasealekua, num. 2, 20009 Donostia-San Sebastián (ES). CABALLERO ROMÁN, Susana; Parque Tecnológico de San Sebastián, Mikeletegi Pasealekua, num. 2, 20009 Donostia-San Sebastián (ES). DÍAZ DE GUEREÑU ZABARTE, María del Mar; Parque Tecnológico de San Sebastián, Mikeletegi Pasealekua, num. 2, 20009 Donostia-San Sebastián (ES). RINCÓN ARROYO, Inés; Parque Tecnológico de San Sebastián, Mikeletegi Pasealekua, num. 2, 20009 Donostia-San Sebastián (ES). OCHOA GÓMEZ, José Ramón; Parque Tecnológico de Álava, C. Leonardo da Vinci, 11, 01510 Miñano (ES).

(74) Agent: ZBM PATENTS - Zea, Barlocchi & Markvardsen; Pl. Catalunya, 1 2nd floor, 08002 Barcelona (ES).

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

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

Declarations under Rule 4.17:

— as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))

Published:

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

(54) Title: METABOLITE PRODUCTION BY LACTIC ACID BACTERIUM

(57) Abstract: It is provided a process for the isolation of a strain of a bacterium *Lactococcus lactis lactis* with increased 2,3-butanediol production capacity, the process comprising subjecting strain CML B4 to two consecutive rounds of mutagenesis. It is also provided a new strain of the bacterium *Lactococcus lactis lactis*, characterized by a high ability to produce 2,3-butanediol, obtainable by the process as defined above, and a method to produce such 2,3-butanediol by culturing the aforementioned bacterium under microaerobiosis.



WO 2016/142419 A1

Metabolite production by lactic acid bacterium

The present invention belongs to the field of microorganisms obtained by mutagenesis. Specifically, this invention is related to a new lactic acid bacteria
5 strain belonging to the species *Lactococcus lactis lactis*, characterized by a high ability to produce 2,3-butanediol, as well as to a method to produce such 2,3-butanediol by the aforementioned bacterium.

BACKGROUND ART

10 2,3-Butanediol (2,3-BDO) is a chemical that can be produced as a result of the metabolic activity of several microorganisms.

2,3-BDO shows a great potential interest in the fields of fuels and chemicals (Voloch, M. et al. "Comprehensive biotechnology", Chapter 45, 2,3-Butanediol, Pergamon Press, New York, 1985. pp. 933-947), including uses as a building
15 block in the manufacture of a wide range of chemicals. It can be used as solvent, antifreeze agent, liquid fuel and monomer for the manufacture of many synthetic polymers and resins. It has a heating value of $27,200 \text{ J g}^{-1}$, similar to that of ethanol ($29,100 \text{ J g}^{-1}$) and methanol ($22,100 \text{ J g}^{-1}$), which would make it suitable as liquid fuel and fuel additive. Moreover, due to its
20 high octane number, it could serve as an octane booster for petrol. 2,3-BDO also finds additional potential applications in the production of printing inks, perfumes, fumigants, spandex, moistening and softening agents, plasticizers and as a carrier for pharmaceuticals.

Of special relevance are the uses of 2,3-BDO as building block, that is, as
25 precursor in the synthesis of valuable industrial chemicals, including acetoin and diacetyl, methyl ethyl ketone, 2-butanol, butenes, 1,3-butadiene and plastics (polyesters, polycarbonates and polyurethanes). Among them, 1,3-butadiene deserves special attention because it is the monomer for the synthesis of synthetic rubber, mainly used in the manufacture of tires. In
30 addition, some of the above derivatives can be converted, by means of oligomerization, condensation and hydrogenation reactions, into higher hydrocarbons with potential uses as (bio)fuels, including jet fuels.

Commercially, the key downstream products of 2,3-BDO have a potential global market of around 32 million tons per annum, valued at approximately \$43 billion in sales. (Kopke M, Mihalcea C, Liew FM, Tizard JH, Ali MS, Conolly JJ, Al-Sinawi B, Simpson SD "2,3-Butanediol production by
5 acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas", *Appl. Environ. Microbiol.*, 2011, Vol. 77, pp. 5467–5475).

The fact that 2,3-BDO can be biotechnologically produced from biomass, that is, from renewable feedstocks, increases its interest because that biobased
10 2,3-BDO would be more environmentally sustainable and would allow to add the prefix bio- to all its derivatives and products.

Many microorganisms are known to produce 2,3-BDO, but only a few of them make it in quantities high enough to be potentially considered as industrially relevant. The best producers are bacteria belonging to the genera *Klebsiella*,
15 *Enterobacter*, *Bacillus* and *Serratia*, especially *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Paenibacillus polymyxa*.

The metabolic pathway involved in the biosynthesis of 2,3-BDO starts with pyruvate resulting from sugar metabolism and comprises three steps. In the first step, the thiamine-dependent enzyme α -acetolactate synthase catalyzes
20 the condensation of two molecules of pyruvate yielding a molecule of α -acetolactate and losing a molecule of CO₂. α -Acetolactate is then converted into acetoin by a decarboxylation reaction catalyzed by α -acetolactate decarboxylase. Finally, acetoin is reduced to 2,3-BDO by acetoin reductase/2,3-BDO dehydrogenase using NADH as cofactor.

25 Regarding an industrial-scale production of 2,3-BDO by fermentation, the fact that the best producers, *K. pneumoniae* and *K. oxytoca*, are pathogenic bacteria (class 2) is a cause of strong concern. Industrial-scale fermentation processes require following strict safety measures, implying that the use of class 2 microorganisms is an obstacle for industrial development of said
30 fermentation processes. Therefore, there is a need for class 1 microorganisms (safe) able to produce 2,3-BDO as efficiently as the aforementioned class 2 bacteria. Several class 1 bacteria have been reported to produce 2,3-BDO,

but, in general, their efficiency was too low for an economic process. As an alternative, production of 2,3-BDO has been engineered in *Escherichia coli*, a bacteria that is not a natural producer of that metabolite and that, therefore, requires the construction of the complete biosynthetic pathway (Nielsen, D.R.,
5 Yoon S.H., Yuan, C.J., and Prather, K.J., "Metabolic Engineering of Acetoin and meso-2,3 Butanediol Biosynthesis in *E. coli*.", *Biotechnol. J.*, 2010, Vol. 5, pp. 274-284). Another possibility to be considered is the use of class 1 natural producers and improving their 2,3-BDO synthesis capacities. Within this last alternative the lactic acid bacterium *Lactococcus lactis* appears as a possible
10 candidate.

