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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
17 April 2014 (17.04.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/057036 A1

(51) International Patent Classification:
C12N 15/52 (2006.01) *C12P 7/52* (2006.01)

(21) International Application Number:
PCT/EP2013/071163

(22) International Filing Date:
10 October 2013 (10.10.2013)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
12188198.1 11 October 2012 (11.10.2012) EP

(71) Applicant: **TECHNICAL UNIVERSITY OF DENMARK** [DK/DK]; Anker Engelunds Vej 1, Building 101 A, DK-2800 Kgs. Lyngby (DK).

(72) Inventors: **BORODINA, Irina**; Anker Engelunds Vej 1, Building 101 A, DK-2800 Kgs. Lyngby (DK). **KILDEGAARD, Kanchana Rueksomtawin**; Anker Engelunds Vej 1, Building 101 A, DK-2800 Kgs. Lyngby (DK). **FÖRSTER, Jochen**; Anker Engelunds Vej 1, Building 101 A, DK-2800 Kgs. Lyngby (DK). **ÖBERG, Fredrik**; Anker Engelunds Vej 1, Building 101 A, DK-2800 Kgs. Lyngby (DK).

(74) Agent: **SMART, Peter**; Beck Greener, Fulwood House, 12 Fulwood Place, London, London WC1V 6HR (GB).

(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, LT, 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, 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:

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

— *with sequence listing part of description (Rule 5.2(a))*

WO 2014/057036 A1

(54) Title: GENETICALLY ENGINEERED YEAST

(57) Abstract: A genetically modified *Saccharomyces cerevisiae* comprising an active fermentation pathway producing 3-HP expresses an exogenous gene expressing the aminotransferase YhxA from Bacillus cereus AH1272 catalysing a transamination reaction between beta-alanine and pyruvate to produce malonate semialdehyde. The yeast may also express a 3-hydroxyisobutyrate dehydrogenase (HIBADH) and a 3-hydroxypropanoate dehydrogenase (3-HPDH) and aspartate 1-decarboxylase. Additionally the yeast may express pyruvate carboxylase and aspartate aminotransferase.

Genetically Engineered Yeast

The present invention relates to genetically engineered yeasts and their use in methods for production of 3-hydroxypropionic acid (3HP).

3HP is a platform chemical, which can be converted to acrylic acid, 1,3-propandiol, malonic acid, and other valuable products. Acrylic acid-derived products include superabsorbent polymers used in baby diapers and incontinence products, various plastics, coatings, adhesives, elastomers, and paints. Currently acrylic acid is derived from propylene, a by-product of ethylene and gasoline production. Establishment of 3HP production from glucose or other renewable carbon source would provide a biosustainable alternative to acrylic acid production from fossil resources. Several methods for production of 3HP from glucose have been described. The specific teachings however primarily use the bacterium *Escherichia coli* as the host. The present invention uses yeast as the host for 3HP production. This allows executing the process at low pH and thus makes it overall more economical.

US2010/0136638 describes, in general terms, production of 3-HP in micro-organisms including yeast by biocatalysis from beta-alanine. It is said that beta-alanine can be synthesized in cells from alpha-alanine by an enzyme having alanine 2,3-aminomutase activity, and sequences are given for relevant enzymes.

Also disclosed are methods of producing 3-HP from beta-alanine using beta-alanine/pyruvate aminotransferase (BAPAT) sequences. Transformed cells having BAPAT activity, which allows the cell to convert beta-alanine to 3-HP through a malonate semialdehyde intermediate, are disclosed.

Although the possibility of conducting such work in yeast is mentioned, there is no practical demonstration of this. We have found that enzymes in this pathway that are effective in E. coli are not effective in *Saccharomyces cerevisiae*. In particular, according to US2010/0136638 enzymes having BAPAT activity can be obtained from *Pseudomonas putida* or *Pseudomonas aeruginosa*. However, we have found that genes encoding these enzymes are not effective in *S. cerevisiae*.

Malonate semialdehyde (or malonic semialdehyde or 3-oxopropanoic acid) is a key intermediate in one pathway leading to 3HP, but many different routes to its production are possible.

