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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
22 August 2019 (22.08.2019)

WIPO | PCT

(10) International Publication Number
WO 2019/159011 A2

(51) International Patent Classification:
C12P 7/56 (2006.01)

(21) International Application Number:
PCT/IB2019/000178

(22) International Filing Date:
15 February 2019 (15.02.2019)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
62/631,541 16 February 2018 (16.02.2018) US

(71) Applicant: **PTT GLOBAL CHEMICAL PUBLIC COMPANY LIMITED** [TH/TH]; 555/1 Energy Complex Vibhavadi Rangsit Road, Building A, 14-18 Floor, Bangkok (TH).

(72) Inventors: **DOLE, Sudhanshu, Vijay**; 23 Suncrest Road, Andover, MA 01810 (US). **HERMANN, Theron**; 151 Warren Street, Arlington, MA 02474 (US). **REGAN, Sean, Joseph**; 33 Alvarado Avenue - Apt 3E, Worcester, MA 01604 (US). **SHEFF, Mark, Andrew**; 85 Hawthorn Street, Westwood, MA 02090 (US). **UDANI, Russell, Lizardo**; 72 Newport Street, Arlington, MA 02476 (US). **YOCUM, R., Rogers**; 4 Orchard Lane, Lexington, MA 02420 (US).

(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, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IR, IS, JO, JP, KE, KG, KH, KN, KP, KR, KW, 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).

Published:
— *without international search report and to be republished upon receipt of that report (Rule 48.2(g))*
— *with sequence listing part of description (Rule 5.2(a))*

(54) Title: MICROORGANISMS AND PROCESSES FOR LACTIC ACID PRODUCTION

(57) Abstract: Improved yeast strains and fermentation process for producing D-lactic acid and L- lactic acid are disclosed. The improvement lead to higher titer, higher yield, shorter time, lower pH, and higher average specific productivity.

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MICROORGANISMS AND PROCESSES FOR LACTIC ACID PRODUCTION

CROSS-REFERENCE TO RELATED APPLICATIONS

(001) This application claims the priority of the U.S. Provisional Application Serial No. 62/631,541, filed on February 16, 2018.

FIELD OF THE INVENTION

(002) The invention relates to the field of genetic engineering of microorganisms for chemical production. More specifically, the invention relates to the production of lactic acid from renewable carbon resources using genetically modified yeasts.

BACKGROUND OF THE INVENTION

(003) Many of the plastics and fibers in current usage are poorly compostable in the natural environment; for example, plastics and fibers derived from polyethylene, polypropylene, polycarbonates, polyesters and nylons remain in the natural environments for a very long time. For the purpose of long-term environmental stewardship, it is desirable to replace traditional plastics and fibers with polymers derived from bio-renewable resources which are easily compostable in the natural environments. Furthermore, as the world's supply of mined petroleum becomes scarcer and more expensive to obtain, it is desirable to replace petrochemical monomers with bio-renewable monomers. Polymers of lactic acid are generally compostable in the natural environments. Lactic acid (2-hydroxy propanoic acid) exists as either of two sterioisomers, L-lactic acid, also known as L(+)-lactic acid or S-lactic acid (L-LAC), or D-lactic acid, also known as D(-)-lactic acid or R-lactic acid (D-LAC). Both isomers can be made from bio-renewable resources, such as sugars, by fermentation. Most of the lactic acid currently produced by fermentation is L-LAC. Poly-L-lactic acid (PLLA) is biodegradable and makes plastics that are useful for some applications; however, its relatively low melting temperature of about 180°C limits its usefulness (Tsuji, 2005). However, the melting temperature of PLLA can be raised by about 45°C to about 225°C by melting together equal weights of PLLA and poly-D-lactic acid (PDLA) to give what is referred to herein as a "stereocomplex PLA" (Tsuji, 2005).

(004) Raising the melting temperature to above the boiling point of water is important for many applications, such as cups for hot drinks, plastic eating utensils and fabrics that can be washed and ironed or pressed at hot temperatures. Most importantly, the higher melting temperature of stereocomplex PLA is necessary for producing high quality "fracking beads", which are pumped into fractured rock formations in deep gas and oil deposits for the purpose of keeping the cracks open enough to extract, remove, or pump out the desired gas or oil.

(005) Lactic acid can be polymerized starting from the free acid or from a lactide. A lactide is a cyclic diester of lactic acid, and can be comprised of two molecules of L-LAC (L,L-lactide), two molecules of D-LAC (D,D-lactide), or one molecule each of L-LAC and D-LAC (D,L-lactide or meso-lactide). Polymerizing a mixture of L-lactic acid and D-lactic acid monomers, or a mixture of lactides (L,L-lactide, D,D-lactide and D,L-lactide) does not lead to a polymer with the desired higher melting temperature (Tsuji, 2005). Instead, in order to obtain the desired polymer with a higher melting temperature, high molecular weight polymers of highly pure L-LAC and highly pure D-lactic acid must be made separately, and mixed only after polymerization. Thus, in order to make the desirable and useful stereocomplex PLA, the starting materials must be pure chiral L-LAC and pure chiral D-LAC. Sometimes the pure isomers are referred to as "optically pure." "Optically pure" or "pure chiral" as used herein means that the lactic acid is greater than 99 % of one isomer by weight or mole percent. Optical purity can be determined by any one of well-known methods, for example analytical high pressure liquid chromatography (HPLC) using a column that is packed with a medium that itself carries an optically pure chiral component on its surface, for example a Phenomenex (Torrance, California, USA) Chirex 3126 column (250 X 4.60 mm) that is packed with beads coated with D-penicillamine. The mobile phase is aqueous 1 mM copper sulfate with a flow rate of 1 ml/minute and UV detection at 254 nm. An alternative method uses a Shodex™ ORpak CRX-853 (8.0 mm I.D. x 50 mm) column (Showa Denko, Tokyo, Japan), eluted with aqueous 0.5 mM $CuSO_4$, a flow rate 1.0 mL/min, detection with UV at 230nm, and column temperature 50°C, as recommended by the manufacturer. If the isomer of lactic acid is not specified in a particular context, then the term "lactic acid" or "lactate" refers to either or both isomers, or a mixture of the two. Lactic acid produced by non-biological chemical synthesis is a racemic mixture of equal portions of L-LAC and D-LAC, so it cannot be used to make stereocomplex PLA.

(006) Historically, most lactic acid manufactured on a large scale has been L-LAC, if produced biologically by fermentation with a micro-organism, or a racemic mixture of L-LAC and D-LAC, if produced chemically (i.e., non-biologically). A process was developed for producing highly pure chiral D-LAC by a genetically engineered strain of *Escherichia coli* (Zhou et al., 2003). However, since *E. coli* only grows well at neutral pH, the D-LAC must be produced as a salt (for example with the ammonium, sodium, potassium, calcium, or magnesium cation), and then the protonated free acid must be separated from the cation to enable polymerization, which adds significant cost to the process. As of November 28, 2017, D-LAC, produced by an undisclosed process, can be purchased from the Purac division of Corbion (Amsterdam, Netherlands). However, the purchase price for D-LAC is significantly higher than that of L-LAC. As a result of the high price of pure D-LAC, stereocomplex PLA has not been widely adopted by large-scale commercial producers.

(007) US Patent Application Publication No. US 2015/0152449 discloses a chemical process for producing D-LAC from either L-LAC or a racemic mixture of L-LAC and D-LAC. This process involves first making a racemic mixture by heating L-LAC. The racemic lactide mix is then dissolved in n-butanol and acetone, and passed over a column of immobilized Novozyme 435 (MilliporeSigma, St. Louis, Missouri, USA), which is a lipase enzyme that is stereospecific for ester formation. The product is a 50-50 mix of the 1-butanol ester of D-lactic acid and the 1-butanol ester of L,L-lactyllactic acid (dimer). These two chemicals can then be separated by distillation and presumably hydrolyzed to give free acid, lactide, and or polymer, although this US Patent Application Publication did not disclose how this last step is done. The yield and purity from the enzymatic and distillation processes were not perfect. In one example, the yield of D-butyl lactate fell from 92% to 79% after 8 runs of 7 hours each over the immobilized enzyme. In one example of the separation of the two esters by distillation, "the distilled product analyzed was 93.9% (R)-butyl lactate; 0.4% (S)-butyl lactate, 5.0% butanol; 0.5% (S,S)-butyl lactyllactate; 0.1% (R,R)-butyl lactyllactate and 0.1% (R,R)-lactide." As such, the lactic ester portion contained about 99 % D-LAC and about 1 % L-LAC. It was not shown in this US Patent Application Publication whether that level of purity is sufficient to make high quality stereocomplex PLA. In any case, the process disclosed in this US Patent Application Publication is rather complicated, requires several unit operations (in addition to fermentation and downstream purification), an expensive

enzyme that might have a limited lifetime, and explosion-proof equipment, and is therefore likely to be more costlier than a simple fermentation process for producing isomeric form of lactic acid in a pure form.

(008) Thus, there remains a need for a process to produce optically pure D-LAC in order to encourage wider adoption of stereocomplex PLA as an economically attractive compostable plastic.

(009) One approach to reducing the cost of producing D-LAC is to use a yeast as the production organism. Many yeast strains can grow well compared to most bacteria at a relatively low pH, so it is possible that D-LAC or L-LAC can be produced by fermentation at a pH that is at or below its pKa, which is published to be pH 3.86. At pH 3.86, only about half as much cation is required compared to a pH 7 fermentation, so that the downstream processing to separate the cations will be commensurately less expensive. If the final pH in the production fermentor can be even lower than 3.86, then production cost can be reduced even further.

(010) There is much prior art in the field of L-lactate production by yeasts, but there is much less prior art in the field of D-lactate production (see Sauer et al, 2010 for a comprehensive review).

(011) Zhou et al disclose increased production of D-LAC by genetically engineered *E. coli,* which naturally produces D-LAC, in which competing anaerobic pathways were deleted, and metabolic evolution was applied (Zhou et al, 2003; US Patent Nos. 7,629,162 and 8,426,191). Dequin and Barre (1994) introduced the concept of engineering the yeast *Saccharomyces cerevisiae,* which does not naturally produce either L-LAC or D-LAC, to produce L-LAC by introducing an L-lactate dehydrogenase, but the resulting yeast still produced ethanol. Porro et al (1995) expanded the concept of producing L-LAC without ethanol from yeast by introducing an L-LAC dehydrogenase and blocking the ethanol pathway by deleting one or more genes that encode pyruvate decarboxylase. Porro et al also introduced the concept of using yeasts from genera other than *Saccharomyces*, such as *Kluyveromyces, Torulopsis, and Zygosaccharomyces* (US Patent No. 6,429,006 B1, 7,049,108 B2 and 7,326,550). Rajgarhia et al introduced the concept of blocking the glycerol biosynthetic pathway and using Crabtree negative yeasts from genera

other than *Saccharomyces,* including *Kluyveromyces, Pichia,* and *Hansenula,* for L-LAC production (US Patent Nos. 6,485,947 and 7,141,410).

(012) A Crabtree positive yeast strain (for example many *S. cerevisiae* strains) is a strain that produces ethanol and carbon dioxide by the "anaerobic" or "fermentative" pathway from a fermentable carbon source such as glucose or other suitable sugar, even in the presence of air oxygen when the concentration of sugar is above about 5 g/L. A Crabtree negative yeast strain (for example many *K. marxianus* and *K. lactis* strains) is a strain that does not produce ethanol and carbon dioxide by the "anaerobic" or "fermentative" pathway from a fermentable carbon source such as glucose or other suitable sugar in the presence of air oxygen when the concentration of sugar is above about 5 g/L.

