HU000031212T2

(19) **HU**

(11) Lajstromszám: **E 031 212**

(13) **T2**

MAGYARORSZÁG
Szellemi Tulajdon Nemzeti Hivatala

EURÓPAI SZABADALOM
SZÖVEGÉNEK FORDÍTÁSA

(21) Magyar ügyszám: **E 09 713531**
(22) A bejelentés napja: **2009. 02. 19.**

(96) Az európai bejelentés bejelentési száma:
EP 20090713531
(97) Az európai bejelentés közzétételi adatai:
EP 2255007 A1 **2009. 08. 27.**
(97) Az európai szabadalom megadásának meghirdetési adatai:
EP 2255007 B1 **2016. 11. 02.**

(51) Int. Cl.: ***C12N 15/52*** *(2006.01)*
C12N 9/04 *(2006.01)*
C12N 9/10 *(2006.01)*
C12P 7/16 *(2006.01)*
C12N 9/88 *(2006.01)*

(86) A nemzetközi (PCT) bejelentési szám:
PCT/EP 09/001191
(87) A nemzetközi közzétételi szám:
WO 09103533

(30) Elsőbbségi adatok:
102008010121 **2008. 02. 20.** **DE**

(72) Feltaláló(k):
FESTEL, Gunter, CH-6331 Hünenberg/Zug (CH)
BOLES, Eckhard, 64293 Darmstadt (DE)
WEBER, Christian, 65779 Kelkheim am Taunus (DE)
BRAT, Dawid, 65929 Frankfurt am Main (DE)

(73) Jogosult(ak):
Butalco GmbH, 6362 Stansstad (CH)

(74) Képviselő:
KOVÁRI Szabadalmi és Védjegy Iroda Kft., Budapest

(54) **Izobutanol fermentációs úton történő előállítása élesztővel**

Az európai szabadalom ellen, megadásának az Európai Szabadalmi Közlönyben való meghirdetésétől számított kilenc hónapon belül, felszólalást lehet benyújtani az Európai Szabadalmi Hivatalnál. (Európai Szabadalmi Egyezmény 99. cikk(1))

A fordítást a szabadalmas az 1995. évi XXXIII. törvény 84/H. §-a szerint nyújtotta be. A fordítás tartalmi helyességét a Szellemi Tulajdon Nemzeti Hivatala nem vizsgálta.

SZTNH-100029367

EP 2 255 007 B1

Fermentative production of isobutanol with yeast

Description

[0001] The invention relates to a fermentative method of producing isobutanol from sugars.

[0002] Isobutanol has excellent properties as fuel. In addition, it is also a useful chemical, e.g. as base chemical for the production of other chemicals or as solvent. Today, isobutanol is predominantly produced by petrochemical methods from fossil resources. However, a much more promising prospect would be to produce it from renewable resources such as, e.g., vegetable sugars or vegetable waste. Recently, two microbial, non-fermentative methods were presented with which isobutanol can be produced from sugars (Atsumi et al., 2008; US patent application 2007/0092957). In both methods host cells were induced, by the insertion of heterologous DNA, to produce isobutanol and also other branch-chained alcohols from the metabolic intermediate pyruvate, which forms as a result of the breakdown of sugars. However, common to both described methods is that they are non-fermentative, i.e. their redox balances are not equilibrated when sugars break down into isobutanol. They can therefore only be used in complex media by simultaneous conversion of co-substrates, through the formation of by-products or under aerobic conditions. This greatly reduces the practicability of the methods and makes them economically unappealing.

[0003] In Villa K.D. et al. (J. Am. Soc. Brew. Chem, 53(2): 49-53, 1995), the amplification of the ILV3 gene or of the ILV5 gene or of both genes together in beer yeasts is

described, wherein the amplification is carried out by transfer of a plasmid which is of bacterial origin and in which either the *ILV3* gene or the *ILV5* gene or both genes together have been cloned. Furthermore, a yeast cell is described in which the genes *ILV3* or *ILV5* or both genes together are overexpressed. As a result, in the yeast cell the metabolic flow of material should be increased by the isoleucine-valine biosynthesis method in order to reduce the production of vicinal diketones and precursor compounds thereof.

[0004] One solution would be the development of a fermentative microbial process which could take place in minimal media, without co-substrates and also under anaerobic or oxygen-limited conditions. In particular, yeasts, and in particular those of the genus *Saccharomyces* such as, e.g., *Saccharomyces cerevisiae*, would be suitable as microorganisms. Interestingly, yeasts already have all the enzymes that are necessary for the formation of isobutanol from sugars. However, these enzymes are located in different compartments of the yeast cells (cytosol and mitochondria), they use different co-factors that are not or not effectively convertible into one another (NAD^+/NADH and $NADP^+$/NADPH) and the enzymes are expressed only weakly or under special conditions or have a low enzyme activity. In order to achieve an effective production of isobutanol from sugars, the metabolic pathways present would have to be modified such that with their help isobutanol could be produced in redox-neutral manner and with energy gain in the form of ATP, including under anaerobic or oxygen-limited conditions. The development of such a fermentative method of producing isobutanol from sugars is the object and aim of this invention.

[0005] Sugars such as, e.g., glucose, are broken down into pyruvate in host cells such as, e.g., yeasts, predominantly through the metabolic pathway of glycolysis. Two molecules of pyruvate are produced from one molecule of glucose. In addition, 2 energy-rich compounds are produced in the form of ATP and 2 molecules of NAD^+ are reduced to $NADH+H^+$. Pyruvate is then usually converted to ethanol either by the pyruvate decarboxylases and alcohol dehydrogenases or it is transported into the mitochondria, where it is converted into acetyl-CoA by pyruvate dehydrogenase and finally funnelled into the citric acid cycle. In addition, pyruvate can also be converted in some other reactions. One of these reaction paths is the biosynthetic pathway to the amino acid valine. On the other hand, however, valine can also be broken down i.a. into the product isobutanol. If the biosynthetic pathway and the catabolic path of valine could be shortened, isobutanol could then be produced direct from sugars via pyruvate. Such a metabolic pathway combines the enzymes which are involved in the biosynthesis of valine (from pyruvate to α-ketoisovalerate) with those which are involved in valine breakdown (from α-ketoisovalerate to isobutanol). The yeast *Saccharomyces cerevisiae* itself contains all the genes required for this. *ILV2* (*YMR108W*) (SEQ.ID. no. 1) encodes the acetolactate synthase which converts two pyruvate molecules into acetolactate. The Ilv2 enzyme (SEQ.ID. no. 2) is activated by the Ilv6 protein (=YCL009C) (SEQ.ID. no. 4). *ILV5* (*YLR355C*) (SEQ.ID. no. 5) encodes the acetohydroxy acid reductoisomerase which converts acetolactate into 2,3-dihydroxy isovalerate. *ILV3* (*YJR016C*) (SEQ.ID. no. 7) encodes the dihydroxy acid dehydratase which converts 2,3-dihydroxy isovalerate into 2-ketoisovalerate. 2-ketoisovalerate is then usually transaminated into valine; by the transaminases Bat1 (SEQ.ID. no. 10) and Bat2 (SEQ.ID. no. 12). But if this reaction is bypassed or reduced, 2-ketoisovalerate could then

