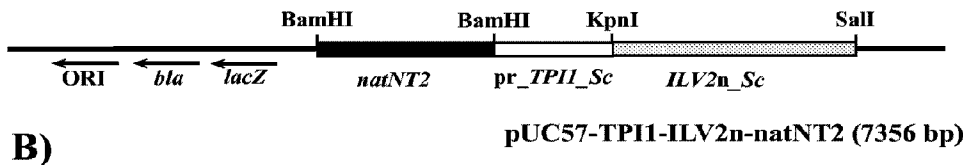
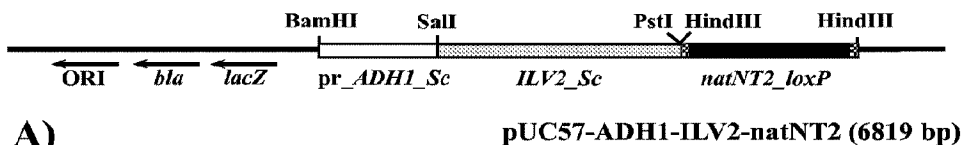




(86) Date de dépôt PCT/PCT Filing Date: 2014/12/05
(87) Date publication PCT/PCT Publication Date: 2015/06/18
(45) Date de délivrance/Issue Date: 2024/06/18
(85) Entrée phase nationale/National Entry: 2016/05/30
(86) N° demande PCT/PCT Application No.: US 2014/068699
(87) N° publication PCT/PCT Publication No.: 2015/088892
(30) Priorité/Priority: 2013/12/12 (US61/915,252)

(51) Cl.Int./Int.Cl. *C12P 7/20* (2006.01)
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(54) Titre : SOUCHE RECOMBINANTE DE SACCHAROMYCES CEREVISIAE SURPRODUCTRICES DE GLYCERINE
(54) Title: RECOMBINANT STRAIN OF SACCHAROMYCES CEREVISIAE OVERPRODUCING GLYCEROL



(57) Abrégé/Abstract:

A truncated version of *Saccharomyces cerevisiae* ILV2 gene encoding a cytosolic form of acetolactate synthase was cloned into an expression cassette under control of a strong constitutive alcohol dehydrogenase (ADH1) promoter. The plasmid was introduced into the *S. cerevisiae* strain and the recombinant strain was tested for ability to overproduce glycerol under anaerobic conditions. It was shown that the recombinant strain was characterized by increased glycerol production and decreased ethanol production under anaerobic conditions.

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

(19) World Intellectual Property
Organization
International Bureau

(43) International Publication Date
18 June 2015 (18.06.2015)



(10) International Publication Number
WO 2015/088892 A3

(51) International Patent Classification:
C12P 7/20 (2006.01)

(21) International Application Number:
PCT/US2014/068699

(22) International Filing Date:
5 December 2014 (05.12.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
61/915,252 12 December 2013 (12.12.2013) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, 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))
- as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))

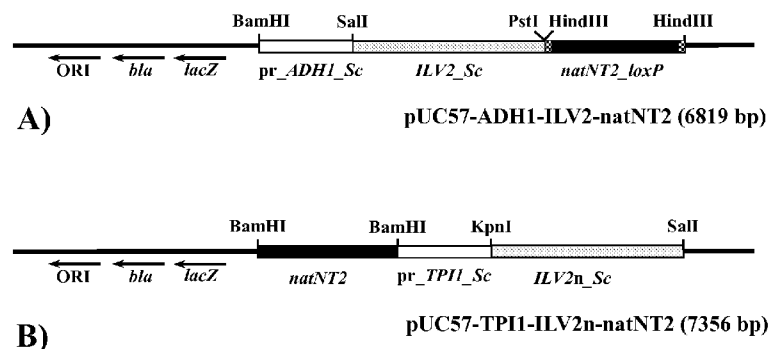
Published:

- with international search report (Art. 21(3))
- before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))

(88) Date of publication of the international search report:
13 August 2015

(54) Title: RECOMBINANT STRAIN OF *SACCHAROMYCES CEREVISIAE* OVERPRODUCING GLYCEROL

FIGURE 1



(57) Abstract: A truncated version of *Saccharomyces cerevisiae* *ILV2* gene encoding a cytosolic form of acetolactate synthase was cloned into an expression cassette under control of a strong constitutive alcohol dehydrogenase (*ADH1*) promoter. The plasmid was introduced into the *S. cerevisiae* strain and the recombinant strain was tested for ability to overproduce glycerol under anaerobic conditions. It was shown that the recombinant strain was characterized by increased glycerol production and decreased ethanol production under anaerobic conditions.

RECOMBINANT STRAIN OF *SACCHAROMYCES CEREVISIAE* OVERPRODUCING GLYCEROL

FIELD OF THE INVENTION

[0001] The present disclosure relates to modified yeast for enhanced production of glycerol, more particularly to yeast modified to overexpress a truncated *ILV2* gene in *S. cerevisiae*, resulting in an increase in glycerol production under anaerobic growth conditions.

BACKGROUND OF THE INVENTION

[0002] Glycerol (1, 2, 3-propanetriol) is used in the cosmetic, paint, automotive, food, tobacco, pharmaceutical, pulp and paper, leather and textile industries. Glycerol has primarily been recovered as a by-product of biodiesel or soap manufacturing or produced from propylene and allyl alcohol. Alternatively, glycerol has also been produced by microbial fermentation, using carbohydrate-based feedstocks. Glycerol has also been considered as a feedstock for new industrial fermentations. For example, glycerol has been fermented to 1, 3-propanediol, dihydroxyacetone and dihydroxyacetone phosphate and other value-added products.

[0003] Due to environmental concerns, the chemical synthesis of glycerol from propylene or allyl alcohol is on the decline. The glycerol obtained as a by-product from biodiesel or soap production contains a lot of impurities, which precludes its further use without additional refining.