(013) Whether a strain is Crabtree positive or negative can be determined by the method of van Dijken using submerged inverted Durham tubes in a rich medium containing 2% glucose, and determining accumulation of gas (carbon dioxide) in the inverted Durham tube after aerobic incubation (van Dijken et al, 1986). Crabtree positive strains produce gas, which visibly collects in the inverted Durham tube, while Crabtree negative strains do not.

(014) There are few disclosures on the topic of D-LAC production at low pH by engineered yeasts. A "low pH" is defined as a pH below the published pKa for D-lactic acid or L-lactic acid, which is 3.86. Winkler introduced the concept of producing D-lactic acid using yeast and showed the production of D-LAC at 37 g/L D-LAC with an average specific productivity of 0.54 g/L-hour by an engineered *S. cerevisiae* strain (US Patent Application Publication No. 2007/0031950). Miller et al produced D-LAC from an engineered *K. marxianus* strain with a specific productivity of 0.58 g/L-hour and a yield of 0.69 g/g from a medium starting with 90 g/L glucose and ending at pH 3.0, but titers and times were not given, so one can only infer a maximum titer of 62 g/L and a fermentation time of at least 107 hours, assuming all glucose was used, and depending on the volume of KOH solution added (US Patent No. 8,137,953). Yocum et al. discloses an engineered strain of *K. marxianus* that produced D-LAC at 49 g/L in 48 hours for an average specific productivity of 1.02 g/L-hour (US Patent Application No. 2015/0240270). However, none of the prior art discloses a strain and processes for producing D-LAC at a low pH in the final fermentation broth that would be economically attractive.

(015) Baek et al (2016) discloses an engineered and evolved strain of *S. cerevisiae* that produced 112 g/L D-LAC in 52 hours with a yield of 0.80 g/g glucose, and a specific productivity of 2.2 g/L-hr. However to achieve these parameters, the authors used a rich medium, YPD (yeast extract, peptone, dextrose), and calcium carbonate for neutralization, both features that are not desirable. The rich medium is costly upfront and adds to the downstream purification costs. Fermentation at neutral pH negates the advantage of using yeast as a production organism. The same group (Baek et al, 2017) discloses further engineered and evolved strains of *S. cerevisiae* that produced 82.6 g/L D-LAC at pH 3.5 with yield of 0.83 g/g glucose in 55 hours, for a specific productivity of 1.50 g/L-hr. However, traces of ethanol were still produced and a rich medium, (YPD medium containing yeast extract, peptone, dextrose) was used, which, as mentioned above, is undesirable for economic reasons. Another Korean group (PCT/KR2015/006225; Bae et al, 2018) discloses production of either L-LAC or D-LAC from engineered *K. marxianus* strains, using glucose or Jerusalem artichoke powder as carbon sources. L-LAC was produced at 130 g/L with a yield of 0.98 in 66 hours. D-LAC was produced at 122 g/L with a yield of 0.95 in 66 hours. However, in both cases, the pH was maintained at 6.0 with NaOH, and the cells were pre-grown to high density in a rich medium (YPD medium containing yeast extract, peptone, dextrose) and concentrated by centrifugation before inoculating the production fermentor. This practice is impractical and too expensive to be used commercially. Furthermore, as mentioned above, fermenting at a pH that is substantially higher than the pKa of lactic acid negates the advantage of using a yeast. Kim et al. (US Patent No. 9,353,388) disclose overexpression of lactic acid transporters encoded by *JEN1* and *ADY2* in *S. cerevisiae*, however the highest L-LAC titer reported was 13.3 g/L, and no details on fermentation conditions were given. Table 1 of the instant patent application summarizes the most relevant prior art references by listing the best published processes for producing L-LAC and D-LAC by fermentation using a yeast strain wherein the final pH is at or below the published pKa for lactic acid.

(016) Production of commodity chemicals by fermentation at a cost that is competitive with chemicals produced from petroleum is difficult. The petrochemical industry has been active for more than 100 years, and highly developed technology now exists for producing useful chemicals at a large scale, including fuels and monomers for polymers, such as ethylene, propylene,

butadiene, isoprene, ethylene glycol, terephthallic acid, adipic acid, hexamethylene diamine, caprolactam, and many others. Historically, very few commodity chemicals have been produced by fermentation and the only large volume examples have been ethanol, L-LAC, citric acid, succinic acid, itaconic acid, and couple of the L-amino acids. There are three highly significant problems with producing chemicals by fermentation. First, the raw product is mostly in water, and the water must be separated from the desired chemical, which is energy intensive and costly. Second, if the chemical is to be used for polymerization, it must be extremely pure in order to prevent chain termination, unwanted coloration, and catalyst poisoning. Microbes typically produce a wide variety of chemicals in addition to the desired product, so these unwanted contaminants must be purified away. Many microbes grow better in a rich medium that contains many nutrients, for example molasses, peptone, Jerusalem artichoke powder, or yeast extract (as opposed to a minimal or chemically defined medium), but rich media are relatively expensive and contain many impurities that remain in the fermentation broth and therefore need to be purified away as well. Third, at the titers required for attractive economics, the desired chemicals are usually toxic to the cells, which limits titers and productivity. Thus, it is quite difficult to profitably produce commodity chemicals by fermentation.

(017) With the advent of genetic engineering, it seemed possible that many more commodity chemicals could be profitably produced, because the producing microbes could be manipulated much more easily and drastically than in the past using classical strain development methods. However, in reality, only a few new commodity chemicals have been successfully produced in commercial scale since the advent of genetic engineering, for example 1,3-propane diol. Several other chemicals have been attempted, but most with only limited success, primarily due to price competition from petrochemicals, for example succinic acid, isoprene, 1,4-butane diol, and isobutanol.

(018) Thus, there is still a need for improved microbes and fermentation processes for producing commodity chemicals such as D-LAC and L-LAC, in which the myriad of problems mentioned above are solved to the point that economically attractive processes may be commercialized. The focus of this invention is to provide such microbes and fermentation processes.

SUMMARY OF THE INVENTION

(019) This invention relates generally to production of D-lactic acid and/or L-lactic acid by fermentation at titers, yields, pH and times that result in an economically attractive process.

(020) The inventors of the invention believe that wide adoption of stereocomplex PLA would require a D-LAC selling at a price of no more than twice the current price of L-LAC. Although it is impossible to pick a particular selling price for L-LAC, since its price varies widely depending on time, place, volume, and purity, it is likely that D-LAC will have to be produced at greater than 90 g/L, in less than 48 hours, at a yield greater than 0.75 g/g, and at a pH of less than 3.86, with an average specific productivity of greater than 1.875 g/L-hr. Disclosed herein are strains and fermentation processes that meet these parameters, where no such strain or process has been disclosed in the prior art.

(021) The prior art discloses that it is theoretically impossible to produce an organic acid such as D-LAC by fermentation at high yield and at low pH under anaerobic or microaerobic conditions because the cell would have to expend all available energy (for example in the form of ATP) to export the acid and keep the acid outside of the cell (van Maris et al, 2004). Thus, it was surprising that the inventors were able to construct yeast strains that could produce D-LAC at high titers and low pH under microaerobic conditions.

(022) Unless otherwise defined, all technical and scientific terms used herein have the same meaning as commonly understood by a person having ordinary skill in the art. Although methods and materials similar or equivalent to those described herein can be used in the practice or testing of the invention, suitable methods and materials are described below. All publications, patent applications, patents, and other references mentioned are hereby incorporated by reference in their entirety. In case of conflict, the specification, including definitions provided in the instant patent application will control.

(023) Other features, structures, components, or characteristics as well as the advantages of the invention will be apparent from the description of the invention.

BRIEF DESCRIPTION OF THE DRAWINGS

(024) FIG. 1. Structure of the plasmid pMS52 containing the cassette used to delete the *KmURA3* gene from the *K. marxianus* strain SD98.

(025) FIG. 2. Structure of the plasmid pSD100 *ldhA* containing the cassette used to install the *ldhA* gene cassette at the *PDC1* locus in the *K. marxianus* strain SD98.

(026) FIG. 3. Structure the plasmid pSD95 *ldhA* containing the cassette used to install the *ldhA* gene cassette at the *GPP1* locus in the *K. marxianus* strain SD98.

(027) FIG. 4. Structure the plasmid pSD104-*PCK1* containing the cassette used to install the *ldhA* gene cassette at the *PCK1* locus in the *K. marxianus* strain SD98.

(028) FIG. 5. Structure the plasmid pSD104-*NDE1* containing the cassette used to install the *ldhA* gene cassette at the *NDE1* locus in the *K. marxianus* strain SD98.

(029) FIG. 6. Structure the plasmid PCR product containing the wild type *KmURA3* gene and 1033bp upstream flanking sequence and 1046bp downstream flanking sequence of the *URA3* gene, of *K. marxianus* strain SD98, used to reinstall the *KmURA3* gene at the *delta-KmURA3* locus in the *K. marxianus* strain.

(030) FIG. 7. Production of D-lactate and pyruvate by SD1555 and SD1566 strains of *K. marxianus* in a BioLector fermentation.

(031) FIG. 8. Production of D-lactate and pyruvate by SD1555 and SD1566 strains of *K. marxianus* as a function of time, in computer controlled 7-liter fermentors.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

(032) Various non-limiting embodiments of the disclosure will now be described herein and illustrated in the accompanying drawings. A person having ordinary skill in the art will understand that the features, structures, components, or characteristics described or illustrated in connection

with one non-limiting embodiment may be combined with the features, structures, components, or characteristics of one or more other non-limiting embodiments. Such combinations are intended to be included within the scope of the disclosure. A person having ordinary skill in the art will also understand that that the features, structures, components, or characteristics described or illustrated in connection with one or more non-limiting embodiments can be modified or varied without departing from the scope and spirit of the invention.

(033) To facilitate understanding of the invention, a description of nomenclature is provided below.

(034) In regards to nomenclature, a bacterial gene or coding region is usually named with lower case letters in italics, for example "*ldhA*" from *E. coli*, while the enzyme or protein encoded by the gene can be named with the same letters, but with the first letter in upper case and without italics, for example "LdhA". A yeast gene or coding region is usually named with upper case letters in italics, for example "*PDC1*", while the enzyme or protein encoded by the gene can be named with the same letters, but with the first letter in upper case and without italics, for example "Pdc1"or "Pdc1p", the latter of which is an example of a convention used in yeast for designating an enzyme or protein. The "p" is an abbreviation for protein, encoded by the designated gene. The enzyme or protein can also be referred to by a more descriptive name, for example, D-lactate dehydrogenase (*ldhA* / LdhA) or pyruvate decarboxylase (*PDC1* / Pdc1), referring respectively to the two above examples. A gene or coding region that encodes one example of an enzyme that has a particular catalytic activity can have several different names because of historically different origins, functionally redundant genes, genes regulated differently, or because the genes come from different species. For example a gene that encodes glycerol-3-phosphate dehydrogenase can be named *GPD1*, *GDP2*, or *DAR1*, as well as other names. To specify the organism from which a particular gene was derived, the gene name can be preceded by two letters indicating the genus and species. For example, the *KmURA3* gene is derived from *Kluyveromyces marxianus* and the *ScURA3* gene is derived from *Saccharomyces cerevisiae*.

(035) Note that all isomers of lactic acid and any lactic acid analog can exist in solid, liquid, or solution form as a protonated acid (also known as a free acid) or as an ionized salt. In aqueous

solution, both protonated and ionic forms co-exist in an equilibrium. Since it would be cumbersome to refer to all forms of such compounds, any mention of either the acid form or the salt form (for example D-lactic acid (D-LAC) or beta-chlorolactate) includes all forms or mixtures of all forms.