also be converted by different 2-keto acid decarboxylases into isobutyraldehyde, e.g. by the enzymes Pdc1 (SEQ.ID. no. 14), Pdc5 (SEQ.ID. no. 16), Pdc6 (SEQ.ID. no. 18), Aro10 (SEQ.ID. no. 20), Thi3 (SEQ.ID. no. 22) (Dickinson et al., 1998; 2003). This direct conversion is usually impeded inter alia by the different compartmentalization of the enzymes (mitochondria, cytosol). Isobutyraldehyde can then finally be reduced to isobutanol by different alcohol dehydrogenases (Dickinson et al., 2003). These include i.a. Adh1-7 (SEQ.ID. no. 24), (SEQ.ID. no. 26), (SEQ.ID. no. 28), (SEQ.ID. no. 30), (SEQ.ID. no. 32), (SEQ.ID. no. 34), (SEQ.ID. no. 36), Sfa1 (SEQ.ID. no. 38), Ypr1 (SEQ.ID. no. 40).

[0006] However, most of the named enzymes are not strongly enough expressed or have low levels of enzyme activity for an efficient production of isobutanol from pyruvate or sugars. Another problem is the co-factor specificity and redox balance. During the reduction of the two molecules of pyruvate produced from glycolysis to isobutanol, one molecule of NADPH from the acetohydroxy acid reductoisomerase and one molecule of NADH or NADPH from the branch-chained alcohol dehydrogenases are required. However, in glycolysis, two molecules of NADH are produced from one molecule of glucose in the glyceraldehyde-3-phosphate dehydrogenase reaction. Thus there is a shortfall of NADPH and an excess of NADH. But NADH is not easily convertible into NADPH. On the other hand, the enzymes Ilv2/Ilv6, Ilv5 (SEQ.ID. no. 6) and Ilv3 (SEQ.ID. no. 8) are at least mainly located in the mitochondria of the yeast cells. The pyruvate must therefore firstly be transported into the mitochondria and finally the 2-ketoisovalerate transported out of the mitochondria into the cytosol. As transport, via membranes can often have a limiting effect on flows of material; it would therefore be desirable to shift all reactions into the cytosol. Equally

disadvantageous for an efficient production of isobutanol is that some intermediates are drawn off for other metabolic reactions on the way from the sugar to the product. This applies above all to pyruvate, which is largely converted to ethanol by the pyruvate decarboxylases and alcohol dehydrogenases. It is therefore important for a more efficient production of isobutanol to reduce or completely eliminate these secondary reactions. The object and aim of this invention is therefore to provide a fermentative method of producing isobutanol from sugars in which (i) the yeast's own set of enzymes is used for the metabolic pathway from pyruvate to isobutanol by increasing their expression or activities, i.e. without heterologous genes having to be introduced into the yeast, (ii)a) the co-factor specificity of acetohydroxy acid reductoisomerase is modified such that this enzyme preferably uses NADH instead of NADPH as a co-factor, or (ii)b) the co-factor specificity of the glyceraldehyde-3-phosphate dehydrogenase is modified such that this enzyme preferably uses $NADP^+$ instead of NAD^+ as co-factor or a heterologous NADP glyceraldehyde-3-phosphate dehydrogenase is expressed in the yeast cells, (iii) the formation of secondary products such as, e.g., ethanol is minimised and (iv) in which as many of the enzymes involved as possible are located in the cytosol of the yeast cells.

[0007] The object is achieved according to the invention by the over-expression of the enzyme activities of Ilv2 with or without its activator Ilv6, Ilv5, Ilv3, at least one 2-keto acid decarboxylase such as, e.g., Aro10, and at least one alcohol dehydrogenase which can also reduce isobutyraldehyde (preferably Adh1 or Adh6, but also Adh2-5, Sfa1, Ypr1 or others). This is carried out firstly through the exchange of the respective promoters of the corresponding genes for stronger promoters, preferably, but not exclusively,

constitutive promoters. Preferably, but not exclusively, promoter sequences are selected from HXT7, shortened HXT7, PFK1, FBA1, TPI1, PGK1, PMA1, ADH1, TDH3. Furthermore, the corresponding nucleic acid sequences of the genes are converted into codon-optimised alleles. Every amino acid is encoded at gene level by a codon. However, for most amino acids there are several different codons which code for a single amino acid. The genetic code is consequently degenerated. The preferred codon choice for a corresponding amino acid differs from organism to organism. Thus in the case of heterologously expressed genes, problems can occur if the host organism or the host cell has a very different codon usage. The gene may not be expressed at all, or only slowly. But a different codon usage can also be detected in genes of different proteins and metabolic pathways within a cell. Glycolysis genes from *S. cerevisiae* are known to be strongly expressed. They have a strongly restrictive codon usage which corresponds approximately to the quantity ratios of the corresponding tRNAs. The adaptation of the codon usage of the genes *ILV2*, (*ILV6*) (SEQ.ID. no. 3), *ILV5*, *ILV3*, one of the above-named 2-keto acid decarboxylase genes and one of the above-named alcohol dehydrogenase genes to the preferred codon usage of *S. cerevisiae* results in an improvement of the isobutanol formation rate in yeast. The preferred codon usage can be defined as described in Wiedemann and Boles (2008) for the glycolytic genes, but need not necessarily be restricted to these examples. The over-expressed, possibly codon-optimised genes can either be inserted cloned on plasmids into the yeast cells, they can be integrated into the genome of the yeast cells or they can genomically replace the naturally occurring alleles.