L. lactis shows several advantageous features to be used in industrial processes: it has a small sized, well characterized, genome, which has been sequenced for several strains; a wide range of tools are available for its genetic manipulation; it shows a fast growth under either aerobic or anaerobic
15 conditions; it displays a high glycolytic flux; it has a rather simple energy and carbon metabolism; it has different metabolic pathways for the production of interesting chemicals; it is not a pathogen and it is considered a GRAS (Generally Recognized As Safe) organism, which allows it to be used in food applications. Leaving aside food uses, that have their own characteristics, all
20 these features make *L. lactis* a highly suitable host for development of cell factories intended for the efficient production of valuable industrial chemicals by fermentation.

L. lactis is a facultative anaerobe characterized by producing lactic acid as the main product of carbohydrate fermentation (homolactic fermentation). Under
25 certain conditions, nevertheless, this bacterium can also carry out a heterolactic or mixed-acid fermentation, producing a different profile of metabolites, including 2,3-BDO. However, although *L. lactis* contains a complete metabolic pathway for 2,3-BDO biosynthesis, natural production of this metabolite is in general residual.

30 There are a few reports describing the use of metabolic engineering to increase the production of 2,3-BDO in *L. lactis* (Platteeuw C, Hugenholtz J, Starrenburg M, van Alen-Boerrigter I, de Vos WM, "Metabolic engineering of

Lactococcus lactis — influence of the overproduction of alpha-acetolactate synthase in strains deficient in lactate-dehydrogenase as a function of culture conditions", *Appl. Environ. Microbiol.*, 1995, Vol. 61, pp. 3967–71; Gaspar P, Neves AR, Gasson MJ, Shearman CA, Santos H, "High yields of 2,3-butanediol and mannitol in *Lactococcus lactis* through engineering of NAD(+) cofactor recycling", *Appl. Environ. Microbiol.*, 2011, Vol. 77, pp. 6826–35) and other lactic acid bacteria (Enhanced pyruvate to 2,3-butanediol conversion in lactic acid bacteria, PCT/US2009/058834). All of them lie, on the one hand, in the inactivation of competitive pathways, specially L-lactate dehydrogenase, and, on the other hand, in the overexpression of the genes of the 2,3-BDO pathway.

Production of 2,3-BDO by these recombinant strains requires that they are cultured in media containing antibiotics to maintain the foreign genetic material introduced into them and/or compounds such as nisin that induce an increased gene expression. In any case, culture media must be supplemented with expensive ingredients that increase operation cost to a level hardly compatible with an economically efficient industrial process.

SUMMARY OF THE INVENTION

Keeping in mind that *L. lactis* contains the complete metabolic pathway for 2,3-BDO, inventors have developed an effective process to manipulate its genome by mutation, that is, without introducing into the bacterium foreign DNA sequences and without requiring the use of chemical inducers, in order to allow an efficient 2,3-BDO production by fermentation using a safe microorganism.

An object of the present invention is to provide a procedure for obtaining new strains of *L. lactis* with increased 2,3-BDO production capacity. Thus, a first aspect of the invention is to provide a process for the isolation of a strain of a bacterium *Lactococcus lactis lactis* with increased 2,3-BDO production capacity, the process comprising subjecting strain CML B4, deposited at the Spanish Type Culture Collection under accession number CECT 7512, to two consecutive rounds of mutagenesis and selecting a mutant strain which is

deficient in ethanol biosynthesis and has a reduced lactic acid synthesis in relation to the non-mutated CML B4 strain, wherein selecting the mutant strain is performed by: after a first round of mutagenesis a strain is selected, which is deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain; and wherein, after a second round of mutagenesis applied to the strain selected in the first round, a strain is selected that has a reduced lactic acid synthesis in relation to the non-mutated CML B4. Therefore, selecting a mutant strain which is deficient in ethanol biosynthesis and has a reduced lactic acid synthesis in relation to the non-mutated CML B4 strain, is to be understood or means that within the two consecutive rounds of mutagenesis, after a first round of mutagenesis a strain is selected that is deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain, and is this strain deficient in ethanol biosynthesis that is submitted to a second round of mutagenesis and, after this second round, a strain is then selected that has a reduced lactic acid synthesis in relation to the non-mutated CML B4, so that the final isolated strain has at least a reduced lactic acid synthesis in relation to the non-mutated CML B4.

Thus, the first aspect can also be formulated as a process for the isolation of a strain of a bacterium *Lactococcus lactis lactis* with increased 2,3-BDO production capacity is provided, the process comprising subjecting strain CML B4 deposited at the Spanish Type Culture Collection under accession number CECT 7512 to two consecutive rounds of mutagenesis, wherein after a first round of mutagenesis a strain is selected, which is deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain; and wherein, after a second round of mutagenesis applied to the strain selected in the first round, a strain is selected that has a reduced lactic acid synthesis in relation to the non-mutated CML B4.

Along this description the strain CECT 7512 is also referred as strain CML B4. "Increased 2,3-BDO production capacity" is to be understood as a production of 2,3-BDO higher than the produced in the same conditions by the CML B4 strain from which it derives. In particular, a 2,3-BDO production of at least 5-fold the 2,3-BDO produced by CML B4 strain in the same conditions.

Another aspect of the invention relates to the strain of the bacterium *Lactococcus lactis lactis*, which is capable of producing 2,3-BDO, obtainable by the process of the invention. This strain is capable of producing increased levels of 2,3-BDO, of at least 5-fold the 2,3-BDO production achieved by the CML B4 strain under the same anaerobiosis conditions. Indeed, strains of the invention are capable of producing 2,3-BDO in all tested conditions of oxygen concentration (anaerobiosis, aerobiosis, and microaerobiosis) and pH.

Specifically, one of these strains is the strain RC2. The strain RC2 is derived from the strain CML B4 (deposited at the Spanish Type Culture Collection under accession number CECT 7512 and described in the Spanish Patent ES2352633B1), following two rounds of random mutagenesis. Thus, another aspect of the invention relates to an isolated strain of the bacterium *Lactococcus lactis lactis*, derived from the acetoin overproducing strain CML B4, which is capable of producing 2,3-BDO with high yield and which is deposited at the Spanish Type Culture Collection (CECT) under accession number CECT 8792. The strain of *Lactococcus lactis lactis* of the invention was deposited, according to the requirements of the Budapest Treaty, on November 18th 2014 in the Spanish Type Culture Collection (CECT), located in the Universidad de Valencia C.P 46980 Catedrático Agustín Escardino, num. 9, Paterna, Valencia (Spain), by the depositor Fundación Tecnalia Research & Innovation, located in the Parque Tecnológico de San Sebastián, Mikeletegi Pasealekua, num. 2, E-20009 Donostia-San Sebastián (Spain). The strain of *Lactococcus lactis lactis* was identified by the depositor with the reference RC2, and received the accession number CECT 8792. It was, in addition, declared viable.