US2012135481 describes a 3HP producing pathway in yeast including genes encoding gabT, 3-HPDH and HIBADH and others. However, other and better 3HP producing yeasts are needed.

We have now found that 3HP production from beta-alanine was obtained in yeast *S. cerevisiae* when an uncharacterized aminotransferase *yhxA* from *Bacillus cereus* AH1272 was heterologously expressed. The amino acid sequence of the said yhxA encoded aminotransferase is set out in SEQ ID NO1 and the DNA sequence is set out in SEQ ID NO2. SEQ ID NO2 is codon-optimized for *S. cerevisiae*.

It is our belief that the said aminotransferase *YhxA* from *Bacillus cereus* AH1272 catalyzes a transamination reaction between beta-alanine and pyruvate leading to L-alanine and malonic semialdehyde, in which case the enzyme would be beta-alanine-pyruvate aminotransferase E.C. 2.6.1.18 (BAPAT)rather than a gabT (E.C. 2.6.1.19).

US2012/0135481 discloses genetically modified yeast cells comprising an active 3-HP fermentation pathway including the BAAT gene (beta alanine amino transferase - EC 2.6.1.19) which catalyzes the conversion of [beta]-alanine to

malonate semialdehyde. BAAT here is therefore synonymous with naturally occurring or genetically modified gabT. However, successful production of 3-HP by this method is not shown.

WO2005/118719 discloses, but does not demonstrate the effectiveness of, methods of producing 3-HP from beta-alanine using beta-alanine/pyruvate aminotransferase (BAPAT) sequences from any organism in a yeast cell. Identified sources for BAPAT here include *Pseudomonas, Arabidopsis, rat* and *Xenopus*. As mentioned above, we have established that a BAPAT genes from *Pseudomonas* is not effective in *S. cerevisiae*.

The Uniprot entry for yhxA provides a sequence but does not identify the enzyme as being a BAPAT.

Accordingly, the present invention now provides a genetically modified yeast cell comprising an active fermentation pathway producing 3-HP, wherein the cell comprises and expresses an exogenous gene coding for the production of an enzyme having at least 80% identity with SEQ ID NO: 1 and catalysing a transamination reaction between beta-alanine and pyruvate to produce malonate semialdehyde.

Preferably, said yeast also expresses 3-hydroxyisobutyrate dehydrogenase (HIBADH), suitably from *Pseudomonas aeruginosa*, *P. putida*, *Bacillus cereus*, or *Candida albicans* and/or 3-hydroxypropanoate dehydrogenase (3-HPDH), optionally from *Metallosphaera sedula*, *Sulfolobus tokadaii* or *E. coli*.

To enable the synthesis of 3-hydroxypropionic acid directly from glucose is it preferred in addition to reconstructing pathway from beta-alanine to 3-hydroxypropionic acid to express heterologous aspartate 1-decarboxylase, preferably from insect, preferably red flour beetle (*Tribolium castaneum*). To further increase the flux

towards 3-hydroxypropinic acid it is preferred to overexpress pyruvate carboxylase and or PEP carboxylase and aspartate aminotransferase. Additionally deletion of pyruvate decarboxylase activity (*PDC1*, *PDC5*, *PDC6*) or alcohol dehydrogenase (*ADH*) activity would allow anaerobic fermentation without formation of ethanol as a by-product.

Strains according to the invention can be evolved using adaptive laboratory evolution methods to improve glucose tolerance, remove acetate dependence and increase 3HP production.

The yeast is preferably *S. cerevisiae* but may be *Saccharomyces kluyveri*, *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Debaryomyces hansenii*, *Cyberlindnera jadinii*, *Rhodotula minuta*, *Rhodotula glutinis*, *Torulaspora delbrueckii*, *Pichia stipitis*, *Pichia pastoris*, *Kluyveromyces lactis*, *Kluyveromyces marxianus*, or other yeast.

Yeast strains suitable for modification according to the invention can be selected for their tolerance to growth in the presence of 3HP.