[0008] The present invention therefore relates in a first embodiment to a yeast cell producing isobutanol, characterised

in that the cell has an increased metabolic flow of material from pyruvate via acetolactate, 2,3-dihydroxy isovalerate, 2-ketoisovalerate, isobutyraldehyde to isobutanol, in that all genes which code for the enzymes which are involved in this conversion are over-expressed and that these genes are homologous to the said yeast cell, wherein Ilv2 catalyses the acetolactate synthase reaction from pyruvate to acetolactate, Ilv5 catalyses the acetohydroxy acid reductoisomerase reaction from acetolactate to 2,3-dihydroxy isovalerate, Ilv3 catalyses the dihydroxy acid dehydratase reaction from 2,3-dihydroxy isovalerate to 2-ketoisovalerate, a 2-keto acid decarboxylase catalyses the reaction from 2-ketoisovalerate to isobutyraldehyde, and an alcohol dehydrogenase catalyses the reaction from isobutyraldehyde to isobutanol, wherein either at least one of the promoters of these genes is exchanged for at least one stronger promoter or the nucleic acid sequences of these genes are converted into codon-optimised alleles, characterised in that the genes *ILV2*, *ILV5* and *ILV3* are overexpressed without the mitochondrial targeting sequence of the enzymes acetolactate synthase, acetohydroxy acid reductoisomerase and dihydroxy acid dehydratase or with a destroyed, inactivated mitochondrial targeting sequence, and characterised in that in addition the expression of the genes *PCC1* (SEQ.ID. no. 13), *PDC5* (SEQ.ID. no. 15) and *PDC6* (SEQ.ID. no. 17) or, respectively, the activity of the encoded enzymes is reduced or, respectively switched off.

[0009] In a preferred embodiment, the yeast cell according to the invention is characterised in that the at least one stronger promoter is a constitutive promoter.

[0010] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the promoter sequence is selected from the group consisting of

HXT7, shortened HXT7, PFK1, FBA1, TPI1, PGK1, PMA1, ADH1 and TDH3.

[0011] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the 2-keto acid decarboxylase is selected from at least one of the enzymes Aro10 or Thi3.

[0012] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the alcohol dehydrogenase is selected from at least one of the enzymes Adh1 (SEQ.ID. no. 24), Adh2 (SEQ.ID. no. 26), Adh3 (SEQ.ID. no. 28), Adh4 (SEQ.ID. no. 30), Adh5 (SEQ.ID. no. 32), Adh6 (SEQ.ID. no. 34), Adh7 (SEQ.ID. no. 36), Sfa1 (SEQ.ID. no. 38) or Yprl (SEQ.ID. no. 40).

[0013] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the over-expressed gene is over-expressed in a codon-optimised variant.

[0014] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the over-expressed gene is over-expressed in a codon-optimised variant, wherein the codon optimization is aligned with the codon usage of the highly-expressed glycolysis genes of yeast.

[0015] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the cell expresses an acetohydroxy acid reductoisomerase which has an increased specificity for NADH compared with NADPH.

[0016] In a further preferred embodiment, the yeast cell according to the invention is characterised in that this NADH-

preferring acetohydroxy acid reductoisomerase is a mutated variant of the Ilv5 enzyme of the yeast.

[0017] In a further preferred embodiment, the yeast cell according to the invention is characterised in that an NADH-preferring alcohol dehydrogenase of yeast which converts isobutyraldehyde into isobutanol is simultaneously over-expressed.

[0018] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the cell also expresses a phosphorylative glyceraldehyde-3-phosphate dehydrogenase which has an increased specificity for $NADP^+$ compared with NAD^+.

[0019] In a further preferred embodiment, the yeast cell according to the invention is characterised in that this NADP-preferring glyceraldehyde-3-phosphate dehydrogenase is heterologous to the yeast host cell.

[0020] In a further preferred embodiment, the yeast cell according to the invention is characterised in that this NADP-glyceraldehyde-3-phosphate dehydrogenase is encoded by mutated alleles of one, two or all three *TDH1-3* (SEQ.ID. no. 41), (SEQ.ID. no. 43), (SEQ.ID. no. 45) genes of yeast. In a further preferred embodiment, the yeast cell according to the invention is characterised in that this NADP-glyceraldehyde-3-phosphate dehydrogenase is expressed in a yeast cell which displays no or a reduced expression or activity of the NAD-glyceraldehyde-3-phosphate dehydrogenases.

[0021] In a further preferred embodiment, the yeast cell according to the invention is characterised in that an NADPH-preferring alcohol dehydrogenase which converts

isobutyraldehyde into isobutanol is simultaneously over-expressed.

[0022] In a further preferred embodiment, the yeast cell according to the invention is characterised in that, in addition, the Ilv6 protein is over-expressed in the same cell compartment as Ilv2.

[0023] In a further preferred embodiment, the yeast cell according to the invention is characterised in that additional mutations increase the production of isobutanol. In a further preferred embodiment, the yeast cell according to the invention is characterised in that additional mutations increase the resistance to toxic concentrations of isobutanol.

[0024] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the cell is selected from the following group: *Pichia*, *Candida*, *Hansenula*, *Kluyveromyces*, *Yarrowia* and *Saccharomyces*.

[0025] In a further preferred embodiment, the yeast cell according to the invention is characterised in that the host cell is *Saccharomyces cerevisiae*.