[0004] Several processes are known for the microbial synthesis of glycerol from carbohydrates that use osmotolerant yeasts, algae and a number of bacteria. However, all these utilize aerobic conditions, so the glycerol production demands air or oxygen purging throughout the fermentation, which considerably increases production costs.

[0005] It is known that wild-type strains of *S. cerevisiae* are able to produce substantial amounts of glycerol after adding sulfites to the fermentation medium, which results in trapping of acetaldehyde at an alkaline pH. However, glycerol yield from this sulfite process is typically low, and this mode of production results in large buildup of sulfite, leading to

waste stream byproduct disposal problems. The metabolic engineering of *S. cerevisiae* strains with high yield of glycerol that accumulates under anaerobic conditions without buildup of waste byproducts would be much more efficient. However, whereas anaerobic glucose conversion to ethanol is energy positive (net yield of 2 ATP per mole of glucose), anaerobic glycerol production from glucose presents a major challenge as it is an energy-negative process (minus 2 moles of ATP per mole of glucose). This is because glucose first has to be phosphorylated twice, with no production of ATP during glycerol synthesis. This means that yeast cannot survive anaerobically when it accumulates only glycerol, as it needs energy for cell maintenance. To be able to survive, cells either need to get some oxygen for ATP production by oxidative phosphorylation, or need to accumulate some by-product, the synthesis of which is coupled to ATP production. If the cells accumulate equal amounts of glycerol and ethanol, ATP balance is zero and this ratio of glycerol to ethanol can theoretically support strict anaerobic growth. Demonstration of this hypothesis can be demonstrated through the construction of yeast strains through the use of genetic engineering that allow for the production of glycerol and ethanol under anaerobic conditions where the strains can derive sufficient ATP and NADH.

[0006] There are a number of publications that describe the increase in glycerol production by *S. cerevisiae* mutants deleted in one or several *ADH* genes coding for alcohol dehydrogenases (Drewke et al., 1990) or *PDC1* and *PDC5* genes coding for pyruvate decarboxylases (Nevoigt and Stahl, 1996). Mutants defective in alcohol dehydrogenase typically accumulate large amounts of acetaldehyde and acetic acid which are toxic to yeast cells. Mutants defective in pyruvate decarboxylase look more promising. However, they cannot grow without adding exogenous ethanol or acetate, as pyruvate decarboxylase is the only source of two-carbon compounds in cytosol that are needed mainly for lipid biosynthesis (Pronk et al., 1996). The largest amount of glycerol is accumulated by *S. cerevisiae* mutants deleted in the *TPI1* gene coding for triose phosphate isomerase (Overkamp et al., 2002). Unfortunately, such mutants fail to grow on glucose as the primary carbon source. Metabolic engineering of an alternative NADH reoxidation pathway (isocitrate lyase) has allowed

partial restoration of the growth on glucose, however the robustness of strains was low. Additionally, glucose inhibited growth of the analyzed *TPI1* strains.

[0007] Therefore, there is continuing need in the field to develop yeast that are capable of efficient glycerol production from glucose under anaerobic conditions.

BRIEF SUMMARY OF THE INVENTION

[0008] The present disclosure provides methods for increasing glycerol production by increasing expression of acetolactate synthase in recombinant yeast and genetic tools for producing said recombinant yeasts. One embodiment of the disclosure comprises recombinant nucleic acid with a truncated portion of a gene encoding a cytosol located acetolactate synthase activity, wherein said gene is operably linked to a non-native promoter to express said acetolactate synthase activity in the cytosol.

[0009] A further embodiment comprises a nucleic acid molecule with a gene encoding the protein of a cytosol located acetolactate synthase, wherein said gene is at least 75% identical to SEQ ID NO:2 and does not contain a mitochondrial targeting signal.

[0010] A further embodiment includes the recombinant nucleic acid molecule with the cytosol located acetolactate synthase activity is gene is a SEQ ID NO: 1.

[0011] One embodiment of the disclosure contains the recombinant nucleic acid molecule with an *ADH1* promoter operably linked to the truncated ILV2 gene. A further embodiment comprises a vector containing the truncated ILV2 gene operably linked to a strong constructive promoter. An additional embodiment comprises the vector including a selective marker. An even further embodiment contains the selective marker of the vector being a natNT2 gene.

[0012] One embodiment of the disclosure is a host cell includes a vector containing the truncated ILV2 gene operably linked to a strong constructive promoter. An additional embodiment comprises a host cell comprising the vector containing the truncated ILV2 gene

operably linked to a strong constructive promoter and including a selective marker. A further embodiment comprises the host cell is *S. cerevisiae* cell.

[0013] One embodiment of the disclosure is a yeast strain containing a recombinant nucleic acid molecule comprising a truncated portion of a gene encoding a cytosol located acetolactate synthase activity that is operably linked to a non-native promoter to express the acetolactate synthase activity in the cytosol.

[0014] A further embodiment includes a yeast strain with a truncated gene that does not comprise a mitochondrial targeting signal. A further embodiment includes a yeast strain comprising a truncated portion of a gene encoding a cytosol located acetolactate synthase activity that is operably linked to a non-native promoter to express the acetolactate synthase activity in the cytosol when the truncated gene is according to SEQ ID NO: 1. A further embodiment is when promoter element of this yeast strain is an *ADH1* promoter. An even further embodiment is when this yeast strain is *S. cerevisiae*.

[0015] One embodiment of this disclosure includes a method for enhancing glycerol production in yeast comprising growing the yeast strain comprising a recombinant nucleic acid molecule comprising a truncated portion of a gene encoding a cytosol located acetolactate synthase activity that is operably linked to a non-native promoter to express the acetolactate synthase activity in the cytosol anaerobically in a culture medium, under conditions that cause the yeast strain to make glycerol.