(036) To facilitate understanding of the invention, a number of terms are defined below, and others are found elsewhere in the specification.

(037) "Yeast" means any fungal organism that is capable of growing in a single cell state under some conditions. Some yeast strains can also grow in a hyphal state or psuedohyphal (i.e., short hyphae) state under some conditions, such as under starvation. In particular, yeast includes, but is not limited to, organisms in the genera *Saccharomyces*, *Kluyveromyces*, *Issatchenkia*, *Pichia*, *Hansenula*, *Candida*, *Yarrowia*, *Zygosaccharomyces*, *Schizosaccharomyces*, and *Lachancea*.

(038) "Cassette" or "expression cassette" means a deoxyribose nucleic acid (DNA) sequence that is capable of encoding and producing, or alternatively eliminating or reducing, one or more desired proteins or enzymes when installed in a host organism. A cassette for producing a protein or enzyme typically comprises at least one promoter, at least one coding sequence, and optionally at least one terminator. If a gene to be expressed is heterologous or exogenous, the promoter and terminator are usually derived from two different genes or from a heterologous gene, in order to prevent double recombination with the native gene from which the promoter or terminator was derived. A cassette can optionally and preferably contain one or two flanking sequence(s) on either or both ends that is/are homologous to a DNA sequence in a host organism, such that the cassette can undergo homologous recombination with the host organism, either with a chromosome or a plasmid, resulting in integration of the cassette into said chromosome or plasmid. If only one end contains a flanking homology, then the cassette in a circular format can integrate by single recombination at the flanking sequence. If both ends of a cassette contain flanking homologies, then the cassette in a linear or circular format can integrate by double recombination with the surrounding flanks. A cassette can be constructed by genetic engineering, where for example a coding sequence is expressed from a non-native promoter, or it can use the naturally associated promoter. A cassette can be built into a plasmid, which can be circular, or it can be a linear DNA

created by polymerase chain reaction (PCR), primer extension PCR, or by in vivo or in vitro homologous recombination. A cassette can be designed to include a selectable marker gene or DNA sequence that upon integration is surrounded by a direct repeat sequence of about 30 base pairs or more (the same sequence, in the same orientation present both ends of the integrated selectable gene), such that the selectable marker can be deleted by homologous recombination between the direct repeats after the cassette is integrated into a chromosome or plasmid. Useful selectable marker genes include, but are not limited to, antibiotic G418 resistance (*kan* or *kanR*), hygromycin resistance (*hyg* or *hygR*), zeocin resistance (*zeo* or *zeoR*), naturicin resistance (*nat* or *natR*), *URA3*, *TRP1*, *TRP5*, *LEU2*, and *HIS3*. For the biosynthetic genes to be used as a selectable marker, the host strain must, of course, contain a mutation in the corresponding gene, preferably a null mutation. For the antibiotic resistance genes, the resistance gene usually requires a promoter that functions well enough in the host yeast strain to enable selection. Although a gene that is desired to be expressed can be installed in a host strain in the form of a cassette, a gene, for example a coding sequence from start codon to stop codon can be integrated into a host chromosome or plasmid without a promoter or terminator such that the incoming coding sequence precisely or approximately replaces the coding sequence of a gene native to the host strain, such that after integration, the incoming coding region is expressed from the remaining promoter of the host coding sequence that was replaced by the incoming coding sequence.

(039) "D-lactate dehydrogenase" means any enzyme that catalyzes the formation of D-lactate from pyruvate. "L-lactate dehydrogenase" means any enzyme that catalyzes the formation of L-lactate from pyruvate. The necessary reducing equivalent for either of these reactions can be supplied by NADH, NADPH, or any other reducing equivalent donor.

(040) "Gibson method" means a method for joining together two or more linear DNA fragments that have short (about 15-40 base pairs) overlapping homology at their ends. This method can be used to construct plasmids from synthetic linear DNA fragments, PCR fragments, or fragments generated by restriction enzymes. Kits can be purchased to perform the Gibson method, for example the NEBuilder® kit (New England BioLabs, Ipswitch, Massachusetts, USA), and used as instructed by the manufacturer.

(041) "Transformant" means a cell or strain that results from installation of a desired DNA sequence, either linear or circular, and either autonomously replicating or not, into a host or parent strain.

(042) "Titer" means the concentration of a compound in a fermentation broth, usually expressed as grams per liter (g/L) or as % weight per volume (%). Titer is determined by any suitable analytical method, such as quantitative analytical chromatography, for example high pressure liquid chromatography (HPLC) or gas chromatography (GC), with a standard curve made from external standards, and optionally with internal standards.

(043) "Yield" means the grams of product per gram of carbon source used during fermentation. This is typically calculated based on titer, final liquid volume, and amount of carbon source supplied, with the final volume corrected for volumes sampled, fed, and/or evaporated. It is usually expressed as grams per gram (g/g) or as a % weight per weight (%).

(044) "Time" means the time elapsed from inoculation to sampling or harvesting in a fermentation, typically measured in hours.

(045) "Specific productivity" means the rate of product formation in grams of product produced in given volume of fermentation broth in a given period of time, typically expressed in grams per liter-hours (g/L-hr). The "average specific productivity" means the specific productivity where the period of time is the entire fermentation from inoculation to sampling or harvest. The average specific productivity is lower than the specific productivity from the middle of a fermentation, since specific productivity is lower than average during the early growth period and during the later stages. Average specific productivity can be calculated by dividing final titer by the number of hours at harvest. Note that some published specific productivities are clearly not average specific productivities, although the period of measurement is not explicitly given (see Table 1 for some examples).

(046) "pKa" means the pH at which an acid in solution is half in the conjugate base state, which is typically an ionic or salt form. The pKa for L-LAC and D-LAC is published to be from 3.78 to 3.86, although the exact pKa can vary slightly with temperature, concentration, and concentration

of other solutes. For lactic acid, the conjugate base state is the lactate ion, so the pKa is the pH where the concentration of the lactate ion equals the concentration of the protonated or "free acid" state. The pKa can be measured by the well-known method of performing an acid-base titration and taking the midpoint of the titration curve. One skilled in the art will know that in aqueous solution, D-lactic acid exists to some extent in two forms, the protonated acid form and the ionized salt (i.e., conjugate base) form. As such, depending on context, the terms "D-lactate", "D-lactic acid", and "D-LAC" can mean either form, or a mixture of the two forms. In particular, when discussing titers and yields, the sum of both forms is meant to be included, but it is expressed in terms of the free acid, in other words, titer and yield is expressed as if any salt form that is present is converted to the free acid form.

(047) "Heterologous" means a gene or protein that is not naturally or natively found in an organism, but which can be introduced into an organism by genetic engineering, such as by transformation, mating, or transduction. A heterologous gene can be integrated (i.e., inserted or installed) into a chromosome, or contained on a plasmid. The term "exogenous" means a gene or protein that has been introduced into, or altered, in an organism for the purpose of increasing, decreasing, or eliminating an activity, by genetic engineering, such as by transformation, mating, transduction, or mutagenesis. An exogenous gene or protein can be heterologous, or it can be a gene or protein that is native to the host organism, but altered by one or more methods, for example, mutation, deletion, change of promoter, change of terminator, duplication, or insertion of one or more additional copies in the chromosome or in a plasmid. Thus, for example, if a second copy of a DNA sequence is inserted at a site in the chromosome that is distinct from the native site, the second copy would be exogenous.

(048) "Plasmid" means a circular or linear DNA molecule that is substantially smaller than a chromosome, is separate from the chromosome or chromosomes of a microorganism, and replicates separately from the chromosome or chromosomes. A plasmid can be present in about one copy per cell or in more than one copy per cell. Maintenance of a plasmid within a microbial cell usually requires growth in a medium that selects for presence of the plasmid, for example using an antibiotic resistance gene, or complementation of a chromosomal auxotrophy. However,

some plasmids require no selective pressure for stable maintenance, for example the 2 micron circle plasmid in many *Saccharomyces* strains.

(049) "Chromosome" or "chromosomal DNA" means a linear or circular DNA molecule that is substantially larger than a plasmid and usually does not require any antibiotic or nutritional selection. In the invention, a yeast artificial chromosome (YAC) can be used as a vector for installing heterologous and/or exogenous genes, but it would require selective pressure for maintenance.

(050) "Overexpression" means causing the enzyme or protein encoded by a gene or coding region to be produced in a host microorganism at a level that is higher than the level found in the wild type version of the host microorganism under the same or similar growth conditions. This can be accomplished by, for example, one or more of the following methods: installing a stronger promoter, installing a stronger ribosome binding site, installing a terminator or a stronger terminator, improving the choice of codons at one or more sites in the coding region, improving the mRNA stability, or increasing the copy number of the gene either by introducing multiple copies in the chromosome or placing the cassette on a multi-copy plasmid. An enzyme or protein produced from a gene that is overexpressed is said to be "overproduced." A gene that is being overexpressed or a protein that is being overproduced can be one that is native to a host microorganism, or it can be one that has been transplanted by genetic engineering methods from a different organism into a host microorganism, in which case the enzyme or protein and the gene or coding region that encodes the enzyme or protein is called "foreign" or "heterologous." Foreign or heterologous genes and proteins are by definition overexpressed and overproduced, since they are not present in the native, wild type, parent or precursor host organism.

(051) "Homolog" means a gene, DNA sequence, or protein that performs a similar biological function to that of another gene, DNA sequence, or protein, and that has at least 25% sequence identity (when comparing protein sequences or comparing the protein sequence derived from gene sequences) with said another gene, DNA sequence, or protein, as determined by the Basic Local Alignment Search Tool (BLAST) computer program for sequence comparison (Altschul et al, 1990; Altschul et al, 1997) and allowing for deletions and insertions. An example of a homolog of the *K. marxianus PDC1* gene would be the *PDC1* gene from *S. cerevisiae*.

(052) "Analog" means a gene, DNA sequence, or protein that performs a similar biological function to that of another gene, DNA sequence, or protein, but where there is less than 25% sequence identity (when comparing protein sequences or comparing the protein sequence derived from gene sequences) with said another gene, DNA sequence, or protein, as determined by the BLAST computer program for sequence comparison (Altschul et al, 1990; Altschul et al, 1997), and allowing for deletions and insertions. An example of an analog of the *K. marxianus Gpd1* protein would be the *K. marxianus Gut2* protein, since both proteins are enzymes that catalyze the same reaction, but there is no significant sequence homology between the two enzymes or their respective genes. A person having ordinary skill in the art will know that many enzymes and proteins that have a particular biological function (in the immediately above example, glycerol-3-phosphate dehydrogenase), can be found in many different organisms, either as homologs or analogs, and since members of such families of enzymes or proteins share the same function, although they may be slightly or substantially different in structure. Different members of the same family can in many cases be used to perform the same biological function using current methods of genetic engineering. Thus, for example, a gene that encodes D-lactate dehydrogenase could be obtained from any of many different organisms.

(053) "Mutation" means any change from a native or parent DNA sequence, for example, an inversion, a duplication, an insertion of one or more base pairs, a deletion of one or more base pairs, a point mutation leading to a base change that creates a premature stop codon, or a missense mutation that changes the amino acid encoded at that position. "Null mutation" means a mutation that effectively eliminates the function of a gene. A complete deletion of a coding region would be a null mutation, but single base changes can also result in a null mutation. "Mutant", "mutated strain", "mutated yeast strain", or a strain "that has been mutated" means a strain that comprises one or more mutations when compared to a native, wild type, parent or precursor strain.