[0026] In a possible embodiment of the invention (see Fig. 1), the enzyme Ilv5 (acetohydroxy acid reductoisomerase) is modified such that it preferably uses NADH instead of NADPH as co-factor. At the same time, an alcohol dehydrogenase which also uses NADH as co-factor (e.g. Adh1 or Adh2-5 or Sfa1) is preferably but not necessarily over-expressed. Ilv5 catalyses the reduction of acetolactate to 2,3-dihydroxy isovalerate accompanied by the simultaneous oxidation of $NADPH+H^+$ to $NADP^+$. As a result of the glycolytic breakdown of sugars, however, no or only small quantities of NADPH form. However, NADH does

form. But NADH is not easily convertible into NADPH (Boles et al., 1993). For this reason, it would be desirable to modify the co-factor specificity of acetohydroxy acid reductoisomerase such that this enzyme prefers NADH instead of NADPH. This can be achieved by replacing specific amino acids of Ilv5, which are required for the exclusive use of NADPH, by others which also or preferably allow a use of NADH. Such amino acids are preferably but not exclusively the amino acids Arg108, Gly111, Ala112 and/or Ser113 of the non-processed precursor enzymes which can be derived by comparing the yeast-Ilv5 enzyme with the structure of the acetohydroxy acid reductoisomerase of spinach (Biou et al., 1997). Arg108 can preferably but not exclusively be converted to Met, Trp, Phe, Glu or Asp, Gly111 preferably but not exclusively into Glu or Asp, Ala112 preferably but not exclusively into Ser or Gly and Ser113 preferably but not exclusively into Glu or Asp. However, it is not to be ruled out that the exchange of further or different amino acids also leads to a modification of the co-factor specificity of Ilv5 in favour of NADH.

[0027] In another possible embodiment of the invention (see Fig. 2), the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) of the yeast is modified such that it prefers $NADP^+$ instead of NAD^+, or is replaced or supplemented by a glyceraldehyde-3-phosphate dehydrogenase which prefers $NADP^+$ compared with NAD^+. At the same time, an alcohol dehydrogenase which prefers NADPH as co-factor (e.g., Adh6 or Yprl) is preferable but not necessarily over-expressed. GAPDH is encoded e.g. in *S. cerevisiae* by the genes *TDH1* (SEQ.ID. no. 41), *TDH2* (SEQ.ID. no. 43) and *TDH3* (SEQ.ID. no. 45) and catalyses the oxidation of glyceraldehyde-3-phosphate accompanied by simultaneous phosphorylation to 1,3-diphosphoglycerate. During the glycolytic breakdown of sugars, NAD^+ is usually used as co-factor, and $NADH+H^+$ forms. NADH is not easily convertible into

NADPH (Boles et al., 1993). However, as the acetohydroxy acid reductoisomerase NADPH is used as co-factor, it would be desirable to modify the co-factor specificity of GAPDH such that this enzyme prefers $NADP^+$ instead of NAD^+. A modification of the co-factor specificity of the yeast-GAPDH can be achieved by replacing specific amino acids of Tdh1 (SEQ.ID. no. 42), Tdh2 (SEQ.ID. no. 44) and/or Tdh3 (SEQ.ID. no. 46), which are required for the exclusive use of NAD^+, by others which also or preferably allow a use of $NADP^+$. Such amino acids are preferably but not exclusively the amino acids Asp33 and/or Gly188-Pro189, which can be derived by comparing the yeast-GAPDH enzymes with the structure of $NADP^+$-preferred GAPDHs (Fillinger et al., 2000). Asp33 can preferably but not exclusively be converted to Asn, Gly, Ala or Ser, Gly188-Pro189 preferably but not exclusively into Ala-Ser, Val-Arg, Asn-Pro or Thr-Lys. However, it is not to be ruled out that the exchange of further or different amino acids also leads to a modification of the co-factor specificity of Tdh1-3 in favour of $NADP^+$. Alternatively, a heterologous GAPDH which preferably uses $NADP^+$, e.g., but not exclusively Gdp1 (SEQ.ID. no. 48) from *Kluyveromyces lactis* (Verho et al., 2002) or GapB (SEQ.ID. no. 50) from *Bacillus subtilis* (Fillinger et al., 2000), could be over-expressed in yeast. The NADP-GAPDH can be over-expressed codon-optimised in a preferred embodiment. The mutated or heterologous NADP-GAPDHs can be expressed in addition to the NAD-GAPDHs present or in a preferred version in yeast mutants with reduced or switched-off NAD-GAPDH expression or activity.

[0028] Proteins that are transported into the mitochondrial matrix are synthesised as precursor proteins in the cytosol and then transported via translocases into the mitochondrial matrix. The N-terminal presequences are split from a mitochondrial peptidase during translocation. In a preferred

embodiment, but not necessarily, the genes *ILV2*, (*ILV6*), *ILV5* or *ILV5*$^{(NADHmut.)}$ and *ILV3* are over-expressed without the mitochondrial targeting sequence of the corresponding proteins or with a broken, inactivated mitochondrial targeting sequence, with the result that the produced proteins are preferably located in the cytosol of the yeast cells (Pang and Duggleby, 1999; Omura, 2008). This can be carried out with the natural or codon-optimised alleles.

[0029] Pyruvate can be further converted by various reaction paths. The quantitatively strongest of these reaction paths is its conversion into ethanol. Pyruvate is decarboxylated to acetaldehyde and further to ethanol by the pyruvate decarboxylases (Pdcs). Pyruvate is lost to the production of isobutanol. In a preferred embodiment of the invention, but not necessarily, the flow of the pyruvate to ethanol is therefore blocked or reduced by switching off or reducing the pyruvate decarboxylase expression or activities. This is carried out e.g. by deleting or reducing the expression of the genes *PDC1*, *PDC5* and/or *PDC6*. However, as yeast requires the acetyl-CoA produced in the cytosol from acetaldehyde, this must also be made available when the pyruvate decarboxylases are completely switched off. This is carried out either (i) by an incomplete switching-off of the expression or activity of the pyruvate decarboxylases, (ii) by expression of a heterologous pyruvate-formate lyase with its activating enzyme including the over-expression of a formate dehydrogenase, (iii) by heterologous expression of a reversible mitochondrial carnitine carrier or (iv) by the introduction of spontaneous suppressor mutations. In addition, the possibility of reducing or switching off further metabolic reactions in order to intensify the flow of the intermediate metabolites to isobutanol still remains.

[0030] Furthermore, the production of isobutanol as well as the resistance to toxic concentrations of isobutanol in the recombinant yeast cells can be further increased by random mutagenesis or the "Evolutionary Engineering" or "Directed Evolution" methods (Sauer, 2001).

[0031] The present invention furthermore relates to a method for the production of isobutanol with yeast cells, comprising the provision of a yeast cell as defined above as well as bringing the yeast cell into contact with a fermentable carbon source.