This microorganism, belonging to the group of lactic acid bacteria, advantageously shows an increased ability to produce 2,3-BDO and has been obtained through a process of random mutagenesis and selection, as explained below. Taxonomically, the strain of the present invention (hereinafter referred to as strain RC2) is classified in the genus *Lactococcus*, species *lactis*, subspecies *lactis*. Therefore, the strain object of the present invention is specifically the strain *L. lactis lactis* RC2.

Another aspect of the invention is a method for the production of 2,3-BDO comprising carbohydrate fermentation under microaerobiosis by the strain RC2 as defined above.

5 DETAILED DESCRIPTION OF THE INVENTION

In a particular embodiment, the strains of the invention as defined above are obtained by genetic manipulation, namely by mutagenesis of the strain CML B4.

10 In a more particular embodiment the mutagenesis is done by random mutagenesis.

Random mutations are induced using chemical mutagens, such as ethyl methanesulfonate (EMS) and others, although the way the mutations are obtained is not limited to this procedure and any other method available in the art can be equally valid, including physical, chemical, DNA recombinant and
15 other procedures.

L. lactis lactis CML B4 is characterized by a reduced L-lactate dehydrogenase activity and an increased NADH oxidase activity, which results in an aerobic acetoin overproduction with reduced lactic acid (and other metabolites) generation. So, due to its low L-lactate dehydrogenase activity and enhanced
20 acetoin/2,3-BDO pathway the CML B4 is used in the present invention as the mutagenesis recipient strain for 2,3-BDO overproduction.

2,3-BDO synthesis requires the consumption of NADH. Thus, the invention propose the inactivation of two of the main competing NADH-consuming reactions, those of L-lactate and ethanol biosynthesis, to obtain an increased
25 2,3-BDO production.

In the process for the isolation of a strain of a bacterium *Lactococcus lactis lactis* according to the invention, after a first round of mutagenesis a strain is selected, which is deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain; after that, a second round of mutagenesis is applied
30 to the strain selected in the first round, and a strain is selected that has a reduced lactic acid synthesis in relation to the non-mutated CML B4 strain. By

this way, strains of *L. lactis* with increased 2,3-BDO production capacity in relation to CML B4 strain are obtained.

Selection of a mutant strain during its process of obtention by means of two consecutive rounds of mutagenesis starting from CML B4, in which after a first round of mutagenesis a strain is selected being deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain, and then submitting this mutant strain to a second round of mutagenesis and selecting one, which has a reduced lactic acid synthesis, does not mean that the final resulting strain cannot produce ethanol or lactic acid in all culture conditions (i.e. anaerobiosis, aerobiosis, microaerobiosis) as illustrated in the examples. Distinguishing features of the strains of the invention are, indeed, the surprising capability of producing increased levels of 2,3-BDO, of at least 5-fold the 2,3-BDO production achieved by the CML B4 strain under the same anaerobiosis conditions. These strains produce 2,3-BDO even under microaerobiosis, in which conditions the highest yield of 2,3-BDO from carbohydrate transformation (1% glucose in the culture) are attained. Other particular features of the strains of the invention are disclosed in more detail below.

In another particular embodiment of the process of the invention, optionally in combination with any embodiment above or below, the same is carried out in such a way that, after a first round of mutagenesis, an ethanol biosynthesis deficient mutant is selected under strict anaerobiosis in an allyl alcohol-supplemented xylose medium as an allyl alcohol resistant growing colony.

Yet in another particular embodiment of the process, also optionally in combination with any embodiment above or below, the ethanol biosynthesis deficient mutant obtained after the first round of mutagenesis is subjected to a second round of mutagenesis, and a strain that has reduced lactic acid synthesis in relation to non-mutated CML B4 is selected in an acidic low buffering capacity medium supplemented with 2,3,5-triphenyl tetrazolium, as a red-colored colony.

The process of isolation according to the invention is in another particular embodiment carried out by mutagenesis selected from the group consisting of

random, site-directed mutagenesis, and combinations thereof, in order to obtain a first phenotype characterized by a deficient in ethanol biosynthesis and a second phenotype, from the second round of mutagenesis with reduced lactic acid synthesis in relation to non-mutated CML B4 strain.

- 5 In another more particular embodiment, the process is carried out by random mutagenesis.

With all these embodiments of the process for isolating strains of *L. lactis* with increased 2,3-BDO production capacity in relation to CML B4 strain, there are obtained strains with a 2,3-BDO production that is at least 5-fold the 2,3-BDO
10 production achieved by the CML B4 strain under the same anaerobiosis conditions. In a particular embodiment, optionally in combination with any embodiment above or below, the strains of *L. lactis* have a 2,3-BDO production that is at least 10-fold the 2,3-BDO production achieved by the CML B4 strain under the same anaerobiosis conditions. A particular example
15 of a strain with this capacity is the above mentioned strain of the bacterium *Lactococcus lactis lactis* CECT 8792 (also named RC2 in this description).

All these strains with these advantageous features are thus isolated mutant strains of the bacterium *Lactococcus lactis lactis* CML B4 deposited at the Spanish Type Culture Collection under accession number CECT 7512, said
20 isolated mutant strains capable of producing 2,3-BDO, and obtainable by the process as defined in any of the aspects and embodiments disclosed above.

The strains obtainable by the process of the invention are in particular characterized by a lactic acid production capacity equal or lower than 10 mM in a culture medium containing 1% glucose.

- 25 Moreover, these strains of the invention are capable of producing 2,3-BDO with a yield, relative to the maximum theoretical one, greater than 70% in batch culture under microaerobiosis. For "maximum theoretical yield is to be understood "the stoichiometric amount of 2,3-BDO that could be obtained from pyruvate in the batch culture if all pyruvate would be transformed to this
30 compound".

As mentioned above, the process for the isolation of the particular strain of the invention, strain RC2, comprises subjecting strain CML B4 to two consecutive

rounds of mutagenesis, and particularly two consecutive rounds of random mutagenesis.