The amino acid sequence of the native *yhxA* expression product of *B. cereus* AH1272 and the DNA sequence coding for it can be modified for use in this invention in various ways. First, the DNA sequence can be codon optimised for expression in the appropriate yeast. Secondly, the amino acid sequence may be modified by deletion, addition, or substitution of amino acids whilst not interfering with, or indeed whilst increasing, the enzyme activity. Such a modified enzyme may have at least 80%, more preferably at least 85%, or 90% or 95% homology with the native amino acid sequence.

The invention includes a method for the production of 3HP comprising culturing a yeast cell of the invention, and optionally recovering 3HP from the culture. The culture may

be conducted in a culture medium including beta-alanine or a source thereof other than said yeast. Said source may be another micro-organism. However, the yeast of the invention may be engineered to produce beta-alanine, e.g. from L-aspartate, suitably by incorporating exogenous genes producing aspartate-1-decarboxylase (EC 4.1.1.11) or glutamate decarboxylase (EC 4.1.1.15) or from L-alanine by 2,3-alanine aminomutase. Due to its role in the synthesis of pantothenate, aspartate 1-decarboxylase is also known as PanD. A gene for this enzyme is not present in the genome of wild-type *S. cerevisiae*.

We have found that superior results are obtained using certain exogenous PanD genes encoding aspartate-1-decarboxylase compared to others. In particular, we have found that PanD genes from insects, especially flour beetles, more especially red flour beetle (*Tribolium castaneum*), provides better production titres and better yields of 3-HP compared to bacterial PanD genes.

Preferably, the production of 3HP by said yeast is such that at least 100mg of 3HP per litre of culture medium is produced or is recovered from said culture medium, more preferably at least 200, or 300, or 400 or 500 or 1000 or 2000 or 14000 mg/l.

The invention will be further described and illustrated in the following non-limiting examples, in which reference will be made to the following Tables.

Table 1. Primers

Primer name	Primer sequence, 5'->3'	
pE1_fw	AGTGCAGGU GGTACCAAAACAATG	SEQ ID NO26
pE1_rv	CGTGCGAU GTCGACTCA	SEQ ID NO27
EcRutE_U1_fw	AGTGCAGGU AAAACAATGAACGAAGCCGTTAG	SEQ ID NO28
EcRutE_U1_rv	CGTGCGAU TTACAACAGCCCGCAG	SEQ ID NO29
EcYdfG_U1_fw	AGTGCAGGU AAAACAATGATCGTTTTAGTAACTGG	SEQ ID NO30
EcYdfG_U1_rv	CGTGCGAU TTACTGACGGTGGACATTC	SEQ ID NO31