[0032] In a preferred embodiment, the method according to the invention is characterised in that the fermentable carbon source is a C3-C6 carbon source.

[0033] In a further preferred embodiment, the method according to the invention is characterised in that the carbon source belongs to the group consisting of monosaccharides, oligosaccharides or polysaccharides.

[0034] In a further preferred embodiment, the method according to the invention is characterised in that the carbon source belongs to the group consisting of glucose, fructose, mannose, galactose, saccharose, maltose, xylose or arabinose.

[0035] In a further preferred embodiment, the method according to the invention is characterised in that the host cell is brought into contact with the carbon source in culture medium.

Methods

1. Strains and media

1.1 Bacteria

[0036]

- *E. coli* SURE (Stratagene)
- *E.coli* DH5α (Stratagene)

[0037] Full medium LB 1% Trypton, 0.5 % yeast extract, 0.5% NaCl, pH 7.5 (see Maniatis, 1982).
For the selection for a plasmid-encoded resistance to antibiotics 40µg/ml ampicillin was added to the medium after autoclaving. Solid nutrient media also contained 2% agar. Culture took place at 37°C.

1.2 Yeast

[0038] Strains from the CEN.PK series, industrial yeasts

-synthetic complete selective medium SC:
0.67% yeast nitrogen base w/o amino acids, pH 6.3, amino acid/nucleobase solution, carbon source in the respective given concentration

-synthetic minimal selective medium SM:
0.16% yeast nitrogen base w/o amino acid and ammonium sulphate, 0.5% ammonium sulphate, 20mM potassium dihydrogen phosphate, pH6.3, carbon source in the respective given concentration

-synthetic fermentation medium (mineral medium) SFM: (Verduyn *et al.*, 1992), pH 5.5

Salts: $(NH_4)_2SO_4$, 5g/l; KH_2PO_4, 3g/l; $MgSO_4*7H_2O$, 0.5g/l

Trace elements: EDTA, 15mg/l, $ZnSO_4*4.5$mg/l; $MnCl_2*4H_2O$, 0.1mg/l; $CoCl_2*6H_2O$, 0.3 mg/l; $CuSO_4$, 0.192 mg/l; $Na_2MoO_4*2H_2O$, 0.4 mg/l; $CaCl_2*2H_2O$, 4.5 mg/l; $FeSO_4*7H_2O$, 3 mg/l; H_3BO_3, 1 mg/l; KI, 0.1 mg/l

Vitamins: biotin, 0.05 mg/l; p-aminobenzoic acid, 0.2 mg/l; nicotinic acid, 1 mg/l; calcium pantothenate, 1 mg/l; pyridoxine-HCL, 1 mg/l; thiamine-HCL, 1 mg/l; minositol, 25 mg/l

[0039] Concentration of the amino acids and nucleobases in the synthetic complete medium (according to Zimmermann, 1975): adenine (0.08mM), arginine (0.22mM), histidine (0.25mM), isoleucine (0.44mM), leucine (0.44mM), lysine (0.35mM), methionine (0.26mM), phenylalanine (0.29mM), tryptophan (0.19mM), threonine (0.48mM), tyrosine (0.34mM), uracil (0.44mM), valine (0.49mM). L-arabinose and D-glucose were used as carbon source.

[0040] Solid full and selective media also contained 1.8% agar. Cultivation of the yeast cells took place at 30°C. The synthetic mineral medium used for the fermentations contained salts, trace metals and vitamins in the concentrations listed above and L-arabinose as carbon source. A parent solution of the trace metals and vitamins was prepared. Both solutions were sterile-filtered. Both were stored at 4°C. The pH was a decisive factor for the production of the trace metal solution. The different trace elements had to be fully dissolved in water one after the other in the order given

above. After every addition, the pH had to be adjusted to 6.0 with KOH before the next trace element could be added. At the end, the pH was adjusted to 4.0 with HCL. To avoid foaming, 200µl antifoam (Antifoam2004, Sigma) was added to the medium. As the experiments were carried out under anaerobic conditions, 2.5 ml/l of a Tween80-Ergosterol solution also had to be added to the medium after autoclaving. This consists of 16.8 g Tween80 and 0.4 g Ergosterol, which were made up to 50 ml with ethanol and dissolved therein. The solution was sterile-filtered. The salts and the antifoam were autoclaved jointly with the complete fermenter. The arabinose was autoclaved separately from the rest of the medium. After cooling of the medium, the trace elements as well as the vitamins were added.

2. Transformation

2.1 Transformation of *E. coli*

[0041] The transformation of the *E. coli* cells took place using the electroporation method according to Dower *et al.* (1988) and Wirth (1993) by means of an Easyject prima device (EQUIBO).

2.2 Transformation of *S. cerevisiae*

[0042] The transformation of *S. cerevisiae* strains with plasmid DNA or DNA fragments took place according to the lithium acetate method of Gietz and Woods (1994).

3. Preparation of DNA

3.1 Isolation of plasmid DNA from *E. coli*

[0043] The isolation of plasmid DNA from *E. coli* took place according to the alkaline lysis method of Birnboim and Doly (1979), modified according to Maniatis *et al.* (1982) or alternatively with the Qiagen "QIAprep Spin Miniprep Kit". High-purity plasmid DNA for sequencing was prepared with the Qiagen "Plasmid Mini Kit" according to the manufacturer's instructions.

3.2 Isolation of plasmid DNA from *S. cerevisiae*

[0044] The cells of a stationary yeast culture (5 ml) were harvested by centrifugation, washed and resuspended in 400µl buffer P1 (Plasmid Mini Kit, Qiagen). Following the addition of 400µl buffer P2 and $^2/_3$ volume glass beads (Ø 0.45 mm), cells were broken by 5 minutes' shaking on a Vibrax (Vibrax-VXR from Janke & Kunkel or IKA). ½ volume buffer P3 was added to the supernatant, and the whole mixed and incubated on ice for 10 min. After 10 minutes' centrifugation at 13000 rpm, the plasmid DNA was precipitated at room temperature by adding 0.75ml isopropanol to the supernatant. The DNA pelleted by centrifugation for 30 min at 13000 rpm was washed with 70% ethanol, dried and resuspended in 20µl water. 1µl of DNA was used for the transformation into *E. coli*.