[0016] A further embodiment includes a method of growing the yeast strain comprising a recombinant nucleic acid molecule comprising a truncated portion of a gene encoding a cytosol located acetolactate synthase activity that is operably linked to a non-native promoter to express the acetolactate synthase activity in the cytosol anaerobically in a culture medium, under conditions that cause the yeast strain to make glycerol, wherein an amount of glycerol produced by said yeast strain is more than four times greater than an amount of glycerol produced by a corresponding yeast strain that does not contain said recombinant nucleic acid molecule but is otherwise identical to said yeast strain.

[0017] A further embodiment includes the yeast strain to be *S. cerevisiae*.

BRIEF DESCRIPTION OF THE DRAWINGS

[0018] FIG. 1 shows a linear scheme of recombinant plasmid pUC57-ADHI-ILV2-natNT2 (A) and a linear scheme of recombinant plasmid pUC57-natNT2 -TPI1-ILV2n (B).

[0019] FIG. 2 shows the complete sequence of recombinant plasmid pUC57-ADHI-ILV2-natNT2. Components of complete sequence are represented by font style and bold differences, as follows: pUC57 in Calibri 11 (CGGAT), ADHI in Calibri 8 (CGGAT), ILV2 in Calibri 11 Bold (CGGAT), natNT2 in Tahoma 12 Italic (CGGAT), loxP in Tahoma 12 Bold (CGGAT)

[0020] FIG. 3 shows the complete sequence of recombinant plasmid pUC57-natNT2-TPI1-ILV2n. Components of complete sequences are represented by font style and bold differences as follows: pUC57 in Calibri 11 (CGGAT), TPI1 in Calibri 8 (CGGAT); ILV2 in Calibri 11 Bold (CGGAT), natNT2 in Tahoma 12 Italic (CGGAT).

[0021] FIG. 4 shows yeast metabolism of glucose and catalytic action of acetolactate synthase to yield glycerol and ethanol.

SEQUENCE LISTING

[0022] SEQ ID NO: 1 - The nucleic acid sequence of the truncated ilv2 gene from *S. cerevisiae*.

[0023] SEQ ID NO: 2 - The amino acid sequence of the truncated ilv2 gene from *S. cerevisiae*.

[0024] SEQ ID NO: 3 - The nucleic acid sequence of the full length native ilv2 gene from *S. cerevisiae*.

[0025] SEQ ID NO: 4 - The amino acid sequence of the full length native ilv2 gene from *S. cerevisiae*.

[0026] SEQ ID NO: 5 - The nucleic acid sequence of the ADH1 promoter.

- [0027]** SEQ ID NO: 6 - The nucleic acid sequence of the TPI1 promoter.
- [0028]** SEQ ID NO: 7 - The nucleic acid sequence of the natNT2 marker.
- [0029]** SEQ ID NO: 8 - The nucleic acid sequence of loxP, used to facilitate marker rescue.
- [0030]** SEQ ID NO: 9 - The nucleic acid sequence of pUC57-ADHI-ILV2-natNT2.
- [0031]** SEQ ID NO: 10 - The nucleic acid sequence of pUC57-natNT2 -TPI1-ILV2n.
- [0032]** SEQ ID NO: 11 - Ko574 Primer used to PCR amplify truncated ILV2.
- [0033]** SEQ ID NO: 12 - Ko575 Primer used to PCR amplify truncated ILV2.
- [0034]** SEQ ID NO: 13 - Ko572 Primer used to PCR amplify ADH1 promoter.
- [0035]** SEQ ID NO: 14 - Ko573 Primer used to PCR amplify ADH1 promoter.
- [0036]** SEQ ID NO: 15 - OK19 Primer used to PCR amplify natNT2 marker for cloning into plasmid pUC57-ADHI-ILV2-natNT2.
- [0037]** SEQ ID NO: 16 - OK20 Primer used to PCR amplify natNT2 marker for cloning into plasmid pUC57-ADHI-ILV2-natNT2.
- [0038]** SEQ ID NO: 17 - LY7 Primer used to PCR amplify TPI1 promoter.
- [0039]** SEQ ID NO: 18 - LY8 Primer used to PCR amplify TPI1 promoter.
- [0040]** SEQ ID NO: 19 - LY9 primer to amplify the full length native ilv2 gene from *S. cerevisiae*.
- [0041]** SEQ ID NO: 20 - LY10 primer to amplify the full length native ilv2 gene from *S. cerevisiae*.
- [0042]** SEQ ID NO: 21 - Ko446 Primer used to amplify natNT2 for cloning into pUC57 natNT2 -TPI1-ILV2n.
- [0043]** SEQ ID NO: 22 - Ko448 Primer used to amplify natNT2 for cloning into pUC57natNT2 -TPI1-ILV2n.