The first round of random mutagenesis, particularly with EMS, of the strain CML B4 seeks to inactivate one of these NADH-consuming pathways, the biosynthesis of ethanol. Following mutagenesis, mutant clones unable to produce ethanol can be selected on plates containing allyl alcohol. This compound is converted into toxic acrolein by ethanol producing *L. lactis* strains, preventing any growth of these strains. However, mutants deficient in ethanol biosynthesis would be unable to convert allyl alcohol into acrolein, growing as visible colonies on allyl alcohol plates. Accordingly, in a particular embodiment of the process for the isolation of strain of the invention as defined above, after a first round of random mutagenesis, an ethanol biosynthesis deficient mutant is selected under strict anaerobiosis in an allyl alcohol-supplemented xylose medium as an allyl alcohol resistant growing colony.

Once a mutant strain lacking the ability to produce ethanol is obtained the next step is directed to inactivate or reduce lactic acid synthesis. Therefore, the above CML B4-derived mutant strain is subjected to a second round of random mutagenesis, particularly with EMS, and low lactate producers can be selected on tetrazolium salts-containing plates. This selection procedure is carried out using an acidic agar nutritive medium with reduced buffering capacity and supplemented with 2,3,5-triphenyl tetrazolium, modified from those described in patents FR2777905 and ES2352633B1. High lactate producing bacteria growing in this selection medium form pink colonies and it is expected that low lactate producers appear as red/brown colonies.

Accordingly, in a particular embodiment of the process for the isolation of strain of the invention, the ethanol biosynthesis deficient mutant obtained after the first round of random mutagenesis is subjected to a second round of random mutagenesis, and a low lactate producer (in other words a strain with reduced lactic acid synthesis in relation to non-mutated CML B4 strain) is selected in an acidic low buffering capacity medium supplemented with 2,3,5-

triphenyl tetrazolium, as a dark red-colored colony clearly differentiated over a background of high lactate-producing pink colonies.

After the first round of random mutagenesis applied to the CML B4 acetoin-overproducing strain a total of 26 allyl alcohol resistant (AAR) clones were isolated, with one them, named AAR21, showing the best features. When metabolite production by the AAR21 strain is measured it is found that it does not produce measurable amounts of ethanol and that the prevailing product of glucose metabolism under anaerobiosis is lactate, with no acetoin or 2,3-BDO. But, unexpectedly, this metabolite profile occurs even under aerobic culture conditions, where acetoin is expected to be the main product.

Random mutagenesis applied to the AAR21 strain resulted in the isolation of seven red-colored (RC) colonies on 2,3,5-triphenyl tetrazolium-supplemented plates. The analysis of the isolated clones shows that one of them, named RC2, displays the best features, with reduced lactic acid production under all culture conditions, as is the aim of this second round of mutagenesis

Analysis of the RC2 strain shows that its metabolite profile depends mainly on the oxygen availability. Under aerobiosis the main metabolite is acetoin, with minor levels of other products, which resembles the behaviour of the CML B4 acetoin-overproducing strain, although the RC2 strain produces slightly more 2,3-BDO. On the other hand, surprisingly, under anaerobiosis metabolism is shifted to 2,3-BDO synthesis, at the expense of acetoin, with low production of lactate and acetate and appearing again relevant levels of ethanol. Even more surprisingly, culture of the RC2 strain under microaerobiosis results in the highest 2,3-BDO production, reaching 38 mM from 1% glucose, which represents a yield of 70% of theoretical maximum. Accordingly, in a particular embodiment the strain of the invention is capable of producing 2,3-BDO with a yield, relative to the maximum theoretical one, greater than 70%, preferably greater than 80%, in batch culture under microaerobiosis. Moreover, additional 10 mM of acetoin are produced, which means that the joint yield of acetoin and 2,3-BDO is 87% of theoretical maximum. Again, as occurs under anaerobiosis, ethanol is appreciably produced (24 mM).

The strain of the present invention, *L. lactis lactis* RC2, is characterized by a reduced lactic acid production, whatever the oxygen availability is. Growth of this strain in a culture medium containing 1% glucose results in a lactic acid concentration equal or lower than 10 mM. This behaviour of the strain RC2
5 differs from that of the strain CML B4, the mutagenesis recipient strain, since the latter only produces low levels of lactic acid under aerobiosis, whereas under anaerobiosis lactic acid is the prevailing product.

Other clones from the second random mutagenesis applied to the AAR21 strain resulted in the isolation of strains showing also a high 2,3-BDO
10 production, in particular of at least 5-fold the production of the non-mutated CML B4 strain under the same anaerobiosis conditions.

As a result of the genetic alterations introduced into the CML B4 strain, which are a particular object of this invention, a particular new strain was obtained, the RC2 strain, characterized by an increased production of 2,3-BDO, that
15 was specially expressed under microaerobic growth conditions. Therefore, in a particular embodiment, the strain of the present invention displays a production of 2,3-BDO greater than 30 mM, preferably between 35 and 40 mM, in a batch culture under microaerobiosis carried out in a medium containing 1% glucose. For comparison purposes, the original CML B4 strain,
20 under the same culture conditions, produces almost exclusively lactic acid, with undetectable levels of 2,3-BDO.

As mentioned above, the present invention also provides a method for the efficient production of 2,3-BDO by fermentation, which comprises carbohydrate fermentation by the strain of the invention, particularly of RC2
25 strain, as defined above under microaerobiosis.

The abovementioned procedure includes the following steps:

- (a) preparation of a pre-culture of the strain of the invention, particularly RC2 strain, as defined above;
- (b) inoculation of a carbohydrate-rich fermentation broth with the pre-culture
30 obtained in (a);
- (c) fermentation of carbohydrates from the inoculated broth;
- (d) separation of the resulting fermentation broth from cells; and

(e) optionally, purification from the fermentation broth of the 2,3-BDO produced.

The term "pre-culture", as used herein, refers to a preliminary small-scale culture of the strain used to inoculate the broth wherein fermentation will be carried out. It is carried out in the same medium as the one used to carry out the fermentation step. The pre-culture of the strain of the invention can be carried out for 24 hours in YEC medium comprising 10 g/L glucose, 5 g/L yeast extract, and 20 mM sodium citrate buffer (pH 6.5), under the aerobic culture conditions explained below in the section of General Procedures.

10 The term "carbohydrate-rich fermentation broth", as used herein, refers to any culture medium containing assimilable energy, carbon and nitrogen sources, that allows growth of the RC2 strain and a high 2,3-BDO production. For example and without limiting purposes, among suitable carbon and energy sources are found glucose, xylose, fructose, sucrose, lactose, galactose, molasses, and mixtures thereof. As suitable nitrogen sources can be included, but without being limited to them, yeast extract, meat extract, corn steep liquor (CSL), meat, soybean or casein peptones, protein hydrolysates from protein-rich agro-food by-products, such as soybean meal/flour, whey, dry distillers grains with solubles (DDGS), and mixtures thereof.