3.3 Determination of the DNA concentration

[0045] The DNA concentration was spectrophotometrically measured in a wavelength range of from 240-300 nm. If the purity of the DNA, determined by the quotient E_{260nm}/E_{280nm}, is 1.8, the absorbance E_{260nm}=1.0 corresponds to a DNA concentration of 50µg dsDNA/ml (Maniatis *et al.*, 1982).

3.4 DNA amplification by means of PCR

[0046] Use of the Phusion™ High Fidelity system

The polymerase chain reaction was carried out in a total volume of 50µl with the Finnzymes "Phusion™ High Fidelity PCR System" in accordance with the manufacturer's instructions. Each batch consisted of 1-10ng DNA or 1-2 yeast colonies as synthesis template, 0.2mM dNTP-Mix, 1xbuffer 2 (contains 1.5mM $MgCl_2$), 1U polymerase and 100pmol of each of the corresponding oligonucleotide primer. The PCR reaction was carried out in a Techne thermocycler and the PCR conditions selected as follows according to the requirements:

1	1x	30 sec, 98°C	denaturation of DNA
2	30x	10 sec, 98°C	denaturation of DNA
		30 sec, 56-62°C	annealing/binding of the oligonucleotides to the DNA
		0.5-1 min, 72°C	DNA synthesis/elongation
3	1x	7 min, 72°C	DNA synthesis/elongation

[0048] After the first denaturation step, the polymerase was added ("hot start PCR"). The number of synthesis steps, the annealing temperature and the elongation time were adapted to the specific melting temperatures of the oligonucleotides used or to the size of the expected product. The PCR products were examined by an agarose gel electrophoresis and then purified.

3.5 DNA purification of PCR products

[0049] The purification of the PCR products was carried out with the "QIAquick PCR Purification Kit" from Qiagen in accordance with the manufacturer's instructions.

3.6 Gel electrophoretic separation of DNA fragments

[0050] The separation of DNA fragments measuring 0.15-20kb was carried out in 0.5-1% agarose gels with 0.5µg/ml ethidium bromide. 1xTAE buffer (40mM TRIS, 40mM ethyl acetate, 2mM EDTA) was used as gel and running buffers (Maniatis et *al.*, 1982). A lambda phage DNA cut with the restriction endonucleases *Eco*RI and *Hin*dIII served as marker. $^1/_{10}$ volume blue marker (1xTAE buffer, 10% glycerol, 0.004% bromophenol blue) was added to the DNA samples before application and made visible after separation by irradiation with UV light (254nm).

3.7 Isolation of DNA fragments from agarose gels

[0051] The desired DNA fragment was cut out from the TAE agarose gel under long-wave UV light (366 nm) and isolated with the Qiagen "QIAquick Gel Extraction Kit" in accordance with the manufacturer's instructions.

4. Enzymatic modification of DNA

4.1 DNA restriction

[0052] Sequence-specific splitting of the DNA with restriction endonucleases was carried out under the manufacturer's recommended incubation conditions for 1 hour with 2-5 U enzyme per µg DNA.

[0053] Further possible expression vectors are from the pRS303X, p3RS305X and p3RS306X series. These are integrative vectors which have a dominant antibiotic marker. Further details about these vectors are to be found in Taxis and Knop (2006).

5. Cloning of DNA fragments by *in vivo* recombination

[0054] For an *in-vivo* cloning of DNA fragments in *S. cerevisiae*, first the corresponding gene or DNA sequence is synthesised by a PCR reaction. The therein used oligonucleotides each contain in the 5` region 36-39 nucleotides comprising specific appendages which are homologous to the 5`- or 3`-flanking sequences of the integration region in the target vector. In the 3` region, the oligonucleotides contain 20-22 bases homologous to the 3` or 5` ends of the gene to be amplified. The PCR product produced was transformed into yeast together with the vector linearised and purified by restriction in the integration region. The cells were plated out onto synthetic selective medium which lacked the corresponding amino acid or nucleotide base for the selection on the auxotrophic marker of the vector. In this way, only transformants which had again formed a stable, circular plasmid due to homologous recombination of the DNA fragment in the linearised vector were obtained. The plasmids were isolated, amplified in *E. coli* and examined by subsequent restriction analysis, or by sequencing.

6. Exchange of and integration into genomic DNA

[0055] This was carried out as described in Becker and Boles (2003) and Wieczorke et al. (1999).

SZTNH-100029366

Izobutanol fermentációs úton történő előállítása élesztővel

Szabadalmi igénypontok

1. Élesztősejt, amely izobutanolt termel, azzal jellemezve, hogy emelt piruvát metabolikus anyagáramma1 rendelkezik az acetolaktát, 2,3-dihidroxi-izovalerát, 2-ketoizovalrát, izobutiraldehid, izobutanol útvonalon, ahol minden gén, amely az ebben az átalakulásában résztvevő enzimeket kódolja túlexpresszált, és gének az említett élesztősejttel homológok, ahol a 2. sz. szekvenciával rendelkező Ilv2 katalizálja az az acetolaktát szintáz reakciót piruvátból acetolaktáttá, a 6. sz. szekvenciával rendelkező Ilv5 katalizálja az acetohidroxisav reduktoizomeráz reakciót acetolaktátból 2,3-dihidroxiizovaleráttá, a 8. sz. szekvenciával rendelkező Ilv3 katalizálja a dihidroxisav dehidratáz reakciót 2,3-dihidroxiizovalerátból 2-ketoizovaleráttá, egy 2-ketosavdekarboxiláz katalizálja a 2-ketoizovalerát izobutiraldehiddé történő reakcióját, és egy alkohol-dehidrogenáz katalizálja az izobutiraldehid izobutanollá történő reakcióját, ahol ezen gének vagy legalább egy promoterje legalább egy erősebb promoterrel kicserélésre kerül vagy ezen gének nukleinsav-szekvenciáit kodon-optimalizált allélekké alakítjuk,
azzal jellemezve, hogy az ILV2, ILV5 és ILV3 gének az acetolaktát szintáz, acetohidroxisav reduktoizomeráz és dihidroxisav dehidratáz enzimek mitokondriális célzó szekvenciája nélkül, vagy egy roncsolt, inaktivált mitokondriális célzó szekvenciával kerülnek túlexpresszá-lásra, és
azzal jellemezve, hogy továbbá a 13. sz. szekvenciával rendelkező PDC1 gén, a 15. sz. szekvenciával rendelkező PDC5 gén, és a 17. sz. szekvenciával rendelkező PDC6 gén, illetve a kódolt enzimek aktivitása redukált vagy kikapcsolt.