[0044] SEQ ID NO: 23 - full length ILV2 gene from *Saccharomyces kudriavzevii* (The truncated version of this gene (amino acid 56- 687) has 94.5% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0045] SEQ ID NO: 24 - full length ILV2 gene from *Naumovozyma castellii* (The truncated version of this gene (amino acid 52- 684) has 85.2% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0046] SEQ ID NO: 25 - full length ILV2 gene from *Naumovozyma dairenensis* (The truncated version of this gene (amino acid 54- 685) has 83.4% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0047] SEQ ID NO: 26 - full length ILV2 gene from *Candida glabrata* (The truncated version of this gene (amino acid 41- 677) has 82.8% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0048] SEQ ID NO: 27 - full length ILV2 gene from *Torulaspora delbrueckii* (The truncated version of this gene (amino acid 53- 682) has 80.9% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0049] SEQ ID NO: 28 - full length ILV2 gene from *Kazachstania africana* (The truncated version of this gene (amino acid 51- 682) has 80.8% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0050] SEQ ID NO: 29 - full length ILV2 gene from *Kazachstania naganishii* (The truncated version of this gene (amino acid 53- 693) has 80.6% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0051] SEQ ID NO: 30 - full length ILV2 gene from *Vanderwaltozyma polyspora* (The truncated version of this gene (amino acid 46- 671) has 79.8% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0052] SEQ ID NO: 31 - full length ILV2 gene from *Zygosaccharomyces rouxii* (The truncated version of this gene (amino acid 72- 700) has 79.2% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0053] SEQ ID NO: 32 - full length ILV2 gene from *Zygosaccharomyces bailii* (The truncated version of this gene (amino acid 55- 685) has 78.5% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0054] SEQ ID NO: 33 - full length ILV2 gene from *Kluyveromyces lactis* (The truncated version of this gene (amino acid 46- 691) has 77.7% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0055] SEQ ID NO: 34 - full length ILV2 gene from *Kluyveromyces marxianus* (The truncated version of this gene (amino acid 68- 700) has 77.25% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0056] SEQ ID NO: 35 - full length ILV2 gene from *Lachancea thermotolerans* (The truncated version of this gene (amino acid 49- 678) has 76.8% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0057] SEQ ID NO: 36 - full length ILV2 gene from *Tetrapisispora phaffia* (The truncated version of this gene (amino acid 53- 688) has 76.6% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

[0058] SEQ ID NO: 37 - full length ILV2 gene from *Tetrapisispora blattae* (The truncated version of this gene (amino acid 79- 710) has 75.4% homology to the truncated ILV2 from *S. cerevisiae*= SEQ ID NO: 2.).

DETAILED DESCRIPTION OF PREFERRED EMBODIMENTS

[0059] It has been discovered that overexpression of the truncated version (deficient 5'-165 bp and lacking a mitochondrial targeting signal) (SEQ ID NO: 1) of the yeast *ILV2* gene, encoding for acetolactate synthase, strongly activates glycerol production under anaerobic conditions. One aspect of the present invention is directed to a recombinant nucleic acid

molecule formed by fusing the truncated *ILV2* gene with a strong constitutive promoter element. The source of the *ILV2* gene could be selected from, but not limited to, a eukaryotic microorganism. One suitable promoter element is the promoter of gene *ADH1* encoding alcohol dehydrogenase. The truncated *ILV2* gene, operably linked to the promoter element, is cloned into a vector. Plasmid pUC57 is one suitable vector. Preferably a selective marker is also cloned into the plasmid. Dominant selective marker natNT2, conferring resistance to antibiotic nourseothricin (NTC) for selection in yeast, is one suitable selection marker. One embodiment of the invention is directed to recombinant plasmid pUC57-ADH1-*ILV2*-natNT2 (SEQ ID NO: 9) (FIG. 1 (A): FIG. 2 shows the DNA sequence) harboring an expression cassette for overexpression of the truncated version of the *S. cerevisiae* *ILV2* gene.

[0060] The recombinant nucleic acid molecule comprising the truncated *ILV2* gene, operably linked to a promoter, is transformed into a host cell. In one preferred embodiment, a *Saccharomyces cerevisiae* strain is used as the host strain, more preferably, *S. cerevisiae* strain BY4742.

[0061] As discussed in more detail below, the novel recombinant strain BY4742/*ILV2* of *S. cerevisiae*, when grown anaerobically in a suitable medium, under conditions that cause the yeast strain to make glycerol, was shown to produce as much as 4 g of glycerol / L, which was a 4.4-fold improvement in glycerol production as compared to parental strain BY4742.

[0062] In a preferred embodiment, a nucleic acid molecule encoding the truncated *ILV2* gene encoding a protein sequence would be at least 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98%, or 99% identical to SEQ ID NO: 2.

[0063] The novel recombinant strain BY4742/*ILV2* of *S. cerevisiae* expresses a presumably cytosolic form of *ILV2*. While not wanting to be bound by any particular mode of increased glycerol production, the following is offered as a possible mode. The acetolactate synthase encoded by *ILV2* is normally transported into the mitochondria where

it is sequestered from the cytosolic pool of NAD(H) and pyruvate that is used during anaerobic fermentation. The activity of the enzyme is to catalyze the condensation of two pyruvate molecules into an acetolactate molecule with release carbon dioxide. We suggest that with the transit peptide removed, the enzyme activity is retained in the cytosol where it reduces the available pyruvate pool that ordinarily would proceed predominantly to ethanol and acetate production under anaerobic conditions. The last step in ethanol production is the conversion of acetaldehyde to ethanol by alcohol dehydrogenase with oxidation of NADH to NAD⁺, which depletes the NADH pool. Because less pyruvate is available for conversion to ethanol, less cytosolic NADH is consumed thereby allowing more of the NADH to be used upstream by NADH dependent glycerol 3-phosphate dehydrogenase, which in turns leads to increased production of glycerol-3-phosphate and its subsequent dephosphorylation to glycerol (FIG. 2). Unlike the case where ADH activity is completely eliminated, there is still reduction in toxic levels of acetaldehyde produced by the action of alcohol dehydrogenase. Since pyruvate decarboxylase is not eliminated, there still remains adequate production of cytosolic acetate from pyruvate, with the acetate being necessary for fatty acid synthesis. Accordingly, the increased expression of cytosolic acetolactate synthase is believed to reduce, but not eliminate, the cytosolic pool of pyruvate, leaving sufficient levels of this important metabolic intermediate to maintain fatty acid synthesis. This also permits increased flow of the phosphorylated 3 carbon metabolite into glycerol production due to higher NADH availability because not as much pyruvate is being shunted to ethanol via alcohol dehydrogenase.

A. DEFINITIONS

[0064] The term "gene" refers to a DNA sequence that comprises coding sequences and optionally control sequences necessary for the production of a polypeptide from the DNA sequence.