20 If necessary, the culture medium can be supplemented with additional nutrients and growth factors, such as vitamins and mineral salts, in order to assist bacterial growth and promote 2,3-BDO production. Vitamins to be used include, among others, pyridoxamine, biotin, nicotinic acid, calcium pantothenate, riboflavin and lipoic acid, that can be added either as artificial mixtures of pure vitamins with known composition or as complex natural preparations or extracts containing them, such as yeast extract, CSL or others. Mineral salts can be preferably selected from the group of phosphates, and potassium and magnesium salts.

30 Culture medium can also be supplemented, if necessary because of an excessive foam formation throughout the fermentation, with an antifoam or surfactant agent, such as silicone-based ones, vegetable oils, polyethylene glycol and the like, according to known procedures in the art.

Culture of the RC2 strain in the fermentation broth can be carried out using any standard culture technology. Culture methods in liquid media are preferred, specially shaken cultures or cultures in fermenter- or chemostat-like reactors.

- 5 Cultures, namely the fermentation of carbohydrates from the inoculated broth, can be performed in a temperature range between 15 °C and 40 °C, preferably between 25 °C and 35 °C. Besides, they can be performed in a pH range between 5.0 and 7.5, preferably between 6.0 and 7.0. Medium pH control, when necessary to maintain pH at a fixed value, can be achieved with
10 the use of a buffering agent, selected among those usually used for that purpose, or by the addition of either an alkali or acid, depending on the particular needs of the culture, in order to counteract acidification or alkalization, respectively, that could cause microbial growth.

- Microaerobic characteristics of the culture can be achieved by means of a
15 limited oxygen supply to the culture medium, either in the form of air, pure oxygen or mixtures of them. In the case of shaken cultures, recipients must be hermetically sealed, leaving an air-filled headspace, with a volumetric ratio of headspace to liquid culture medium between 1.5 and 3.0, and with a reciprocating shaking at frequencies between 50 and 500 rpm, preferably
20 between 100 and 150 rpm.

- 2,3-BDO is accumulated in the fermentation broth for 15-48 hours, preferably for 20-30 hours from the start of the culture, that occurs at the moment of inoculation. When maximal 2,3-BDO is attained, that occurs shortly after microorganism has consumed all the carbohydrate available, fermentation can
25 be considered completed.

Once fermentation is finished, cells of the strain RC2 can be separated from fermentation broth. This process can be done preferably by means of physical methods, such as centrifugation, filtration or any other available in the art.

- Fermentation produced 2,3-BDO, that is dissolved in the clarified fermentation
30 broth, can be then recovered or purified or used as it is as an aqueous solution, in the latter case either without further modification or after a process of concentration. Purification, when carried out, can be done using any

method known in the art, either alone or combining some of them, including but not limited to distillation, precipitation, crystallization, pervaporation, liquid-liquid extraction, solid phase extraction, supercritical fluid extraction, chromatography, and the like.

- 5 2,3-BDO produced according to the method described in this invention can be used either directly or as a building block in the chemical industry for the synthesis of other chemicals.

EXAMPLES

10

The present invention is further illustrated by means of the following examples, which are only given with illustrative purposes and are not intended to limit the invention in any way.

15 General procedures

Microorganism: Microorganisms used in this invention were the bacteria *Lactococcus lactis* subsp. *lactis* strains RC2 and CML B4. The strain RC2 is derived from the strain CML B4 following two rounds of random mutagenesis.

- 20 Culture media: Cultures were carried out in YEC medium, whose composition is as follows: 10 g/L glucose, 5 g/L yeast extract, and 20 mM sodium citrate buffer (pH 6.5).

Cultures for mutagenesis purposes were done in M17 medium, with 1% glucose as carbon and energy source. The composition of M17 medium is as follows (Terzaghi BE, Sandine WE, "Improved medium for Lactic Streptococci and their bacteriophages", *Appl. Microbiol.*, 1975, Vol. 29, pp. 807–813) (in g/L): tryptone, 2.5; meat peptone, 2.5; soybean peptone, 5.0; meat extract, 5.0; yeast extract, 2.5; sodium glycerophosphate, 19.0; magnesium sulfate, 0.25; and ascorbic acid, 0.50.

- 30 Culture conditions: The following types of cultures were used throughout this invention:

A. Aerobic shaken flask cultures: Aerobic shaken flask cultures were carried out at room temperature and 200 rpm rotary shaking in 100 mL Erlenmeyer flasks containing 10 mL YEC medium.

5 B. Anaerobic cultures: Anaerobic cultures were carried out at room temperature in hermetically sealed 13-mL plastic culture tubes containing 5 mL YEC medium, incubated under static conditions.

C. Microaerobic shaken cultures: Microaerobic shaken cultures were carried out at room temperature and 100 rpm reciprocating shaking in horizontal hermetically sealed 13-mL plastic culture tubes containing 5 mL YEC medium,
10 with the remainder of the volume filled with air.

The above three types of cultures were started by inoculating the medium with a 1% (v/v) inoculum, obtained by suspending in 1 mL of YEC medium several bacterial colonies, grown on a plate of YEC agar medium for 3 days.

Metabolite concentration: 2,3-BDO, acetoin, lactate, acetate, ethanol and
15 glucose concentrations were measured by HPLC using an Aminex HPX-87H 300 x 7,8 mm (Bio Rad) column and a Microguard Cation H Refill Cartridge precolumn, with the following conditions: mobile phase, 0.01 N H₂SO₄; flow rate, 0.4 mL/min; temperature, 25 °C. Peak quantification was done with a refractive index detector.

20 Biomass concentration: Biomass concentration was measured spectrophotometrically, as optical density at 600 nm (OD₆₀₀).

Random mutagenesis of *L. lactis* subsp. *lactis* strains: A 100 mL Erlenmeyer flask containing 10 mL of 1% glucose-supplemented M17 medium was inoculated with a single colony of *L. lactis* grown for 3 days on a plate of YEC
25 agar medium, and cultured for 24 hours at room temperature and 250 rpm rotary shaking. Bacterial cells were washed twice by centrifugation/resuspension in 100 mM potassium phosphate buffer (pH 7.5) and finally resuspended in 1 mL of the same buffer. To this concentrated cell suspension 120 µL of the chemical mutagen ethyl methanesulfonate (EMS)
30 were added, and the resulting suspension was incubated at room temperature for 15 minutes with gentle shaking. Then, cells were washed twice again with 10 mL of the same potassium phosphate buffer, resuspended in 10 mL of M17

medium with 1% glucose and incubated with shaking for 1 hour. This mutagenized bacterial suspension can be either used directly or stored at -80 °C with 10% glycerol until further use.