(054) The phrase "a mutation that eliminates or reduces the function of" means any mutation that lowers any assayable parameter or output, of a gene, protein, or enzyme, such as mRNA level, protein concentration, or specific enzyme activity of a strain, when said assayable parameter or output is measured and compared to that of the unmutated parent strain. Such a mutation is

preferably a deletion mutation, but it can be any type of mutation that accomplishes a desired elimination or reduction of function.

(055) "Strong constitutive promoter" means a DNA sequence that typically lies upstream (to the 5' side of a gene when depicted in the conventional 5' to 3' orientation), of a DNA sequence or a gene that is transcribed by an RNA polymerase, and that causes said DNA sequence or gene to be expressed by transcription by an RNA polymerase at a level that is easily detected directly or indirectly by any appropriate assay procedure. Examples of appropriate assay procedures include quantitative reverse transcriptase plus PCR, enzyme assay of an encoded enzyme, Coomassie Blue-stained protein gel, or measurable production of a metabolite that is produced indirectly as a result of said transcription, and such measurable transcription occurring regardless of the presence or absence of a protein that specifically regulates the level of transcription, a metabolite, or an inducer chemical. By using well-known methods, a strong constitutive promoter can be used to replace a native promoter (a promoter that is otherwise naturally existing upstream from a DNA sequence or gene), resulting in an expression cassette that can be placed either in a plasmid or chromosome and that provides a level of expression of a desired DNA sequence or gene at a level that is higher than the level from the native promoter. A strong constitutive promoter can be specific for a species or genus, but often a strong constitutive promoter from a yeast can function well in a distantly related yeast. For example, the *TEF1* (translation elongation factor 1) promoter from *Ashbya gossypii* functions well in many other yeast genera, including *K. marxianus*.

(056) "Microaerobic" or "microarobic fermentation conditions" means that the supply of air to a fermentor is less than 0.1 volume of air per volume of liquid broth per minute (vvm).

(057) "Chemically defined medium", "minimal medium", or "mineral medium" means any fermentation medium that is comprised of purified chemicals such as mineral salts (for example sodium, potassium, ammonium, magnesium, calcium, phosphate, sulfate, chloride, etc.) which provide necessary element such as nitrogen, sulfur, magnesium, phosphorus (and sometimes calcium and chloride), vitamins (when necessary or stimulatory for the microbe to grow), one or more pure carbon sources, such as a pure sugar, glycerol, ethanol, etc., trace metals as necessary or stimulatory for the microbe to grow (such as iron, manganese, copper, zinc, molybdenum, nickel, boron and cobalt), and optionally an osmotic protectant such as glycine betaine, also known

as betaine. Except for the optional osmoprotectant and vitamin(s), such media do not contain significant amounts of any nutrient or mix of more than one nutrient that is not essential for the growth of the microbe being fermented. Such media do not contain any significant amount of rich or complex nutrient mixtures such as yeast extract, peptone, protein hydrolysate, molasses, broth, plant extract, animal extract, microbe extract, whey, Jerusalem artichoke powder, and the like. For producing a commodity chemical by fermentation where purification of the desired chemical by simple distillation is a not an economically attractive option, a minimal medium is preferred over a rich medium because a minimal medium is usually less expensive, and the fermentation broth at the end of fermentation usually contains lower concentrations of unwanted contaminating chemicals that need to be purified away from the desired chemical.

(058) "Fermentation production medium" means the medium used in the last tank, vessel, or fermentor, in a series comprising one or more tanks, vessels, or fermentors, in a process wherein a microbe is grown to produce a desired product (for example D-LAC or L-LAC). For production of a commodity chemical by fermentation such as D-LAC or L-LAC, where extensive purification is necessary or desired, a fermentation production medium that is a minimal medium is preferred over a rich medium because a minimal medium is often less expensive, and the fermentation broth at the end of fermentation usually contains lower concentrations of unwanted contaminating chemicals that need to be purified away from the desired chemical. Although it is generally preferred to minimize the concentration of rich nutrients in such a fermentation, in some cases it is advantageous for the overall process to grow an inoculum culture in a medium that is different from the fermentation production medium, for example to grow a relatively small volume (usually 10% or less of the fermentation production medium volume) of inoculum culture grown in a medium that contains one or more rich ingredients. If the inoculum culture is small relative to the production culture, the rich components of the inoculum culture can be diluted into the fermentation production medium to the point where they do not substantially interfere with purification of the desired product. A fermentation production medium must contain a carbon source, which is typically a sugar, glycerol, fat, fatty acid, carbon dioxide, methane, alcohol, or organic acid. In some geographic locations, for example in the Midwestern United States, D-glucose (dextrose) is relatively inexpensive and therefore is useful as a carbon source. Most prior art publications on lactic acid production by a yeast use dextrose as the carbon source. However,

in some geographic locations, such as Brazil and much of Southeast Asia, sucrose is less expensive than dextrose, so sucrose is a preferred carbon source in those regions.

(059) "Final pH" means the pH of a fermentation broth at the end of a fermentation when the fermentation is considered complete, fermentation is stopped, and the broth is harvested. Although it is preferred that the final pH of a lactic acid fermentation be below the pKa of lactic acid, it is also preferred that the pH during fermentation be controlled by addition of a "base" (an alkaline substance), to prevent the pH from falling too quickly or ending too low. The "base" can be in a solution, suspension, slurry, or solid form. The "base" can be a hydroxide, oxide, carbonate, or bicarbonate salt of sodium, ammonium, potassium, magnesium, or calcium. For production of lactic acid, a preferred base is a slurry of calcium hydroxide or powdered calcium hydroxide, which leads to the formation of some calcium lactate mixed with the protonated acid form in the fermentation broth. The resulting fermentation broth at the end of fermentation can be treated with sulfuric acid, which causes precipitation of calcium sulfate (gypsum), which aids in the removal of calcium, to increase the proportion of the lactic acid that is present in the protonated form. The feeding of the base to control pH can be done manually or by an automatically controlled pump or auger, as called for by pH measurements, which can be obtained manually or by continuous monitoring through a pH probe immersed in the fermentation vessel.

(060) To facilitate understanding of the invention, various genes are listed in Table 2 below. The sequence information for plasmid and exogenous genes used in the present invention are listed in the Table 3 below.

(061) The prior art (described above) discloses several genetically engineered yeasts that produce D-LAC, but as mentioned above, none of the published parameters come close to those necessary for an economically attractive process. Furthermore, D-LAC is generally more toxic than L-LAC to organisms, so techniques and approaches for developing strains and processes for producing L-LAC cannot be assumed to be directly applicable to techniques and approaches for producing D-LAC.

(062) *K. marxianus* strains were grown at 37°C and stored at -80°C in 20 to 40% glycerol. Transformation of *K. marxianus* was done by the "Transformation protocol for gene targeting" method of Abdel-Banat et al (2010) with the modification that the growth medium for making transformation-competent cells was altered to prevent the cultures from becoming too acidic. The growth medium for making competent cells contained, per liter, 10 g yeast extract, 20 peptone, 3 g glucose, and 20 g glycerol.

(063) DNA cassettes were constructed in plasmids in *E. coli DH 5-alpha* strain (New England BioLabs, Ipswitch, Massachusetts, USA) using standard DNA manipulation methods well known to those skilled in the art, including synthetic "g-block" DNA sequences (Integrated DNA Technologies, Woodland, Texas, USA), high fidelity PCR, restriction enzymes, DNA ligase, and the Gibson method using the NEBuilder kit (New England BioLabs, Ipswitch, Massachusetts, USA).

(064) The general design of cassettes for integration have the following features, in the following order: (1) upstream homology to target locus, (2) strong constitutive promoter, (3) coding region to be expressed, (4) terminator, (5) homology to downstream of target locus, (6) selectable (and optionally counter-selectable) marker gene, and (7) homology to a middle sequence of target locus. Selection for the marker gene causes integration between the upstream homology and middle homology of the target locus. Transformants containing the correct integration are identified by diagnostic PCR that shows both an upstream and a downstream junction fragment of the correct expected size. Counter selection, for example against a *URA3* gene, leads to looping out of the selectable and counter-selectable marker *URA3* gene and the middle homology sequence by recombination of the downstream homology component in the cassette with the same chromosomal sequence that occurs just downstream of the integrated cassette. If the target locus is intended to be deleted without a concomitant insertion, then the "(2) strong constitutive promoter, (3) coding region to be expressed, (4) terminator" portion of the general design described above is omitted.

EXAMPLES

(065) The following examples are provided to further explain the invention but are not intended to limit the scope of the invention.

Example 1

Construction of strain SD1555

(066) The starting strain was *K. marxianus* SD98, a wild Crabtree positive strain isolated from rotting bagasse explained in the US Patent Application Publication No. 2015/0240270. The native *KmURA3* gene was deleted from SD98 to give strain KMS95 in order to use a *URA3* gene as a selectable marker for integrative transformation. The *KmURA3* gene was deleted by integrating a cassette constructed on plasmid pMS52 (SEQ ID No. 1). The cassette was obtained from the plasmid as a linear fragment, and integrated into SD98, selecting for hygromycin resistance (300 mg/L hygromycin B in YPD medium after a 3-hour grow out period in YPD without hygromycin before plating on hygromycin plates). The hygromycin resistance gene was driven by the *Ashbya gossypii TEF1* promoter. After correct integration of the cassette at the *KmURA3* locus, a portion of the interrupted *KmURA3* gene and the hygromycin resistance gene were flanked by direct repeats to allows the sequences between the direct repeats to be looped out by homologous recombination with a second selection for resistance to 5-fluoro-orotic acid (5-FOA), which counter-selects the *URA3* gene; in other words, it selects for loss of the *URA3* gene. Selection for 5-FOA resistance was done by plating about 100 million cells on CM glucose minus uracil medium (Teknova, Hollister, California, USA) supplemented with 1 g/L 5-FOA and 24 mg/L uracil.

(067) Four different cassettes designed to express the *E. coli ldhA* gene were constructed on plasmids. In all four cases, the *ldhA* gene was expressed from the *K. marxianus PDC1* promoter. The four different cassettes were designed to insert into four different loci in KMS95: the *PDC1* (pyruvate decarboxylase) locus (SEQ ID No. 2), the *GPP1* (glycerol-3-phosphate phosphatase) locus (SEQ ID No. 3), the *PCK1* (phosphoenol pyruvate carboxykinase) locus (SEQ ID No. 4), and the *NDE1* (NADH dehydrogenase 1) locus (SEQ ID No. 5). The cassettes were designed to integrate at the target loci by homologous recombination, selecting for the *S. cerevisiae URA3*

gene on CM glucose minus uracil medium (Teknova, Hollister, California, USA), and then in a second step for the *URA3* gene to be looped out by homologous recombination between direct repeats of a downstream flank, selecting for resistance to 5-FOA, as described above, in order to reuse the *URA3* gene for subsequent transformations. At each transformation step and each loopout step, single colonies were restreaked one or more times as necessary to free the correct strain from background cells, and to eliminate heterozygous diploids. Correct insertions and correct loop-outs were identified by PCR using appropriate primers that bracketed the borders between the ends of the cassettes and the chromosomal sequences at the target locus that are just upstream or just downstream of the integrated cassette. PCR diagnostics could not distinguish correctly integrated cassettes in haploids from correctly integrated homozygous diploids; so this distinction was not made at any step of the constructions. Starting with strain KMS95, the four *ldhA* cassettes were installed, one at a time in the order listed above. After each initial integration of a cassette, the *URA3* gene was looped out by the 5-FOA counter-selection. After the fourth cassette was thus installed, the native *KmURA3* gene (SEQ ID No. 6) was re-installed by transformation of a linear DNA fragment obtained by PCR from SD98 chromosomal DNA as a template to give a uracil prototroph by selection on CM glucose minus uracil plates. The resulting strain, which now contains four copies of the integrated *ldhA* gene, was named SD1555. Insertion of the cassette into the *PDC1* gene blocks the unwanted synthesis of ethanol. Insertion of the cassette into the *GPP1* gene blocks the unwanted synthesis of glycerol. Insertion of the cassette into the *PCK1* gene blocks the unwanted growth of the yeast on D-LAC or L-LAC as a carbon source, because strains that have reduced or eliminated Pck1 activity cannot perform glucogenesis, and gluconeogenesis is necessary for growth on non-fermenatble carbon sources such as D-LAC or L-LAC. Inability to grow on D-LAC or L-LAC is a desirable trait, because it prevents a loss of titer at the end of fermentation when the concentration of sugar is low or zero. Although a *pck1* mutation is preferable, because *PCK1* encodes the first committed step in the gluconeogenic pathway, any other mutation that reduces or eliminates gluconeogenesis, for a example a mutation that reduces or eliminates fructose 1,6-bisphosphate phosphatase activity, could also lead to a similar desirable result, namely reduced or eliminated gluconeogenesis. Insertion of the cassette into the *NDE1* gene causes conservation of cytoplasmic NADH, one of the substrates for biosynthesis of D-LAC.