[0065] The term "recombinant DNA" molecule means a hybrid DNA sequence comprising at least two nucleotide sequences not normally found together in nature.

[0066] The term "vector" is used in reference to nucleic acid molecules into which fragments of DNA may be inserted or cloned and can be used to transfer DNA segments into a cell and capable of replication in a cell. Vectors may be derived from plasmids, bacteriophages, viruses, cosmids, and the like.

[0067] The terms "recombinant vector", "expression vector" or "construct" as used herein refer to DNA or RNA sequences containing a desired coding sequence and appropriate DNA or RNA sequences necessary for the expression of the operably linked coding sequence in a particular host organism. Prokaryotic expression vectors include a promoter, a ribosome binding site, an origin of replication for autonomous replication in a host cell and possibly other sequences, e.g. an optional operator sequence, optional restriction enzyme sites. A promoter is defined as a DNA sequence that directs RNA polymerase to bind to DNA and to initiate RNA synthesis. Eukaryotic expression vectors include a promoter, optionally a polyadenylation signal and optionally an enhancer sequence.

[0068] A polynucleotide having a nucleotide sequence "encoding a peptide, protein or polypeptide" means a nucleic acid sequence comprising a coding region for the peptide, protein or polypeptide. The coding region may be present in either a cDNA, genomic DNA or RNA form. When present in a DNA form, the oligonucleotide may be single-stranded (i.e., the sense strand) or double-stranded. Suitable control elements such as enhancers/promoters, splice junctions, polyadenylation signals, etc. may be placed in close proximity to the coding region of the gene if needed to permit proper initiation of transcription and/or correct processing of the primary RNA transcript. Alternatively, the coding region, utilized in the expression vectors of the present invention may contain endogenous enhancers/promoters, splice junctions, intervening sequences, polyadenylation signals, etc. In further embodiments, the coding region may contain a combination of both endogenous and exogenous control elements.

[0069] Promoters and enhancers consist of short arrays of DNA sequences that interact specifically with cellular proteins involved in transcription. Promoter and enhancer elements

have been isolated from a variety of eukaryotic sources including genes from yeast, insect and mammalian cells. Promoter and enhancer elements have also been isolated from viruses and analogous control elements, such as promoters, are also found in prokaryotes. The selection of a particular promoter and enhancer depends on the cell type used to express the protein of interest. The enhancer/promoter may be "endogenous" or "exogenous" or "heterologous." An "endogenous" enhancer/promoter is one that is naturally linked with a given gene in the genome. An "exogenous" or "heterologous" enhancer/promoter is one that is placed in juxtaposition to a gene by means of genetic manipulation (i.e., molecular biological techniques) such that transcription of the gene is directed by the linked enhancer/promoter. A "constitutive promoter" is an unregulated promoter that allows for continual transcription of its associated gene.

[0070] The term "expression system" refers to any assay or system for determining (e.g., detecting) the expression of a gene of interest. Those skilled in the field of molecular biology will understand that any of a wide variety of expression systems may be used.

[0071] The term "recombinant protein" or "recombinant polypeptide" as used herein refers to a protein molecule expressed from a recombinant DNA molecule. In contrast, the term "native protein" is used herein to indicate a protein isolated from a naturally occurring (i.e., a non-recombinant or wild type) source. Molecular biological techniques may be used to produce a recombinant form of a protein with identical properties as compared to the native form of the protein.

[0072] The terms "cell," "cell line," "host cell," as used herein, are used interchangeably, and all such designations include progeny or potential progeny of these designations. By "transformed cell" is meant a cell into which (or into an ancestor of which) has been introduced a nucleic acid molecule of the invention. Optionally, a nucleic acid molecule of the invention may be introduced into a suitable cell line so as to create a stably transfected cell line capable of producing the protein or polypeptide encoded by the nucleic acid molecule. Vectors, cells, and methods for constructing such cell lines are well known in the

art. The words "transformants" or "transformed cells" include the primary transformed cells derived from the originally transformed cell without regard to the number of transfers. All progeny may not be precisely identical in DNA content, due to deliberate or inadvertent mutations. Nonetheless, mutant progeny that have the same functionality as screened for in the originally transformed cell are included in the definition of transformants.

[0073] The term "operably linked" as used, herein refer to the linkage of nucleic acid sequences in such a manner that a nucleic acid molecule capable of directing the transcription of a given gene and/or the synthesis of a desired protein molecule is produced. The term also refers to the linkage of sequences encoding amino acids in such a manner that a functional (e.g., enzymatically active, capable of binding to a binding partner, capable of inhibiting, etc.) protein or polypeptide, or a precursor thereof, e.g., the pre- or prepro-form of the protein or polypeptide, is produced.

B. MATERIALS AND METHODS USED FOR EXEMPLARY EMBODIMENTS

Strains.

[0074] The *S. cerevisiae* strain BY4742 (MAT.alpha., his3.DELTA.1, leu2.DELTA.0, lys2.DELTA.0, ura3.DELTA.0; Giaever et al., 2002) was used for overexpression of the truncated version of the *ILV2* gene. *Escherichia coli* DH5 α strain (Φ 80dlacZ Δ M15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_K⁻, m_K⁺), *supE44*, *relA1*, *deoR*, Δ (*lacZYA-argF*)U169 was used for subcloning.

DNA Manipulation

[0075] Genomic DNA from *S. cerevisiae* was isolated using Wizard® Genomic DNA Purification Kit (Promega, Madison, WI, USA). Plasmid DNA from *E. coli* was isolated using Wizard® Plus SV Minipreps DNA Purification System (Promega) and High Fidelity PCR Enzyme Mix and restriction enzymes were used according to recommendation of supplier (Thermo scientific, Vilnius, Lithuania, EU). *S. cerevisiae* transformation was performed by Sambrook and Russell 2001.