5 Example 1

Isolation of a mutant strain deficient in the biosynthesis of ethanol from *L. lactis* CML B4

L. lactis CML B4 was subjected to random mutagenesis as described in the General procedures section and appropriate dilutions of the resulting cell suspension were plated on YEC agar plates containing 1% xilose as carbon and energy source and 1% (v/v) allyl alcohol, and incubated under strict anaerobiosis, where the CML B4 strain was unable to grow. After 10 days of incubation, from a total of 24,000 clones plated, 26 allyl alcohol resistant (AAR) colonies were able to grow, which were isolated on a fresh plate of the same selection medium. From the analysis of metabolite production by isolated clones it was concluded that the strain *L. lactis* AAR21 showed the best features, with no ethanol production, as can be seen in Table 1.

Example 2

20 Isolation of a low lactate producing mutant strain from *L. lactis* AAR21

The strain *L. lactis* AAR21, deficient in ethanol biosynthesis, was subjected to random mutagenesis as described in the General procedures section and appropriate dilutions of the resulting cell suspension were plated on plates containing a modified YEC agar medium supplemented with 100 mg/L 2,3,5-triphenyl tetrazolium. The modified YEC selection medium contains a lower buffering capacity than the standard YEC medium (10 mM instead of 20 mM sodium citrate) and is more acidic (pH 5.5 instead of 6.5). The strain AAR21 forms pink colonies on this selection medium. After 8 days of incubation, from a total of 5,000 clones plated, seven red-colored (RC) colonies were detected, which were isolated on a fresh plate of selection medium. From the analysis of metabolite production by isolated clones it was concluded that the strain *L.*

lactis RC2 showed the best features, with a reduced lactic acid production, as can be seen in Table 1.

Table 1. Metabolite production by different strains of *L. lactis*.

Strain	Oxygen	Acetoin	2,3-BDO	Lactate	Acetate	Ethanol
CML B4	Aerobiosis	38	<2	9	8	–
	Anaerobiosis	5	<2	40	3	13
AAR21	Aerobiosis	–	–	44	7	–
	Anaerobiosis	–	–	46	2	1
RC2	Aerobiosis	32	4	5	14	–
	Anaerobiosis	5	18	8	2	25

5 All concentrations are expressed in mM.

Example 3

Metabolite production by the strain *L. lactis* RC2 in microaerobic shaken cultures

- 10 Microaerobic shaken cultures of the *L. lactis* RC2 strain in 1% glucose-YEC medium were prepared in hermetically sealed 13-mL culture tubes containing different volumes of culture medium. In these cultures, oxygen availability depends on the air-filled headspace volume left empty in the tube. Metabolite production in these cultures is shown in Table 2.
- 15 Culture of the RC2 strain under microaerobiosis with a volumetric ratio of headspace to liquid culture medium of 1.6 (5 mL culture medium) results in the highest 2,3-BDO production, reaching 38 mM from 1% glucose, which represents a yield of 70% of theoretical maximum. Moreover, additional 10 mM of acetoin are produced, which means that the joint yield of acetoin and
- 20 2,3-BDO is 87% of theoretical maximum. Other metabolites, such as lactic and acetic acids, are hardly produced, but ethanol attains a relatively high concentration (24 mM).

Table 2. Metabolite production by *L. lactis* RC2 in microaerobic shaken cultures.

Medium mL	H/M ratio ¹	Glucose	Acetoin	2,3-BDO	Lactate	Acetate	Ethanol
2.5	4.2	55.5	20.2	22.7	6.4	16.6	14.5
5	1.6	55.5	10.2	38.1	10.4	3.9	23.9
6	1.2	47.6	8.1	34.2	11.8	8.4	22.4
10	0.3	32.4	4.9	18.4	11.1	5.9	25.5
13	0	24.2	2.8	9.2	10.5	7.4	23.7

¹H/M ratio: volumetric ratio of headspace to culture medium.

All concentrations are expressed in mM. Glucose concentration refers to glucose consumed.

- 5 For reasons of completeness, various aspects of the invention are set out in the following numbered clauses:

Clause 1. A process for the isolation of a strain of a bacterium *Lactococcus lactis lactis* with 2,3-BDO production capacity, the process comprising
 10 subjecting strain CML B4 to two consecutive rounds of mutagenesis and selecting a mutant strain which is deficient in ethanol biosynthesis and has a reduced lactic acid synthesis in relation to the non-mutated CML B4 strain.

Clause 2. The process according to clause 1 wherein, after a first round of
 15 mutagenesis a strain is selected, which is deficient in ethanol biosynthesis, or alternatively, which has a reduced lactic acid synthesis in relation to the non-mutated CML B4 strain; and wherein, after a second round of mutagenesis applied to the strain selected in the first round, a strain is selected that is both
 20 deficient in ethanol biosynthesis and has a reduced lactic acid synthesis.

Clause 3. The process according to any one of clauses 1 to 2 wherein, after a
 first round of mutagenesis, an ethanol biosynthesis deficient mutant is
 selected under strict anaerobiosis in an allyl alcohol-supplemented xylose
 medium as an allyl alcohol resistant growing colony.

25 Clause 4. The process according to clause 3, wherein the ethanol biosynthesis deficient mutant obtained after the first round of mutagenesis is

subjected to a second round of mutagenesis, and a strain that has reduced lactic acid synthesis in relation to non-mutated CML B4 is selected in an acidic low buffering capacity medium supplemented with 2,3,5-triphenyl tetrazolium, as a red-colored colony.

5

Clause 5. The process according to any one of clauses 1 to 4, wherein mutagenesis is selected from the group consisting of random mutagenesis, site-directed mutagenesis, and combinations thereof.

10 Clause 6. An isolated strain of the bacterium *Lactococcus lactis lactis*, which is capable of producing 2,3-BDO, obtainable by the process as defined in any one of clauses 1 to 5.

Clause 7. The isolated strain of the bacterium *Lactococcus lactis lactis*
15 according to clause 6, which is capable of producing 2,3-BDO and deposited at the Spanish Type Culture Collection (CECT) under accession number CECT 8792.

Clause 8. The strain according to clause 6, obtainable by site-directed
20 mutagenesis of the strain CML B4, or alternatively by random mutagenesis.