Example 2

Construction of strain SD1566

(068) Derivatives of SD1555 that were resistant to beta-chlorolactate (MilliporeSigma, St. Louis, Missouri, USA) were selected as follows. A lawn of about 10^8 cells of SD1555 was spread evenly on a plate containing SDM2 medium containing 20 g/L glucose. A small spec of beta-chlorolactate was placed at the center of the plate. The beta-chlorolactate was not specified as to isomer, so it was assumed to be a racemic mixture of D- and L- isomers. After three days, a lawn grew around a central killing zone. At the edge of the killing zone, several individual colonies appeared. Several such colonies were restreaked to plates containing SDM2 with 20 g/L glucose and 0.75 g/L beta-chlorolactate. After three days at 37°C, the colonies resistant to beta-chlorolactate were visible. The parent strain SD1555 did not give visible single colonies on the same plate at three days.

(069) In addition to beta-chlorolactate, there are many other lactic acid analogs that can be used to select for resistant mutants that have desirable properties. Examples of lactic acid analogs include, but are not limited to 3-chlorolactate (beta-chlorolactate), 3-dichlorolactic acid, 3-trichorolactic acid, 3-fluorolactic acid, 3-difluorolactic acid, 3-trifluorolactic acid, 3-bromolactic acid, 3-dibromolactic acid, 3-tribromolactic acid, all possible 2- halo-substituted derivatives of lactic acid, all chiral forms of any of the above analogs, or any salt of any of the above. "Lactic acid analog" means any compound that is structurally related to lactic acid and inhibits growth of a parent yeast strain under appropriate conditions, and includes the set of compounds disclosed above. Note that the term "analog" in the context of "lactic acid analog" has a meaning different from that when used in the context of a gene or protein sequence. As mentioned above, in the context of a gene or protein, the term "analog" refers to a positively functioning alternative, while in the context of "lactic acid analog", the word means an interfering, toxic compound.

(070) A yeast strain genetically engineered to produce lactic acid that has been "mutated to confer resistance to a lactic acid analog at a higher concentration when compared to an isogenic strain that has not been mutated" means a strain that contains one or more mutations relative to a parent strain, for which a concentration of a lactic acid analog in liquid or agar containing medium can

be found, where said mutated strain gives visibly better growth or larger colonies than the parent strain when the two strains are grown in similar parallel liquid cultures or streaked beside each other on the same Petri plate, after incubation for between about one and five days at a temperature that is appropriate for visible growth of the mutated strain. In the example given above, a beta-chlorolactate concentration of 0.75 g/L and incubation for three days at 37°C was determined to show good contrast in colony size on agar plates, but for other yeast strains and species, other conditions might show better contrast between parents and mutants. Appropriate conditions for establishing resistance to a lactic acid analog in a strain by differentiating said resistant strain from a parent strain can be determined by routine experimentation, by plating or growing a parent strain and a mutant strain in media containing a range of concentrations of said lactic acid analog from 0 g/L to about 10 g/L.

(071) For example, by systematically varying the concentration of a lactic acid analog in petri plates containing a minimal medium that supports growth of the parent strain, incubating at a temperature where the parent strain is known to grow well, and checking the plates on a daily basis to score the plates when a contrast in colony size between mutant and parent is visible, strains that are resistant to a lactic acid analog when compared to a parent strain can be identified. Alternatively, relative resistance to a lactic acid analog can be determined by obtaining growth curves of mutant and parent strains in liquid media that contain a range of lactic acid analog concentrations from 0 to 10 g/L.

(072) "Parent strain" means a starting strain that can be subjected to one or more conditions that leads to a new derivative or descendant strain that comprises a genetic change that ultimately leads to at least one change in a measurable property of the new derivative strain that is significantly different from that property of the parent strain, for example lactic acid titer, lactic acid yield, specific productivity of lactic acid, or titer of any measurable byproduct, such as pyruvic acid. Said "one or more conditions" can be any one or more of a number of manipulations performed on the parent strain, for example genetic engineering involving installation of DNA that alters the genetic makeup of the strain, selection of spontaneous mutants as described above for beta-chlorolactate resistance, and applying any of a number of well-known mutagenesis procedures to a parent strain before subjecting a mutagenized population of cells to a selection or screen for a

desired property, for example for resistance to a lactic acid analog. Well known mutagenesis procedures include exposing cells to a chemical mutagen, for example nitrosoguanidine (also known as NTG, MNNG, and N-methyl-N'-nitro-N-nitrosoguanidine), ethylmethanesulfonate (also known as EMS), or mutagenic radiation, for example X-rays or ultraviolet light. An appropriate dose of mutagen can be determined for a parent strain by exposing a population of live parent cells to a range of mutagen doses, and choosing a dose and conditions that leave a live subpopulation that is sufficiently large that it contains desired mutants of the desired type, for example beta-chlorolactate resistant mutants. In the art, typical mutagen doses are chosen that kill about 10% to 95% of the parent population. Strains resistant to beta-chlorolactate or other lactic acid analog can be selected from a parent strain that has already been genetically engineered to produce lactic acid, but strains resistant to a lactic acid analog can also be selected from parent strains that have not been engineered for lactic acid production. In this latter case, resistant mutants can be engineered for lactic acid production and then screened for improved lactic acid production compared to strains similarly engineered from parent strains that have not been selected for resistance to a lactic acid analog.

(073) Beta-chlorolactate resistant strains isolated as described above were compared to parent strain SD1555 for D-LAC production by fermenting in flower plates in a BioLector minifermentor (m2p-Labs, Hauppauge, New York, USA). Inocula for the BioLector were grown in YPD in which the dextrose concentration was lowered to 3 g/L instead of the usual 20 g/L. The fermentation medium for the BioLector was SDM2 containing 100 or 200 g/L glucose, at 1 ml per well, with a starting OD 600 nm of 0.1- 0.2. The flower plates were shaken at 1200 rpm at 37°C for 48 hours. One particular beta-chlorolactate resistant isolate, named SD1566, performed better than the parent, SD1555. The D-LAC and pyruvate titers are shown in Figure 7. SD1566 produced a higher titer of D-LAC, and a substantially lower titer of the unwanted byproduct pyruvate, than then parent strain SD1555. A "a substantially lower titer of pyruvate when compared to a parent strain that has not been mutated" means a titer that is reproducibly at least 25 % lower than that of the parent strain grown under similar conditions. SD1566 was further characterized in computer controlled 7-liter fermentors (New Brunswick Scientific, Indianapolis, Indiana, USA).

Example 3

Production of D-LAC by strain SD1566 in 7-liter fermenters

(074) Inocula of yeast strain SD1566 were grown at 37°C in 150 ml of YPS-MES medium in 500 ml baffled shake flasks to an OD 600 nm of about 2 to 6. 150 ml was inoculated into 4 liters of AM1S medium in New Brunswick BioFlo 110 or Eppendorf BioFlo 115 fermentors. Impeller speed was 750 rpm and aeration was 260 ml/min, equal to 0.065 vvm of the starting volume. The starting pH was about 6.6. The temperature was set at 37°C. pH was controlled by automatically controlled peristaltic pumping of a slurry of 3 molar calcium hydroxide, which was kept suspended in a vigorously stirred reservoir. The starting set point was pH 6. The pH set point was automatically ramped down (i.e., decreased) to pH 3.5 in a linear fashion from time zero (inoculation time) to 30 hours. The actual pH reached 3.5 at 33 hours. At 36 hours the D-LAC titer was 110 g/L and the calculated yield was 0.81 g/g sucrose (average of duplicate fermentations). The final pyruvic acid titer was 0.55 g/L. The average specific productivity for D-LAC was 3.05 g/L-hr. For comparison, under similar conditions, the parent strain SD1555 produced 78 g/L D-LAC and 9.8 g/L pyruvate with a yield of 0.67 g/g in 39 hours. The average specific productivity for D-LAC from SD1555 was 2.0 g/L-hr. Thus, the beta-chlorolactate resistant strain SD1566 was improved for titer, yield, and average specific productivity, and its pyruvate byproduct titer was lower than that of the parent SD1555. The D-LAC titers are shown as a function of fermentation time in Figure 8.

(075) Using a Chirex 3126 column (Phenomenex, Torrance, California, USA) as recommended by the manufacturer, there was no detectable L-LAC produced by either SD1555 or SD1566. The minimum detectable titer of L-LAC by this HPLC method is 0.01 g/L, so the optical purity of the D-LAC produced by SD1566 was greater than 99.9 %.

(076) A person having ordinary skill in the art would recognize that the methods described herein could be used to add ability to produce lactic acid by genetically engineering other yeast strains, species, and genera that do not naturally produce significant titers of lactic acid, or to improve the ability to produce lactic acid by genetically engineering other yeast strains, species, and genera that already have some capacity to produce lactic acid. The insertion of the *ldhA* cassettes in the

four target genes effectively eliminates or reduces the function of those four target genes, but alternative approaches can be used to achieve the elimination or reduction in function. Any mutation, or combination of more than one mutation, that renders the protein product of the gene to be absent, inactive, or lower in specific activity compared to that of an isogenic wild type strain, can be used to eliminate or reduce the function of any one of the four targeted genes (i.e., *PDC1*, *GPP1*, *PCK1*, and *NDE1*), or homologs or analogs of those genes. In addition, a gene encoding a D-lactate dehydrogenases other than the *E. coli* version can be used instead of the *E. coli* version, installed in one or more copies in a fashion similar to that disclosed herein. In addition, a gene encoding an L-lactate dehydrogenases can be used instead of the *D-lactate dehydrogenase* version, installed in one or more copies in a fashion similar to that disclosed herein.

(077) For the fermentation process, the starting pH can vary, the final pH (when the fermentation is completed and the broth is sampled and/or harvested for downstream processing and purification of the lactic acid product) can vary, and the function, algorithm or program for lowering the pH during fermentation can vary. The pH can be ramped down with a linear function, a step function, or a non-linear, non-stepped function, for example a curved function such as an exponential or parabolic function. Regardless of the type of function, algorithm or program for pH lowering, it is preferable that the final pH is below the pKa of lactic acid in order to reduce the cost of the overall process. A starting pH of between 4.5 and 7.0 is preferable, as yeast strains typically grow faster in this range.