Construction of Plasmid for Overexpression *ILV2*

[0076] Construction of the expression cassette pUC57-ADHI-ILV2-natNT2 for overexpression of *S. cerevisiae* gene *ILV2* encoding acetolactate synthase, lacking a mitochondrial targeting signal, was performed using the *ILV2* gene deficient in 5'-165 bp (SEQ ID NO: 1) and a strong constitutive promoter of gene *ADH1* encoding alcohol dehydrogenase. The truncated *ILV2* gene and promoter were PCR-amplified using pairs of primers Ko574 (CAA TCA ACT ATC TCA TAT ACA GTC GAC ATG GAG CCT GCT CCA AGT TTC AA) (SEQ ID NO: 11) / Ko575 (AAA CTG CAG TCA TCT ATG ACT TAA TTT TAG CC) (SEQ ID NO: 12) and Ko572 (CGC GGA TCC ATA TGG ACT TCC TCT TTT CTG) (SEQ ID NO: 13) and Ko573 (TTG AAA CTT GGA GCA GGC TCC ATG TCG ACT GTA TAT GAG ATA GTT GAT TG) (SEQ ID NO: 14). Both fragments were combined via overlap PCR using primers Ko575 and Ko572. The fused DNA fragment harboring *ADH1* promoter and truncated version of *ILV2* gene was BamHI/PstI double digested and cloned to the BamHI/PstI linearized pUC57 plasmid cloning vector. The resulting plasmid was designated as pUC57-ADHI-ILV2.

[0077] A second marker gene *natNT2*, from *Streptomyces noursei* which provides for resistance to the antibiotic nourseothricin, was PCR-amplified using primer pair OK19 (CCC AAG CTT GGC GCG CCA GAT CTA TAA CTT CGT ATA GCA TAC ATT ATA CGA AGT TAT CTT AAC TAT GCG GCA TCA GAG) (SEQ ID NO: 15) and OK20 (CCC AAG CTT GGC GCG CCA GAT CTA TAA CTT CGT ATA ATG TAT GCT ATA CGA AGT TAT CCG AGA TTC ATC AAC TCA TTG C) (SEQ ID NO: 16), and used with plasmid pRS41N (Taxis and Knop 2006) as a template.

[0078] This 1348 bp DNA fragment was HindIII digested and cloned to HindIII-linearized plasmid pUC57-ADHI-ILV2. The plasmid was transformed into *E. coli* as described above, and selection of *E. coli* transformants was performed on LB medium supplemented with nourseothricin at a concentration of 50 µg/mL. The constructed and verified plasmid was designated as pUC57-ADHI-ILV2-natNT2 (SEQ ID NO:9).

[0079] In order to overexpress the native *ILV2* gene coding for the acetolactate synthase, this target gene was fused with the strong constitutive promoter of gene *TPI1*

encoding triose phosphate isomerase. The native mitochondrial *Ilv2* protein catalyzes the first common step in isoleucine and valine biosynthesis pathway. The *TPI1* promoter and ORF of *ILV2* of *S. cerevisiae* in part with terminator sequence were amplified using pairs of primers LY7 (CGG GAT CCT GAG GGA GAC CTA ACT ACA TAG) (SEQ ID NO:17) / LY8 (TAG CGT AGA TTG TCT GAT CAT GGT ACC TTT TAG TTT ATG TAT GTG TTT TTT) (SEQ ID NO:18) and LY9 (AAA AAA CAC ATA CAT AAA CTA AAA GGT ACC ATG ATC AGA CAA TCT ACG CTA) (SEQ ID NO: 19)/ LY10 (GCG TCG ACG AAG CGT CAG ATC AGA CAC A) (SEQ ID NO: 20). Both fragments were combined using primers LY7 / LY10 by overlap PCR and subsequently cloned to the BamHI/Sall double-digested pUC57 plasmid. This plasmid was designated pUC57-TPII-ILV2n. The gene *natNT2* conferring resistance against the aminoglycoside antibiotic nourseothricin was amplified using primers Ko446 (CCG GGA TCC TCT AGA GTG ATG ACG GTG AAA ACC TCT G) (SEQ ID NO: 21)/ Ko448 (CCG GGA TCC TCT AGA CTG AGG ACA TAA AAT ACA CAC CG) (SEQ ID NO: 22) and plasmid pRS41N as a template. The amplified fragment was digested with BamHI and ligated with BamHI digested and dephosphorylated plasmids pUC57-TPI1-ILV2n. The selection of *E. coli* transformants was performed as described above. Constructed and verified plasmid was designated as pUC57-natNT2-TPI1-ILV2n (FIG. 1 (B), DNA sequence in FIG. 3).

Transformation, Selection and Characterization of Stable Transformants of *S. cerevisiae*

[0080] The constructed plasmids pUC57-ADHI-ILV2-natNT2 and pUC57-natNT2-TPI1-ILV2n were BamHI- and Sall-linearized and used to transform the *S. cerevisiae* BY4742 parental strain as described above. The transformants were selected on a solid YPD medium supplemented with nourseothricin (100 µg/mL). The selected transformants were stabilized by alternating cultivation in a non-selective media for 12-14 generations followed by shifting to a selective media containing nourseothricin. The expression system for detecting the presence of the desired plasmid construct in the genome of the stable transformants was confirmed by diagnostic PCR.

EXAMPLES

[0081] The following examples are intended to guide those skilled in the art in the practice of this invention. They should not be construed to limit the scope of the invention, which is defined by the claims.