Clause 9. The strain according to any one of clauses 6 to 8, characterized by a lactic acid production capacity equal or lower than 10 mM in a culture medium containing 1% glucose.

25

Clause 10. The strain according to any one of claims 6 to 9, which is capable of producing 2,3-BDO with a yield, relative to the maximum theoretical one, greater than 70% in batch culture under microaerobiosis, wherein the maximum theoretical yield is defined as the stoichiometric amount of 2,3-BDO
30 that could be obtained from pyruvate in the batch culture if all pyruvate would be transformed to this compound.

Clause 11. A method for the production of 2,3-BDO comprising carbohydrate fermentation under microaerobiosis by a strain as defined in any one of clauses 6 to 10.

5 Clause 12. The method for the production of 2,3-BDO according to clause 11, comprising the following steps:

(a) preparation of a pre-culture of a strain as defined in any one of clauses 6 to 10;

10 (b) inoculation of a carbohydrate-rich fermentation broth with the pre-culture obtained in (a);

(c) fermentation of a carbohydrate from the inoculated broth;

(d) separation of the resulting fermentation broth from the strain; and

(e) optionally, purification from the fermentation broth of the 2,3-BDO produced.

15

Clause 13. The method for the production of 2,3-BDO according to clauses 11 or 12, wherein the carbohydrate to be fermented is selected from the group consisting of glucose, xylose, fructose, sucrose, lactose, galactose and molasses, and mixtures thereof.

20

Clause 14. The method for the production of 2,3-BDO according to any one of clauses 11 to 13, wherein the fermentation broth comprises a nitrogen source selected from the group consisting of yeast extract, meat extract, corn steep liquor (CSL), meat, soybean or casein peptones, protein hydrolysates from protein-rich agro-food by-products, such as soybean meal/flour, whey, dry distillers grains with solubles (DDGS), and mixtures thereof.

25

Clause 15. The method for the production of 2,3-BDO according to any one of clauses 11 to 14, wherein fermentation is performed in a temperature range from 15 °C to 40 °C; and it is carried out in a pH range from 5.0 to 7.5.

30

REFERENCES CITED IN THE APPLICATION

1. Voloch, M. et al. "Comprehensive biotechnology", Chapter 45, 2,3-Butanediol, Pergamon Press, New York, 1985. pp. 933-947.
- 5 2. Kopke M. et al. "2,3-Butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas", *Appl. Environ. Microbiol.*, 2011, Vol. 77, pp. 5467–5475.
3. Nielsen, D.R. et al. "Metabolic Engineering of Acetoin and meso-2,3 Butanediol Biosynthesis in *E. coli.*", *Biotechnol. J.*, 2010, Vol. 5, pp. 274-284.
- 10 4. Platteeuw C. et al. "Metabolic engineering of *Lactococcus lactis* — influence of the overproduction of alpha-acetolactate synthase in strains deficient in lactate-dehydrogenase as a function of culture conditions", *Appl Environ Microbiol*, 1995, Vol. 61, pp. 3967–71.
5. Gaspar P. et al., "High yields of 2,3-butanediol and mannitol in *Lactococcus*
- 15 *lactis* through engineering of NAD(+) cofactor recycling", *Appl Environ Microbiol*, 2011, Vol. 77, pp. 6826–35.
6. PCT/US2009/058834
7. FR2777905
8. ES2352633B1
- 20 9. Terzaghi B.E. et al., "Improved medium for Lactic Streptococci and their bacteriophages", *Appl Microbiol*, 1975, Vol. 29, pp. 807–813.

CLAIMS

1. A process for the isolation of a strain of a bacterium *Lactococcus lactis*
5 *lactis* with 2,3-Butanediol production capacity, the process comprising
subjecting strain CML B4, deposited at the Spanish Type Culture Collection
under accession number CECT 7512, to two consecutive rounds of
mutagenesis and selecting a mutant strain which is deficient in ethanol
biosynthesis and has a reduced lactic acid synthesis in relation to the non-
10 mutated CML B4 strain, wherein the selection of the mutant strain is
performed by: after a first round of mutagenesis a strain is selected, which is
deficient in ethanol biosynthesis in relation to the non-mutated CML B4 strain;
and wherein, after a second round of mutagenesis applied to the strain
selected in the first round, a strain is selected that has a reduced lactic acid
15 synthesis in relation to the non-mutated CML B4.
2. The process according to claim 1 wherein, after the first round of
mutagenesis, an ethanol biosynthesis deficient mutant is selected under strict
anaerobiosis in an allyl alcohol-supplemented xylose medium as an allyl
20 alcohol resistant growing colony.
3. The process according to claim 2, wherein the ethanol biosynthesis
deficient mutant obtained after the first round of mutagenesis is subjected to a
second round of mutagenesis, and a strain that has reduced lactic acid
synthesis in relation to non-mutated CML B4, deposited at the Spanish Type
25 Culture Collection under accession number CECT 7512, is selected in an
acidic low buffering capacity medium supplemented with 2,3,5-triphenyl
tetrazolium, as a red-colored colony.
4. The process according to any one of claims 1 to 3, wherein mutagenesis is
30 selected from the group consisting of random mutagenesis, site-directed
mutagenesis, and combinations thereof.

5. An isolated strain of the bacterium *Lactococcus lactis lactis*, which is capable of producing 2,3-Butanediol, obtainable by the process as defined in any one of claims 1 to 4.

5 6. The isolated strain of the bacterium *Lactococcus lactis lactis* according to claim 5, which is capable of producing levels of 2,3-Butanediol of at least 5-fold the 2,3-Butanediol production achieved by the CML B4 strain deposited at the Spanish Type Culture Collection under accession number CECT 7512 under the same anaerobiosis conditions.

10

7. The isolated strain of the bacterium *Lactococcus lactis lactis* according to any one of claims 5 to 6, which is capable of producing 2,3-Butanediol and deposited at the Spanish Type Culture Collection (CECT) under accession number CECT 8792.

15

8. The strain according to any one of claims 5 to 6, obtainable by site-directed mutagenesis of the strain CML B4 deposited at the Spanish Type Culture Collection under accession number CECT 7512, or alternatively by random mutagenesis of the strain CML B4 deposited at the Spanish Type Culture
20 Collection under accession number CECT 7512.

9. The strain according to any one of claims 5 to 8, characterized by a lactic acid production capacity equal or lower than 10 mM in a culture medium containing 1% glucose.

25

10. The strain according to any one of claims 5 to 9, which is capable of producing 2,3-Butanediol with a yield, relative to the maximum theoretical one, greater than 70% in batch culture under microaerobiosis, wherein the maximum theoretical yield is defined as the stoichiometric amount of 2,3-
30 Butanediol that could be obtained from pyruvate in the batch culture if all pyruvate would be transformed to this compound.