2. Az 1 igénypont szerinti élesztősejt, azzal jellemezve, hogy a 2-ketosav dekarboxilázt legalább az alábbi enzimek valamelyikéből választjuk: a 20. sz. szekvenciával rendelkező Aro10 enzim vagy a 22. sz. szekvenciával rendelkező Thi3 enzim.

3. Az 1 igénypont szerinti élesztősejt, azzal jellemezve, hogy az alkohol dehidrogenázt legalább az alábbi enzimek valamelyikéből választjuk: a 24. sz. szekvenciával rendelkező Adh1 enzim, a 26. sz. szekvenciával rendelkező Adh2 enzim, a 28. sz. szekvenciával rendelkező Adh3 enzim, a 30. sz. szekvenciával rendelkező Adh4 enzim, a 34. sz. szekvenciával rendelkező Adh6 enzim, a 36. sz. szekvenciával rendelkező Adh7 enzim, a 38. sz. szekvenciával rendelkező Sfa1enzim vagy a 40. sz. szekvenciával rendelkező Ypr1 enzim.

4. Az 1-3. igénypontok bármelyike szerinti élesztősejt, azzal jellemezve, hogy minden említett gén kodon-optimalizált variánsként túlexpresszált.

5. Az 1-4. igénypontok bármelyike szerinti élesztősejt, azzal jellemezve, hogy a sejt egy olyan acetohidroxisav reduktoizomerázt expresszál, amelynek NADH specifitása a NADPH specifitásához képest erősebb.

6. Az 1-4. igénypontok bármelyike szerinti élesztősejt, azzal jellemezve, hogy a sejt továbbá egy foszforiláló glicerinaldehid-3-foszfát dehidrogenázt is expresszál, amelynek $NADP^+$ specifitása a $NADP^+$ specifitásához képest erősebb.

7. A 6. igénypont szerinti élesztősejt, azzal jellemezve, hogy az említett NADP-glicerinaldehid-3--foszfát dehidrogenázt egy olyan élesztősejt expresszálja, amely nem vagy csak redukált NAD-glicerinaldehid-3-foszfát dehidrogenáz expresszióval vagy aktivitással rendelkezik.

8. Az 1-7. igénypontok bármelyike szerinti élesztősejt, azzal jellemezve, hogy továbbá a 4. sz. szekvenciával rendelkező Ilv6 fehérje ugyanabban a sejtrészben kerül túlexpresszálásra, mint az Ilv2.

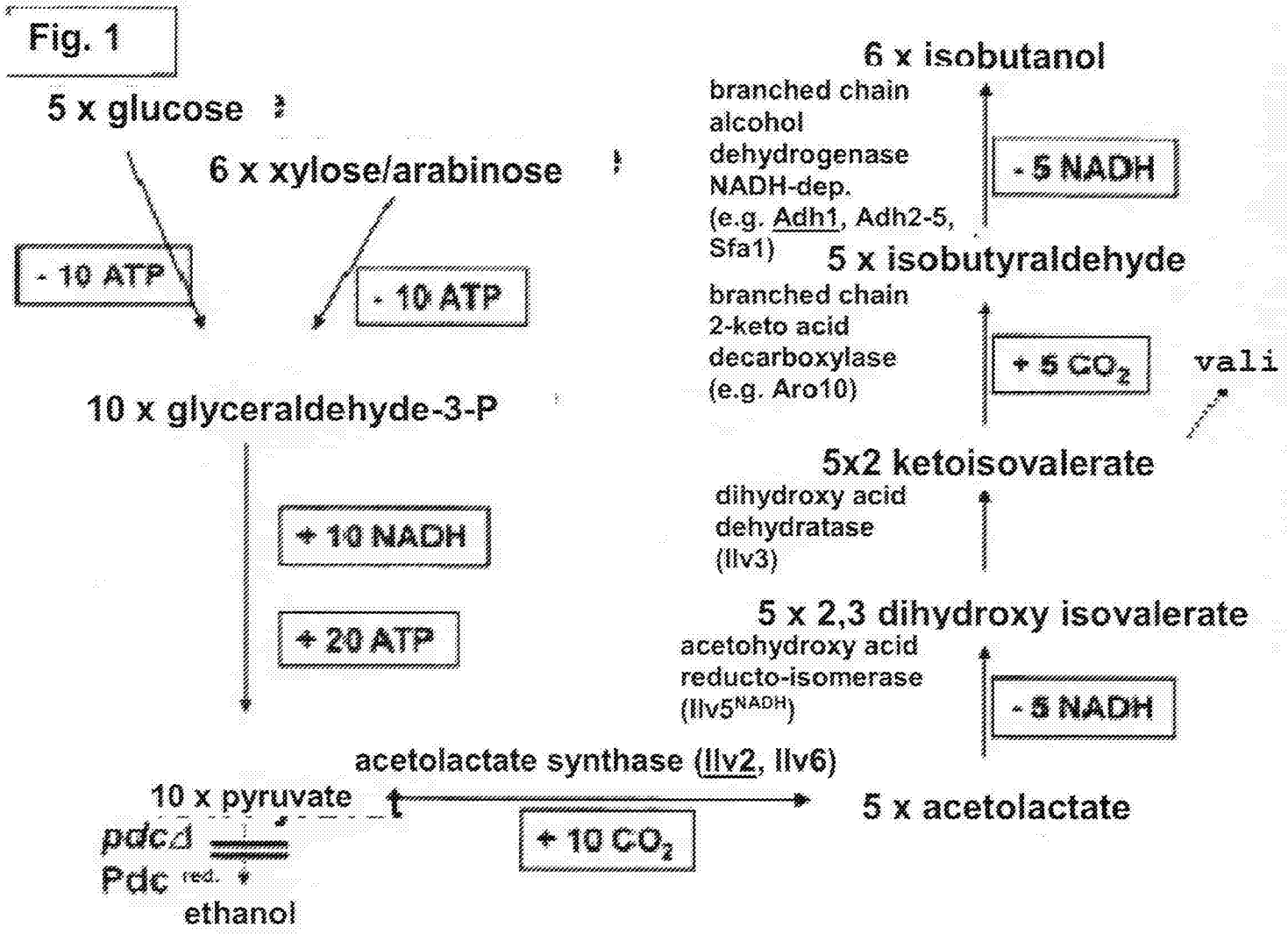
9. Az 1-8. igénypontok bármelyike szerinti élesztősejt, azzal jellemezve, hogy a sejtet az alábbi csoportból választjuk: *Pichia, Candida, Hansenula, Kluyveromyces, Yarrowia* és *Saccharomyces*.