Example 1 - Glycerol Overproduction in Transformed *S. cerevisiae*

[0082] The selected *S. cerevisiae* transformants BY4742/ILV2 and BY4742/ILV2n expressing truncated and native form of *ILV2* gene, respectively, and the parental strain BY4742 as a control, were each incubated in separate fermentation broths comprising yeast extract 5 g/L, peptone 10 g/L, D-glucose 50 g/L. Fermentation was carried out at a temperature of 30°C with limited aeration using a rotary shaker at a setting of 120 revolutions/min. An initial biomass concentration of 1 g/L was used for fermentation. On the second day of fermentation, the recombinant strain BY4742/ILV2 possessed approximately 10% growth retardation as compared to parental strain BY4742 (Table 1). The recombinant strain BY4742/ILV2 produced more than a 4-fold increase of glycerol production as compared to the parental strain, reaching 4 g of glycerol / L (Table 1). Ethanol synthesis of the recombinant strain BY4742/ILV2 was 1.8-fold reduced as compared to the parental strain (Table 1). During fermentation, biomass accumulation of strain BY4742/ILV2n was similar to that of parental strain. Strain BY4742/ILV2n produced a slight increase in the amount of glycerol (1.1 g/L) when compared to parental strain BY4742 (0.9 g/L). Ethanol synthesis of the strain was 1.3-fold reduced reaching 13 g/L (Table 1). Glucose consumption and acetate production were approximately on the same level for all analyzed strains. To prove overexpression of both versions of *ILV2* gene the specific activity of Ilv2 of the constructed strains was assayed. Specific activities of Ilv2 for strains BY4742/ILV2 and BY4742/ILV2n were increased on 46 and 59%, respectively, as compared to that of parental strain (Table 1).

TABLE 1. Biomass, glycerol, ethanol and acetic acid synthesis, glucose consumption, specific activity of Ilv2 of *S. cerevisiae* strains on the second day of 5% glucose fermentation.

Strains	Biomass (g/L)	Analyte (g/L)				Ilv2 specific activity (U/mg of protein)
		Glucose	Acetic acid	Ethanol	Glycerol	
BY4742	2.25	0.5	0.3	17.1	0.9	74.4
BY4742/ILV2	2.01	0.6	0.4	9.4	4.0	108.4
BY4742/ILV2n	2.21	0.3	0.4	13.0	1.1	118.0

Further Transformed Yeast Overproducing Glycerol

[0083] *S. cerevisiae* strains may be engineered using in part the *ILV2* gene with other genes that are involved the synthesis and degradation of glycerol to construct robust *S. cerevisiae* strains that are capable of effective glycerol production from glucose under anaerobic conditions. Strains expressing mitochondrial (native) and cytosolic *ILV2* may be generated on the background of industrial ethanol producing strain AS400. A cytosolic form of *ILV2* may be expressed in strains with altered activity of Pdc (pyruvate decarboxylase), Tpi (triosephosphate isomerase) or Adh (alcohol dehydrogenase) and their combinations.

[0084] From the foregoing it will be seen that this invention is one well adapted to attain all ends and objectives herein-above set forth, together with the other advantages which are obvious and which are inherent to the invention.

[0085] Since many possible embodiments may be made of the invention without departing from the scope thereof, it is to be understood that all matters herein set forth or shown in the accompanying drawings are to be interpreted as illustrative, and not in a limiting sense.

REFERENCES

[0086] The present application cites several references, summarized herein below. Each such citation is to aid one of ordinary skill in the art in better understanding the present invention and to find sources of sequences, recombinant techniques, tests and other routine information that would enable one of ordinary skill to practice any of numerous embodiments of the present invention.

[0087] Wang, Z., Zhuge, J., Fang, H., Prior, B., (2001) Glycerol production by microbial fermentation: A review; *Biotechnology Advances* 19:201-223.

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- [0093] Sambrook J., Russell D. (2001) Rapid isolation of yeast DNA. In: Sambrook J & Russell DW, eds. *Molecular Cloning, a Laboratory Manual*, New York: Cold Spring Harbor Laboratory Press, pp. 631-632.
- [0094] Taxis C, Knop M. (2006) System of centromeric, episomal, and integrative vectors based on drug resistance markers for *Saccharomyces cerevisiae*; Biotechniques. 40:73-78.
- [0095] Urano , et al, US Patent 8232089, Cytosolic isobutanol pathway localization for the production of isobutanol.
- [0096] Chen et al (2011) Increased isobutanol production in *Saccharomyces cerevisiae* by overexpression of genes in valine metabolism; Biotechnology for Biofuels, 4:21.
- [0097] Brat et al (2012) Cytosolic re-localization and optimization of valine synthesis and catabolism enables increased isobutanol production with the yeast *Saccharomyces cerevisiae*; Biotechnology for Biofuels, 5:65.

CLAIMS

1. A method for enhancing glycerol production in yeast comprising:
growing a yeast strain anaerobically in a culture medium, under conditions that cause the yeast strain to make glycerol,
wherein the yeast strain comprises a recombinant nucleic acid molecule comprising a truncated portion of a gene encoding a cytosol located acetolactate synthase, wherein said truncated portion is operably linked to a non-native promoter to express a portion of said acetolactate synthase in the cytosol, wherein said truncated portion does not comprise a mitochondrial targeting signal, and wherein said recombinant nucleic acid molecule comprises SEQ ID NO: 1.
2. The method of claim 1, wherein said truncated portion of a gene consists of SEQ ID NO: 1.
3. The method of claim 1 or 2, wherein said non-native promoter is an ADH1 promoter.
4. The method of any one of claims 1 to 3, wherein an amount of glycerol produced by said yeast strain is more than four times greater than an amount of glycerol produced by a corresponding yeast strain that does not contain said recombinant nucleic acid molecule but is otherwise identical to said yeast strain.
5. The method of any one of claims 1 to 4, wherein said yeast strain is *S. cerevisiae*.