(078) The material for controlling the pH in the fermentor can be any suitable alkaline material, such as the hydroxide, oxide, bicarbonate, or carbonate salt of potassium, sodium, ammonium, magnesium or calcium. The material can be fed as a solution, as a suspension or slurry, or as a solid using an auger.

(079) The foregoing descriptions and examples have been presented for purposes of explanation. In addition to the descriptions and examples provided, numerous combinations, modifications, and variations of the features, structures, components, or characteristics of the non-limiting embodiments are possible in light of the above teachings.

(080) Table 1 provides a summary of the best published lactic acid fermentation runs at a pH below the pKa of 3.86 in *Saccharomyces cerevisiae* (*S. c.*), *Kluveromyces lactis* (*K. m.*) and *Issatchenkia orientalis* (*I. o*). Various genes used in this investigation are listed in Table 2. Table 3 lists the DNA sequence information submitted with this patent application. The compositions of the growth media used are given in Table 4.

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Table 1. Summary of parameters of the best published L-LAC and D-LAC fermentation runs at a final pH below the pKa of 3.86 with *Saccharomyces cerevisiae (S.c.); K.l., Kluveromyces lactis (K. l.); K. marxianus (K.m.; and Issatchenkia orientalis (I.o.).*

Isomer	Yeast	Titer g/L	Yield g/g	Time hours	pH	Specific Productivity g/L-hour	Reference
L	*S.c.*	58			3.6		(Sauer, Porro et al. 2010)
L	*S.c.*	62		76		0.81	(Sauer, Porro et al. 2010)
L	*S.c.*	70		72		0.97	(Sauer, Porro et al. 2010)
L	*K. l.*	35	0.44	97	3.0	0.36	(Porro 2006)
L	*I.o.*	70	0.75	69	3.0	1.5	(Suominen 2009)
D	*S.c.*	37	0.74	68	2.7	0.54	(Winkler 2007)
D	*K.m.*	<62	0.69	>107	3.0	0.58	(Miller 2012)
D	*K.m.*	49		48	3.8	1.02	(Yocum 2014)
D	*S.c.*	82.6	0.83	55	3.5	1.50	(Baek et al. 2017)
D	*K.m.*	110	0.81	36	3.5	3.02	The invention

Table 2. Gene names and descriptions.		
Gene name used herein	Protein or function encoded	Other names encountered in literature (not comprehensive)
ldhA	D-lactate dehydrigenase	*ldhD, D-ldh*
ldhL	L-lactate dehydrogenase	*L-ldh*
PDC1	Pyruvate decarboxylase	*PDC, pdc*
GPP1	Glycerol-3-phosphate phosphatase	
PCK1	Phosphoenolpyruvate carboxykinase	*PCK, pck*
NDE1	NADH dehydrogenase 1	
TEF1	Translation elongation factor 1	*eft, eftu, eftU*
CYC1	Cytochrome C	

Table 3. Sequence information	
SEQ ID 1	Sequence of the plasmid pMS52
SEQ ID 2	Sequence of the plasmid pSD100 *ldhA*
SEQ ID 3	Sequence of the plasmid pSD95 *ldhA*
SEQ ID 4	Sequence of the plasmid pSD100 *PCK1*
SEQ ID 5	Sequence of the plasmid pSD104 *NDE1*
SEQ ID 5	Wt *KmURA3* gene and 1033 bp upstream flanking sequence and 1046 bp downstream flanking sequence of the URA3 gene of strain SD98

Table 4. Composition of growth media. All amounts listed are per liter. For Petri plates, 20 g/L agar was added.

Ingredient	CM minus uracil	SDM2	AM1S	YPD	YPS-MES
Glucose		(20-200 g)		20 g	
sucrose		(20-200 g)	200		20 g
Teknova CM-ura mix	One 1 L pack				
Potassium phosphate monobasic			0.51 g		
Ammonium phosphate monobasic		13.8 g	0.87 g		
Ammonium phosphate dibasic		3.96 g	2.63		
Magnesium sulfate. $7H_2O$		0.493 g	0.37 g		
Yeast extract				10 g	10 g
Peptone				20 g	20 g
Thiamine HCl		200 mcg	0.1 mg		
Niacin		3 mg	4 mg		
Biotin		10 mcg	0.01 mg		
Calcium pantothenate		400 mcg	0.4 mg		
1000 X trace elements*		1 ml	1 ml		
pH (with ammonium hydroxide or phosphoric acid)		6.2	6.6		6.0
MES (2-(*N*-morpholino) ethanesulfonic acid)					19.5
Betaine			0.117 g		
Sodium Chloride		0.234 g			
Potassium Chloride		0.521 g			

*1000 X Trace elements, per liter: 1.6 g $FeCl_3 \cdot 6H_2O$, 0.1 g $CuCl_2 \cdot 2H_2O$, 0.2 g $ZnCl_2$, 0.05 g H_3BO_3, 0.55 g $MnCl_2 \cdot 4H_2O$, 10 mL 85% phosphoric acid

CLAIMS

What is claimed is:

1. A yeast strain genetically engineered to produce lactic acid comprising a chromosomally integrated gene that encodes an exogenous lactate dehydrogenase, wherein said yeast strain produces lactic acid in a fermentation production medium with an average specific productivity of at least 1.875 g/L-hr, and wherein said fermentation production medium has a final pH that is lower than 3.86.

2. A yeast strain of claim 1, wherein said yeast strain produces lactic acid in a fermentation production medium with an average specific productivity of at least 3.00 g/L-hr.

3. A yeast strain of claim 1, wherein said yeast strain produces lactic acid in a fermentation production medium with an average specific productivity of at least 3.00 g/L-hr and a final pyruvic acid titer of less than 1g/L.

4. The yeast strain of claim 1, wherein said yeast strain is selected from a group consisting of the genera *Saccharomyces, Kluyveromyces, Issatchenkia, Pichia, Torulopsis, Candida, Hansenula, Zygosaccharomyces, Schizosaccharomyces, Yarrowia* and *Lachancea.*

5. The yeast strain of claim 1, wherein said yeast strain is selected from a group consisting of the genera *Saccharomyces* and *Kluyveromyces.*

6. The yeast strain of claims 1, wherein said gene that encodes an exogenous lactate dehydrogenase is integrated at least at one chromosomal locus selected from a group consisting of a pyruvate decarboxylase gene or a homolog or analog thereof, a phosphoenolpyruvate carboxykinse gene or a homolog or analog thereof, a glycerol-3-phosphate phosphatase gene or a homolog or analog thereof, and a NADH dehydrogenase 1 gene or a homolog or analog thereof.

7. The yeast strain of claim 1, wherein said gene that encodes an exogenous lactate dehydrogenase is integrated at a pyruvate decarboxylase gene or a homolog or analog thereof, a phosphoenolpyruvte carboxykinse gene or a homolog or analog thereof, a glycerol-3-phosphate phosphatase gene or a homolog or analog thereof, and an NADH dehydrogenase 1 gene or a homolog or analog thereof loci.

8. The yeast strain of claim 1, wherein said yeast strain is resistant to a lactic acid analogue.

9. The yeast strain of claim 8, wherein said lactic acid analogue is selected from a group consisting of 3-chlorolactate (beta-chlorolactate), 3,3-dichlorolactic acid, 3,3,3-trichorolactic acid, 3-fluorolactic acid, 3,3-difluorolactic acid, 3,3,3-trifluorolactic acid, 3-bromolactic acid, 3,3-dibromolactic acid, 3,3,3-tribromolactic acid, all possible 2- halo-substituted derivatives of lactic acid, all chiral forms of any of the above lactic acid analogs and any salt of any of the lactic acid analogs.

10. The yeast strain of claims 1, further comprising a mutation that eliminates or reduces the function of one or more genes selected from the group consisting of *PCK1*, *NDE1*, *PDC1*, and *GPP1*, or eliminates or reduces the function of one or more homologs or analogs of said one or more genes.

11. The yeast strain of claims 1, wherein said lactic acid is optically pure D-lactic acid.

12. The yeast strain of claims 1, wherein said lactic acid is optically pure L-lactic acid.

13. The yeast strain of claims 1, wherein said lactic acid is a mixture of D-lactic acid and L-lactic acid.

14. A process for producing lactic acid comprising the steps of:
    (a) providing the yeast strain and the fermentation production medium of claims 1; and
    (b) culturing said yeast strain in said fermentation production medium; and
    (c) recovering lactic acid from the fermentation production medium.

15. A process for selecting a yeast strain having improved resistance to a lactic acid analog, comprising the steps of:

(a) growing a parent yeast strain on a first medium to generate a population of yeast cells;

(b) optionally exposing said population of yeast cells to a mutagen;

(c) subjecting said population of yeast cells to a second medium, wherein said second medium comprises a lactic acid analog; and

(d) selecting a yeast strain from said population of yeast cells.

16. A yeast strain genetically engineered to produce lactic acid comprising a mutation that eliminates or reduces the function of a *PCK1* gene or a homolog or analog thereof.

17. A yeast strain genetically engineered to produce lactic acid comprising a mutation that eliminates or reduces the function of an *NDE1* gene, or a homolog or analog thereof.

18. A yeast strain genetically engineered to produce lactic acid comprising a mutation that eliminates or reduces the function of a *PDC* gene or a homolog or analog thereof.

19. A yeast strain genetically engineered to produce lactic acid comprising a mutation that eliminates or reduces the function of a *GPP 1* gene or a homolog or analog thereof.