11. A method for the production of 2,3-Butanediol comprising carbohydrate fermentation under microaerobiosis by a strain as defined in any one of claims 5 to 10.
- 5 12. The method for the production of 2,3-Butanediol according to claim 11, comprising the following steps:
- (a) preparation of a pre-culture of a strain as defined in any one of claims 5 to 10;
- (b) inoculation of a carbohydrate-rich fermentation broth with the pre-culture
10 obtained in (a);
- (c) fermentation of a carbohydrate from the inoculated broth;
- (d) separation of the resulting fermentation broth from the strain; and
- (e) optionally, purification from the fermentation broth of the 2,3-Butanediol produced.
- 15 13. The method for the production of 2,3-Butanediol according to claims 11 or 12, wherein the carbohydrate to be fermented is selected from the group consisting of glucose, xylose, fructose, sucrose, lactose, galactose and molasses, and mixtures thereof.
- 20 14. The method for the production of 2,3-Butanediol according to any one of claims 11 to 13, wherein the fermentation broth comprises a nitrogen source selected from the group consisting of yeast extract, meat extract, corn steep liquor (CSL), meat, soybean or casein peptones, protein hydrolysates from
25 protein-rich agro-food by-products, such as soybean meal/flour, whey, dry distillers grains with solubles (DDGS), and mixtures thereof.
15. The method for the production of 2,3-Butanediol according to any one of claims 11 to 14, wherein fermentation is performed in a temperature range
30 from 15 °C to 40 °C; and it is carried out in a pH range from 5.0 to 7.5.

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055012

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/01 C12P7/18 C12N1/20
ADD. C12R1/46

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12N C12R C12P

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

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

EPO-Internal, BIOSIS, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	ES 2 352 633 B1 (FUNDACION LEIA CT DE DESARROLLO TECNOLOGICO [ES]) 2 January 2012 (2012-01-02) cited in the application the whole document figure 1 ----- -/--	5,8, 11-15



Further documents are listed in the continuation of Box C.



See patent family annex.

* Special categories of cited documents :

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

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

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

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

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

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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

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

"&" document member of the same patent family

Date of the actual completion of the international search

24 May 2016

Date of mailing of the international search report

01/06/2016

Name and mailing address of the ISA/

European Patent Office, P.B. 5818 Patentlaan 2
NL - 2280 HV Rijswijk
Tel. (+31-70) 340-2040,
Fax: (+31-70) 340-3016

Authorized officer

van de Kamp, Mart

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2016/055012

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	BOUMERDASSI H ET AL: "Isolation and properties of Lactococcus lactis subsp. lactis biovar diacetylactis CNRZ 483 mutants producing diacetyl and acetoin from glucose", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 63, no. 6, June 1997 (1997-06), pages 2293-2299, XP002086557, ISSN: 0099-2240 the whole document abstract table 2 figure 1	1,6,11
A	----- HENRIKSEN C M ET AL: "Redirection of pyruvate catabolism in Lactococcus lactis by selection of mutants with additional growth requirements", APPLIED MICROBIOLOGY AND BIOTECHNOLOGY, vol. 56, no. 5-6, 24 July 2001 (2001-07-24), pages 767-775, XP002560014, ISSN: 0175-7598, DOI: 10.1007/S002530100694 the whole document page 773, right-hand column, lines 20-28 abstract figure 1	1,6,11
A	----- C PLATTEEUW ET AL: "Metabolic engineering of Lactococcus lactis: influence of the overproduction of alpha-acetolactate synthase in strains deficient in lactate dehydrogenase as a function of culture conditions", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 61, no. 11, November 1995 (1995-11), pages 3967-3971, XP055208739, ISSN: 0099-2240 cited in the application the whole document tables 2,3 abstract figure 1	1,6,11
	----- -/--	

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	P. GASPAR ET AL: "High Yields of 2,3-Butanediol and Mannitol in Lactococcus lactis through Engineering of NAD+ Cofactor Recycling", APPLIED AND ENVIRONMENTAL MICROBIOLOGY, vol. 77, no. 19, 12 August 2011 (2011-08-12), pages 6826-6835, XP055183682, ISSN: 0099-2240, DOI: 10.1128/AEM.05544-11 cited in the application the whole document tables 2,3 -----	1,6,11
A	WO 2014/013330 A2 (BIOPOLIS S L [ES]; UNIV MADRID COMPLUTENSE [ES]) 23 January 2014 (2014-01-23) the whole document	1,6,11
A,P	-& EP 2 876 155 A2 (BIOPOLIS S L [ES]; UNIV MADRID COMPLUTENSE [ES]) 27 May 2015 (2015-05-27) the whole document -----	1,6,11
A	RAFAEL S. COSTA ET AL: "An extended dynamic model of Lactococcus lactis metabolism for mannitol and 2,3-butanediol production", MOLECULAR BIOSYSTEMS, vol. 10, no. 3, 13 January 2014 (2014-01-13), page 628, XP055208709, ISSN: 1742-206X, DOI: 10.1039/c3mb70265k the whole document -----	1,6,11
A	CELINSKA E ET AL: "Biotechnological production of 2,3-butanediol-Current state and prospects", BIOTECHNOLOGY ADVANCES, vol. 27, no. 6, 12 May 2009 (2009-05-12), pages 715-725, XP027205958, ISSN: 0734-9750 the whole document -----	1,6,11
A	KLEEREBEZEMAB M ET AL: "Lactic acid bacteria as a cell factory: rerouting of carbon metabolism in Lactococcus lactis by metabolic engineering", ENZYME AND MICROBIAL TECHNOLOGY, vol. 26, no. 9-10, June 2000 (2000-06), pages 840-848, XP027457445, ISSN: 0141-0229, DOI: 10.1016/S0141-0229(00)00180-0 the whole document -----	1,6,11

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2016/055012

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
ES 2352633	B1	02-01-2012	NONE

WO 2014013330	A2	23-01-2014	CA 2879331 A1 23-01-2014
			CO 7250448 A2 30-04-2015
			EP 2876155 A2 27-05-2015
			US 2015191752 A1 09-07-2015
			WO 2014013330 A2 23-01-2014

EP 2876155	A2	27-05-2015	CA 2879331 A1 23-01-2014
			CO 7250448 A2 30-04-2015
			EP 2876155 A2 27-05-2015
			US 2015191752 A1 09-07-2015
			WO 2014013330 A2 23-01-2014