FIGURE 1

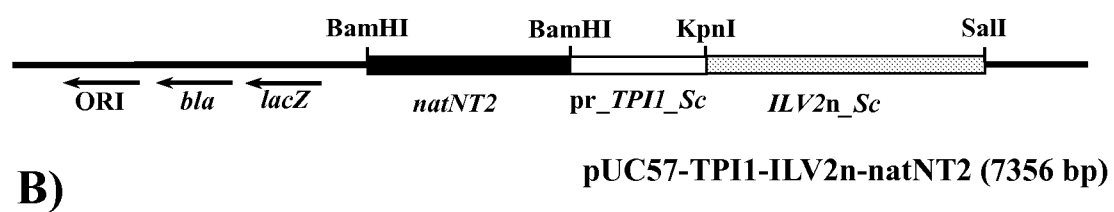
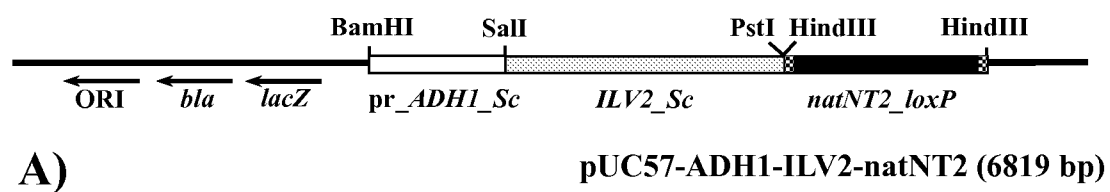


FIGURE 2: >pUC57-ADHI-ILV2-*natNT2*

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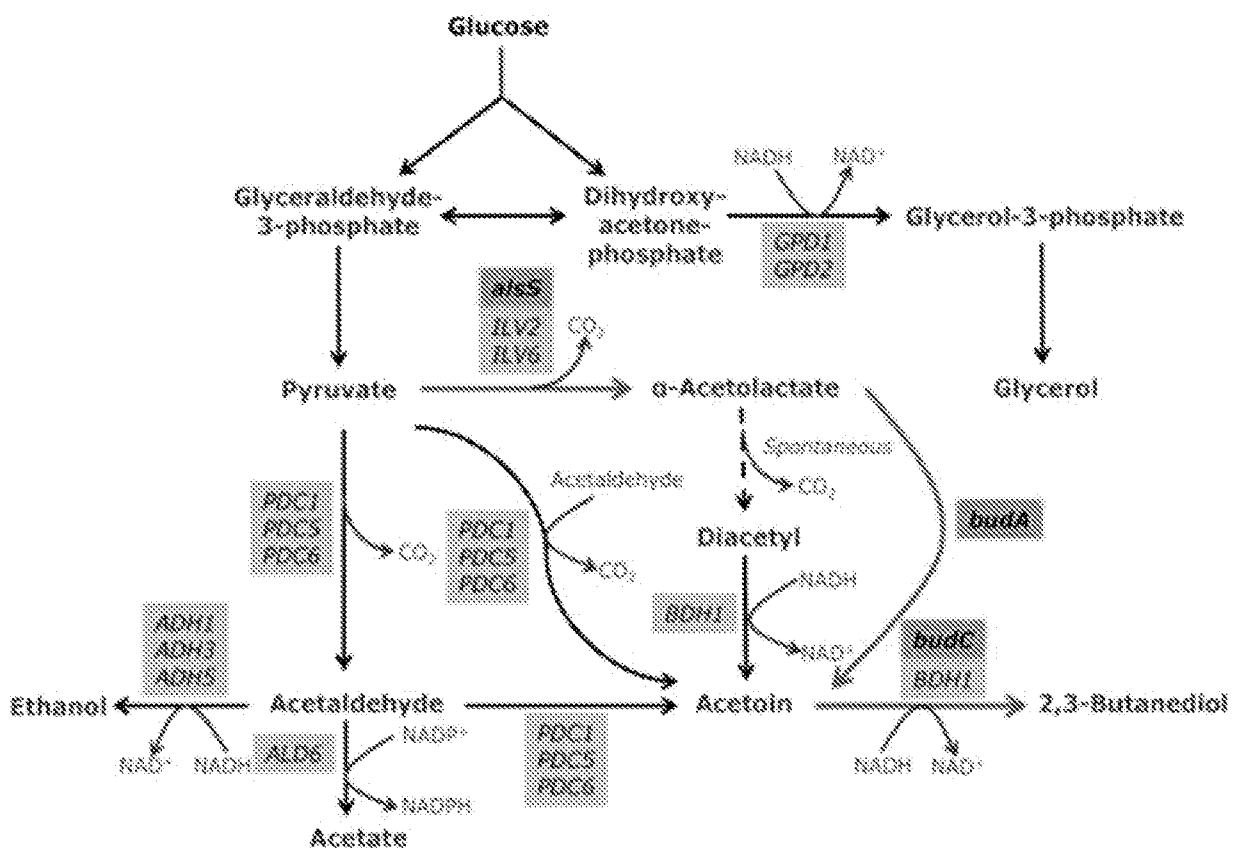
FIGURE 3: >pUC57-*natNT2*-TPI1-ILV2n

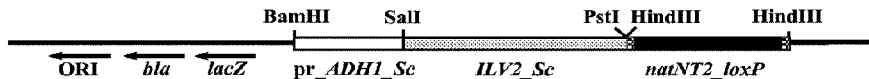
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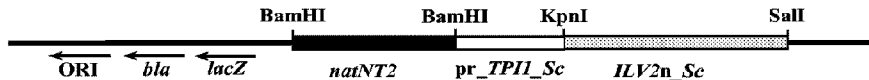
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FIGURE 4:





A) pUC57-ADH1-ILV2-natNT2 (6819 bp)



B) pUC57-TPI1-ILV2n-natNT2 (7356 bp)