Figure 1

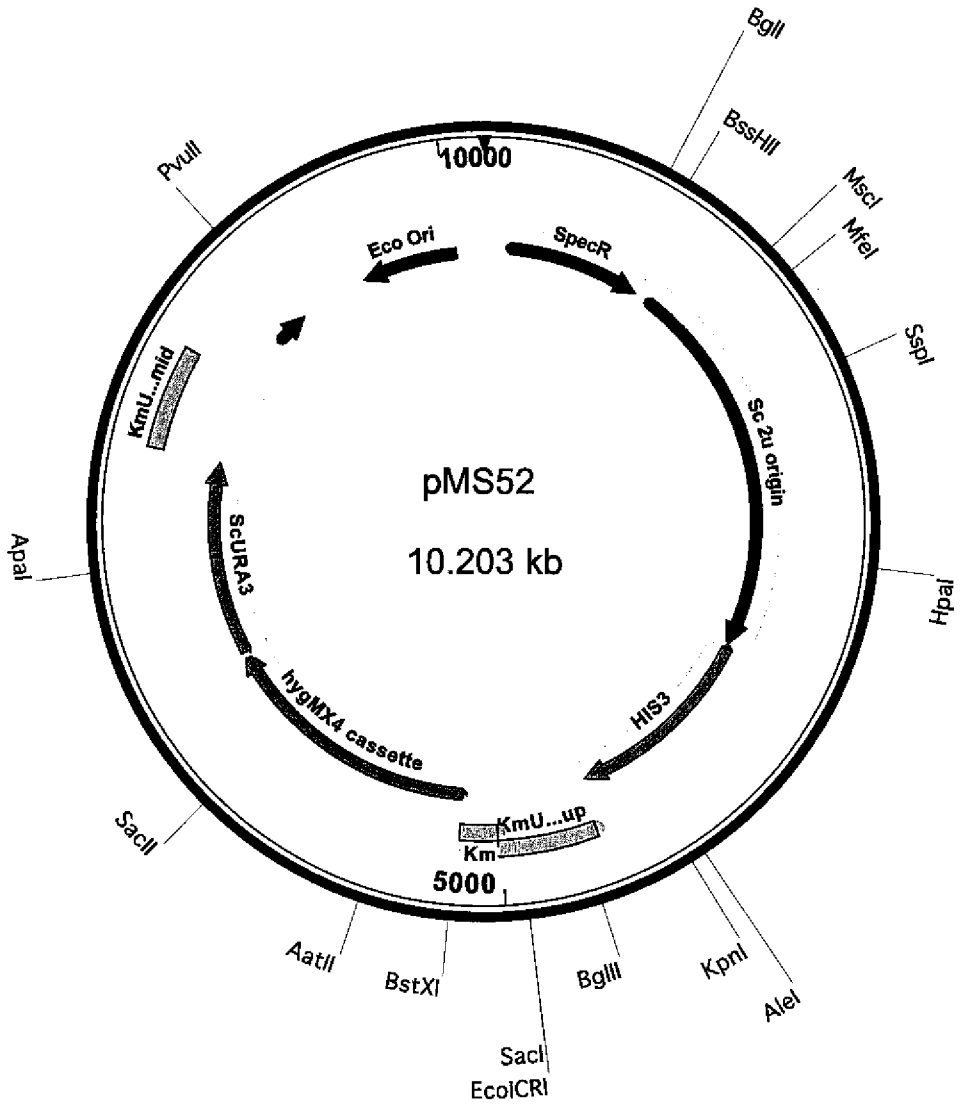

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10.203 kb
10000
5000
Eco Ori
SpecR
Sc 2u origin
HIS3
KmU...up
Km
hygMX4 cassette
ScURA3
KmU...mid
BglI
BssHII
MscI
MfeI
SspI
HpaI
KpnI
AleI
BglII
BstXI
AatII
SacI
EcoICRI
SacII
ApaI
PvuII

Figure 2

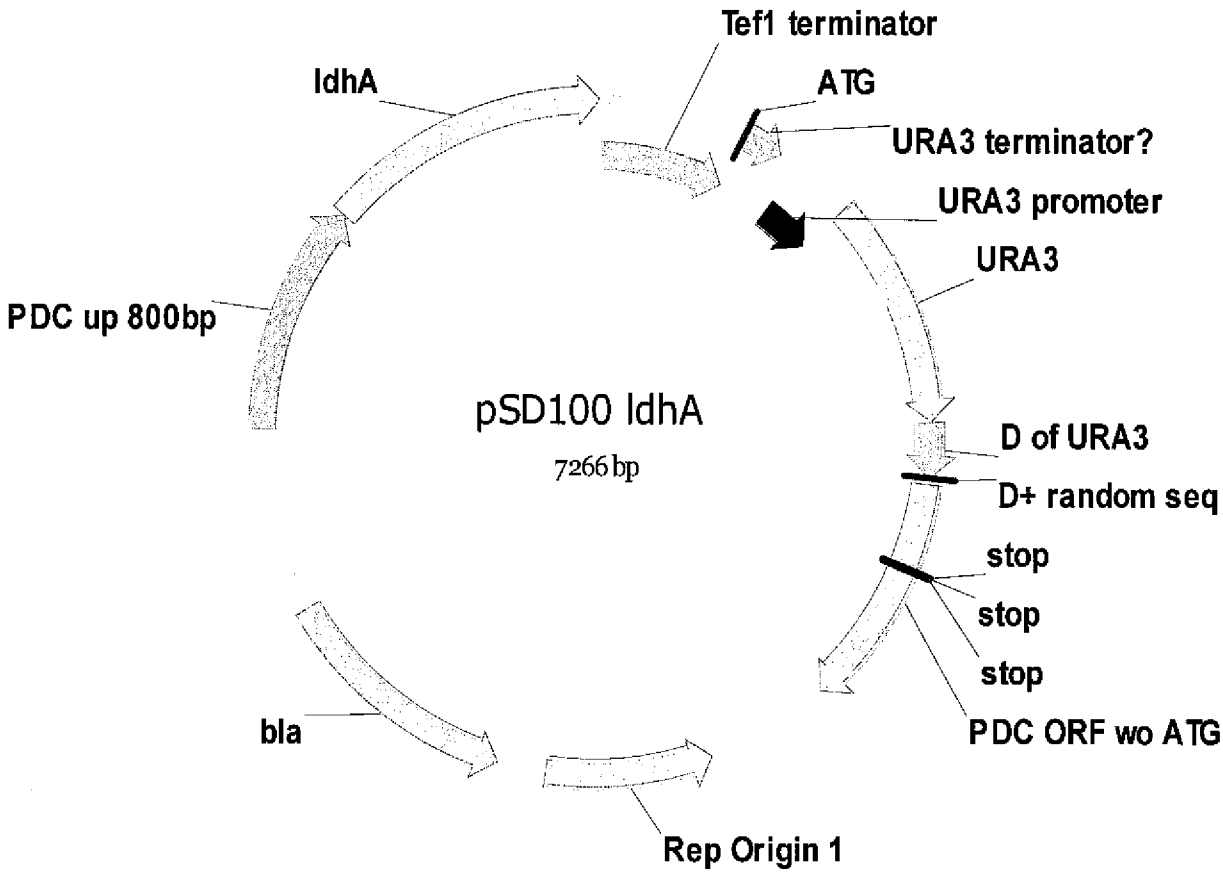

Tef1 terminator
ldhA
ATG
URA3 terminator?
URA3 promoter
URA3
PDC up 800bp
pSD100 ldhA
7266 bp
D of URA3
D+ random seq
stop
stop
stop
PDC ORF wo ATG
bla
Rep Origin 1

Figure 3

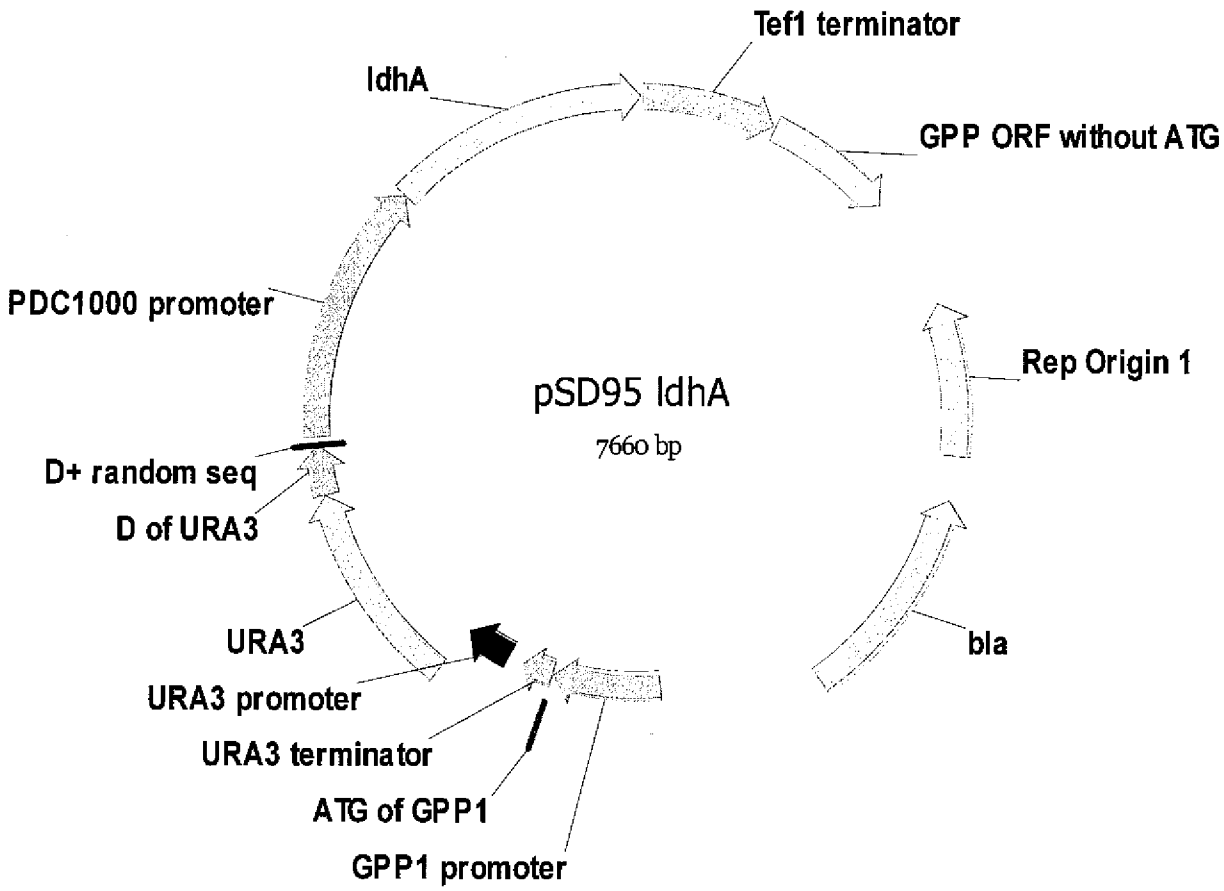

Tef1 terminator
ldhA
GPP ORF without ATG
PDC1000 promoter
Rep Origin 1
pSD95 ldhA
7660 bp
D+ random seq
D of URA3
URA3
bla
URA3 promoter
URA3 terminator
ATG of GPP1
GPP1 promoter

Figure 4

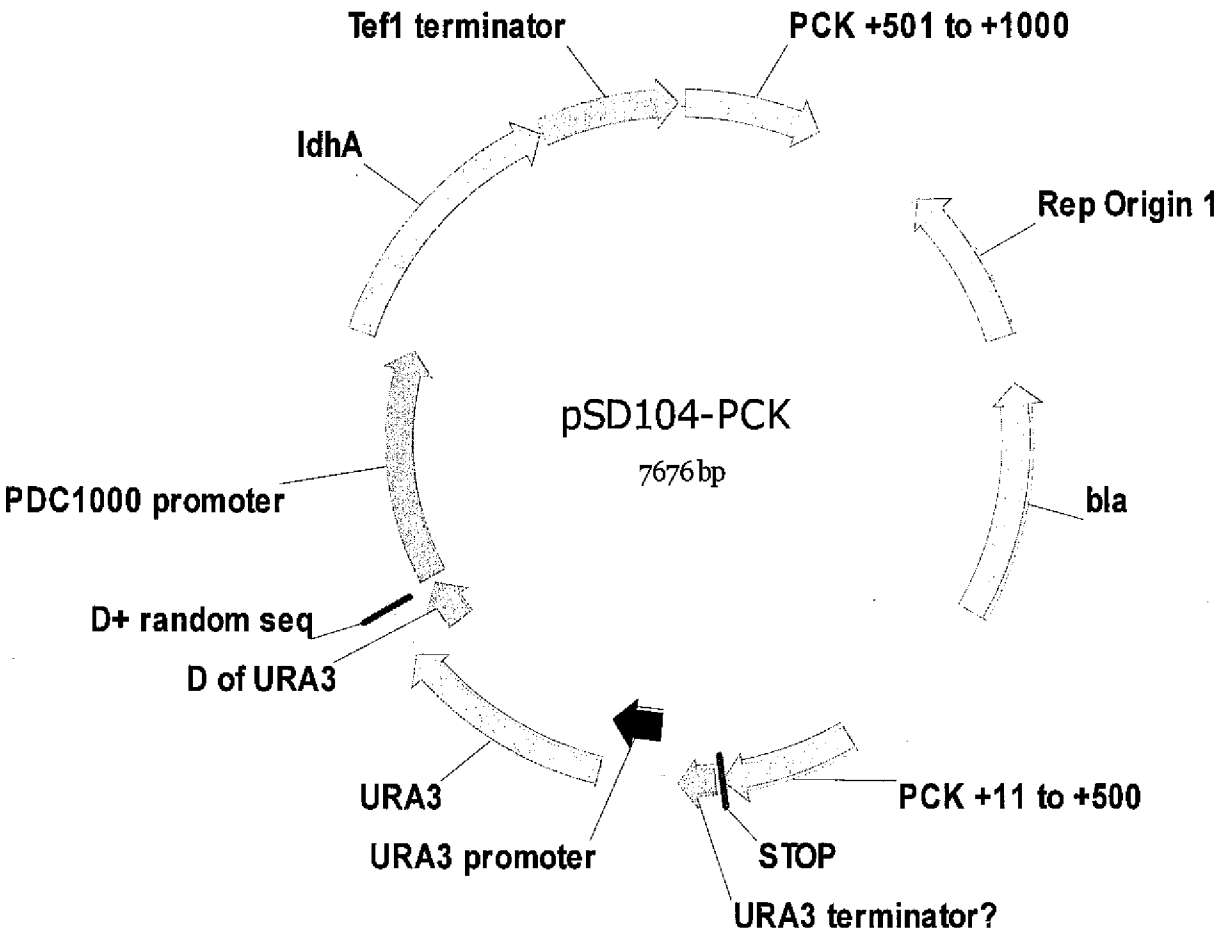

Tef1 terminator
PCK +501 to +1000
IdhA
Rep Origin 1
pSD104-PCK
7676 bp
PDC1000 promoter
bla
D+ random seq
D of URA3
URA3
PCK +11 to +500
URA3 promoter
STOP
URA3 terminator?