10. A 9. igénypont szerinti élesztősejt, azzal jellemezve, hogy a gaztasejt *Saccharomyces cerevisiae*.

11. Eljárás izobutanol előállítására élesztősejtekkel, amelynek során egy az 1-10. igénypontok bármelyike szerinti élesztősejtet állítunk elő, valamint az élesztősejtet egy fermentálható szénhidrátforrással hozzuk kölcsönhatásba.

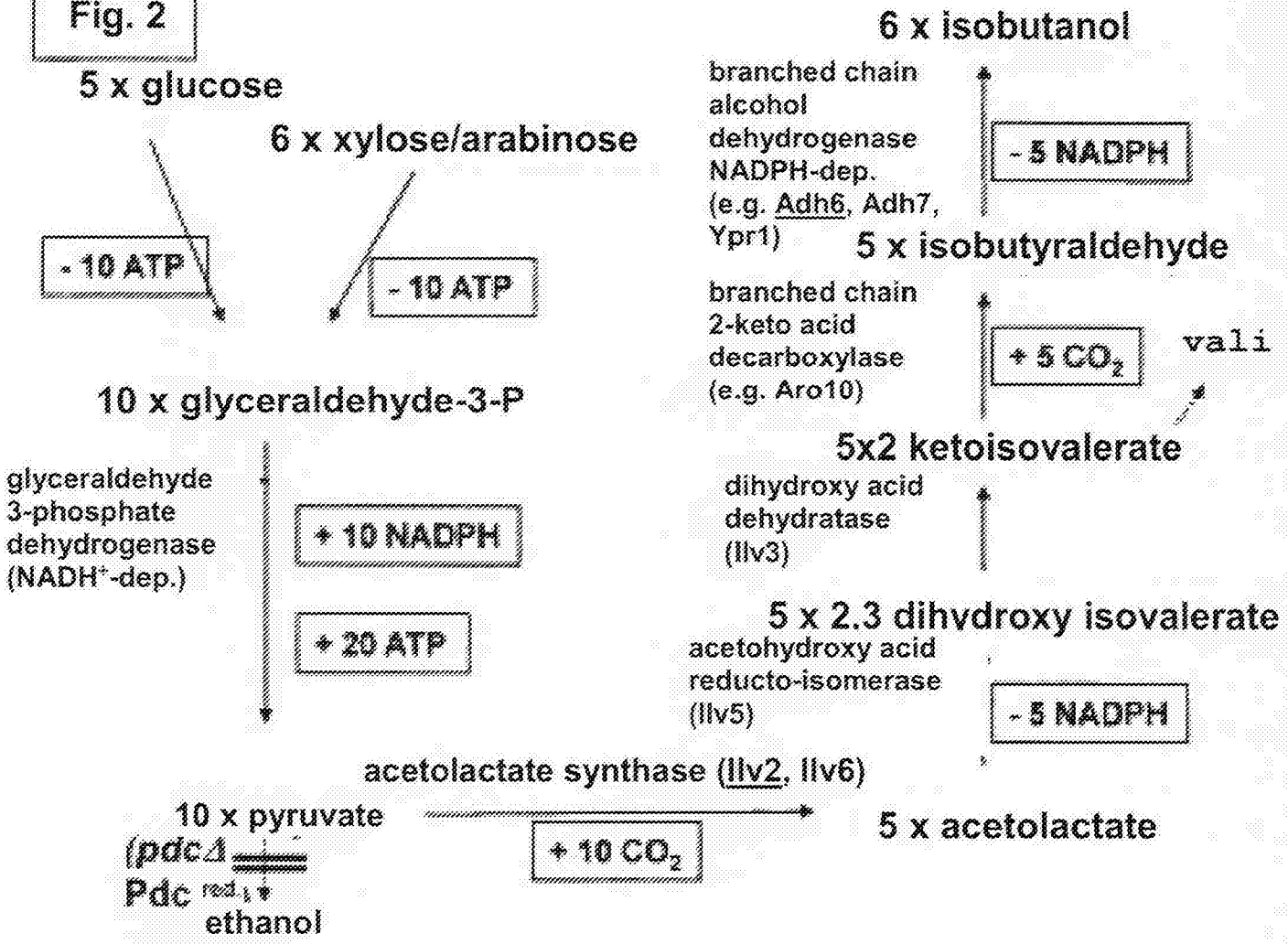
A meghatalmazott

Fig. 1



SZTNH-10002938

Fig. 2


5 x glucose
6 x xylose/arabinose
- 10 ATP
- 10 ATP
10 x glyceraldehyde-3-P
glyceraldehyde 3-phosphate dehydrogenase (NADH+-dep.)
+ 10 NADPH
+ 20 ATP
6 x isobutanol
branched chain alcohol dehydrogenase NADPH-dep. (e.g. Adh6, Adh7, Ypr1)
- 5 NADPH
5 x isobutyraldehyde
branched chain 2-keto acid decarboxylase (e.g. Aro10)
+ 5 CO2
vali
5x2 ketoisovalerate
dihydroxy acid dehydratase (Ilv3)
5 x 2.3 dihydroxy isovalerate
acetohydroxy acid reducto-isomerase (Ilv5)
- 5 NADPH
acetolactate synthase (Ilv2, Ilv6)
10 x pyruvate
(pdc Δ
Pdc red
ethanol
+ 10 CO2
5 x acetolactate