Figure 5

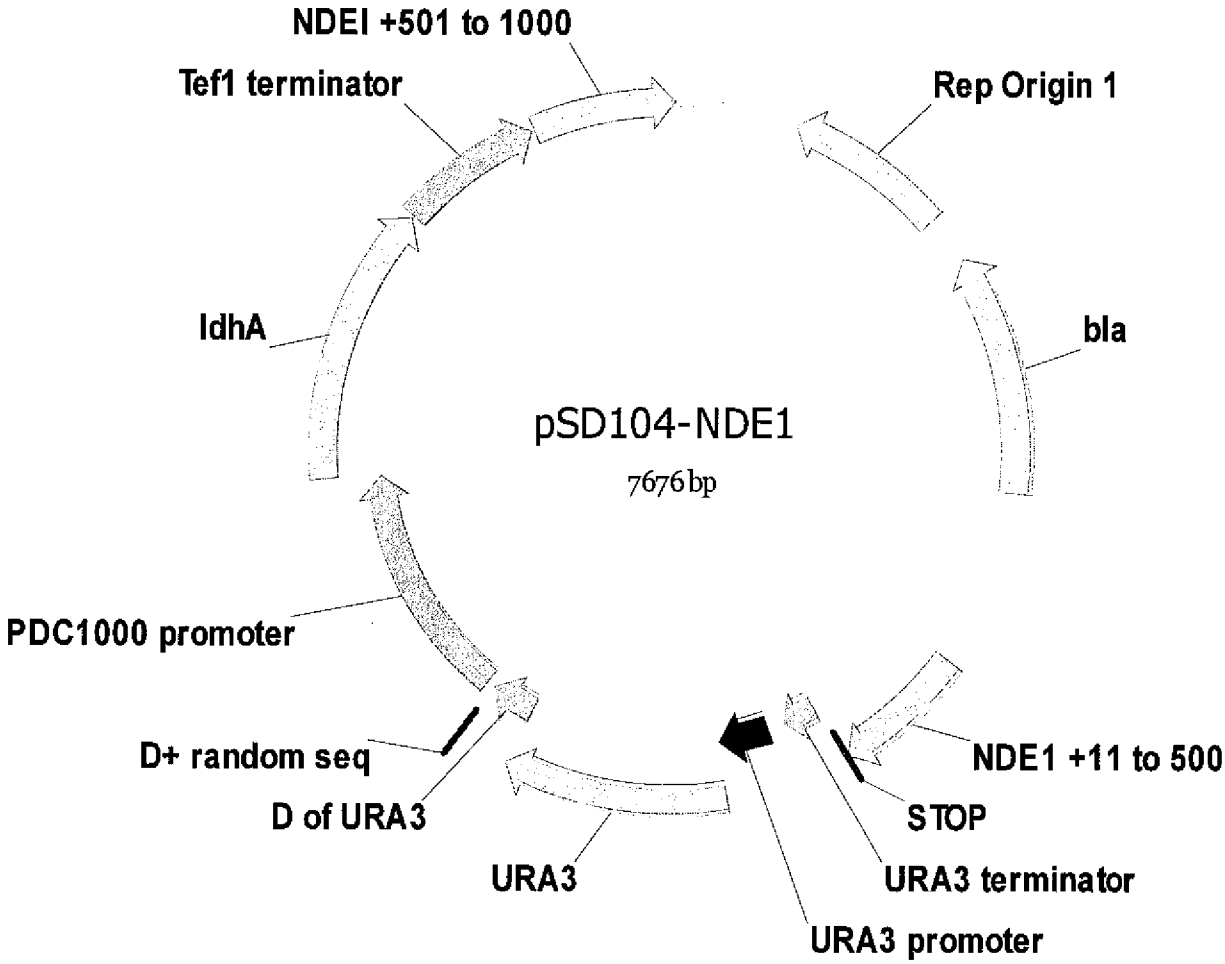
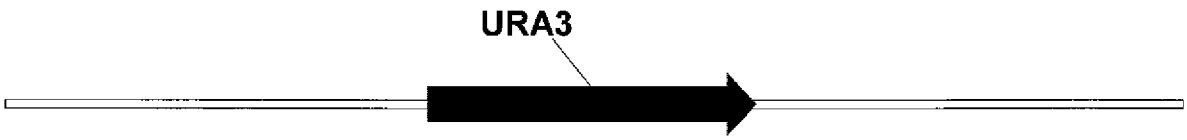

NDEI +501 to 1000
Tef1 terminator
Rep Origin 1
IdhA
bla
pSD104-NDE1
7676 bp
PDC1000 promoter
D+ random seq
D of URA3
URA3
NDE1 +11 to 500
STOP
URA3 terminator
URA3 promoter

Figure 6



wt URA3 PCR product
2883 bp

Figure 7

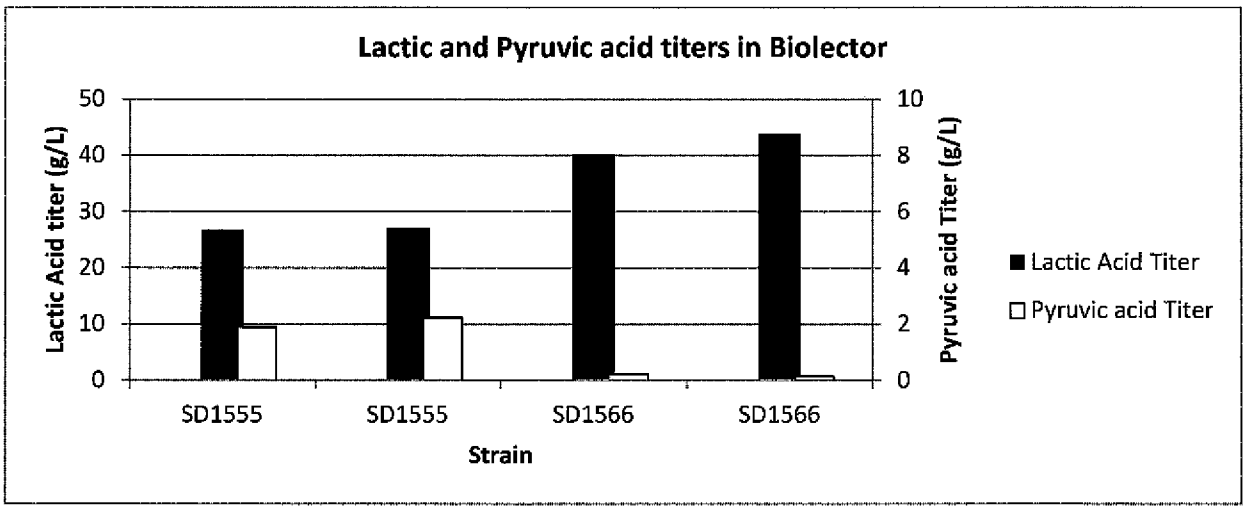
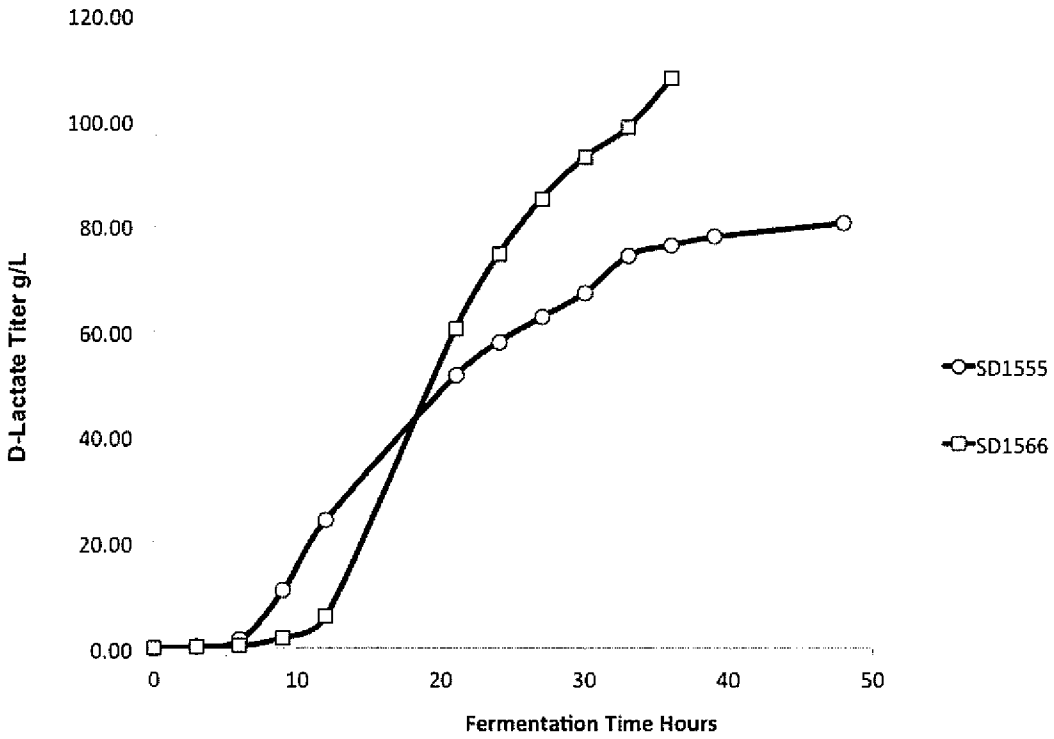

Lactic and Pyruvic acid titers in Biolector
Lactic Acid titer (g/L)
50
40
30
20
10
0
Pyruvic acid Titer (g/L)
10
8
6
4
2
0
SD1555
SD1555
SD1566
SD1566
Strain
Lactic Acid Titer
Pyruvic acid Titer

Figure 8


120.00
100.00
80.00
60.00
40.00
20.00
0.00
D-Lactate Titer g/L
0
10
20
30
40
50
Fermentation Time Hours
SD1555
SD1566