ScGabT_U1_fw	AGTGCAGGU AAAACAATGTCTATTTGTGAACAATACTAC	SEQ ID NO32
ScGabT_U1_rv	CGTGCGAU TCATAATTCATTAACTGATTTGG	SEQ ID NO33
GeneArt_1U_fw	AGTGCAGGU GCATGGTACCAAAACAATG	SEQ ID NO34
GeneArt_1U_rv	CGTGCGAU ATGAGGCCCAGGTCGAC	SEQ ID NO35
PTEF1_fw	ACCTGCACU TTGTAATTAAAACTTAG	SEQ ID NO36
PTEF1_rv	CACGCGAU GCACACACCATAGCTTC	SEQ ID NO37
ydfG_KpnI_express_fw	AAAA GGTACC ATGATCGTTTTAGTAACTGG	SEQ ID NO38
ydfG_PacI_express_rv	AAAA TTAATT AATTACTGACGGTGGACATTC	SEQ ID NO39
EcPAND_U1_fw	AGTGCAGGU AAAACAATGATCAGAACCATG	SEQ ID NO40
EcPAND_U1_rv	CGTGCGAU TCAAGCAACTTGAACTGG	SEQ ID NO41
CgPAND_U1_fw	AGTGCAGGU AAAACAATGTTGAGAACC	SEQ ID NO42
CgPAND_U1_rv	CGTGCGAU TCAAATGGATCTAGAAGTC	SEQ ID NO43
RnGAD1_U1_fw	AGTGCAGGU AAAACAATGGCTTCTTCTACTC	SEQ ID NO44
RnGAD1_U1_rv	CGTGCGAU TCACAAATCTTGACCCAATC	SEQ ID NO45
ScGAD1_U1_fw	AGTGCAGGU AAAACAATGTTACACAGGCACGGTTC	SEQ ID NO46
ScGAD1_U1_rv	CGTGCGAU TCAACATGTTCCTCTATAGTTTCTC	SEQ ID NO47
EcGAD1_U1_fw	AGTGCAGGU AAAACAATGGACCAGAAGCTGTTAAC	SEQ ID NO48
EcGAD1_U1_rv	CGTGCGAU TCAGGTGTGTTTAAAGCTG	SEQ ID NO49
pE2_fw	ATCTGTCAU GGTACCAAAACAATG	SEQ ID NO60
pE2_rv	CACGCGAU GTCGACTCA	SEQ ID NO61
EcYdfg_U2_fw	ATCTGTCAU AAAACAATGATCGTTTTAGTAACTGGAG	SEQ ID NO62
EcYdfg_U2_rv	CACGCGAU TTACTGACGGTGGACATTC	SEQ ID NO63
PTEF1_fw	ACCTGCACU TTGTAATTAAAACTTAG	SEQ ID NO64
PPGK1_rv	ATGACAGAU TTGTTTTATATTTGTTG	SEQ ID NO65
TcPAND_U1_fw	AGTGCAGGU AAAACAATGCCAGCTACTGGTG	SEQ ID 70
TcPAND_U1_rv	CGTGCGAU TCACAAATCGGAACCCAATC	SEQ ID 71
ScPYC1_U1_fw	AGTGCAGGU AAAACA ATGTCGCAAAGAAAATTCG	SEQ ID 72
ScPYC1_U1_rv	CGTGCGAU TCATGCCTTAGTTTCAACAG	SEQ ID 73
ScPYC2_U2_fw	ATCTGTCAU AAAACA ATGAGCAGTAGCAAGAAATTG	SEQ ID 74
ScPYC2_U2_rv	CACGCGAUTTACTTTTTTTGGGATGGG	SEQ ID 75
ScAAT2_U1_fw	AGTGCAGGU AAAACA ATGTCTGCCACTCTGTTCA	SEQ ID 76
ScAAT2_U1_rv	CGTGCGAU TTACAATTTAGCTTCAATAGTATAG	SEQ ID 77

Table 2. Intermediate plasmids

Plasmid name	Parent plasmid	Synthetic gene sequence cloned
pE1-PpBAPAT	pE1	SEQ ID NO4
pE1-PaHIBADH	pE1	SEQ ID NO6
pE1-CaHIBADH	pE1	SEQ ID NO8
pE1-PpHIBADH	pE1	SEQ ID NO10
pE1-BcHIBADH	pE1	SEQ ID NO12
pE1-MsHPDH	pE1	SEQ ID NO14
pE1-StMSR	pE1	SEQ ID NO16
pE1-CaGabT	pE1	SEQ ID NO18
pE2-MsHPDH	pE2	SEQ ID NO14

Table 3. Primers and templates used to generate gene fragments for USER cloning by PCR

Fragment name	Gene	Fw_primer	Rv_primer	Template DNA
PaHIBADH<-	3-hydroxyisobutyrate dehydrogenase from *Pseudomonas aeruginosa*	pE1_fw	pE1_rv	pE1-PaHIBADH
CaHIBADH<-	3-hydroxyisobutyrate dehydrogenase from *Candida albicans*	pE1_fw	pE1_rv	pE1-CaHIBADH
BcHIBADH<-	3-hydroxyisobutyrate dehydrogenase from *Bacillus cereus*	pE1_fw	pE1_rv	pE1-BcHIBADH
PpHIBADH<-	3-hydroxyisobutyrate dehydrogenase from *Pseudomonas putida*	pE1_fw	pE1_rv	pE1-PpHIBADH
MsHPDH<-	3-hydroxypropanoate dehydrogenase from *Metallosphaera sedula*	pE1_fw	pE1_rv	pE1-MsHPDH
StMSR<-	3-hydroxypropanoate dehydrogenase from *Sulfolobus tokadaii*	pE1_fw	pE1_rv	pE1-StMSR
EcRutE<-	3-hydroxypropanoate dehydrogenase from *Escherichia coli*	EcRutE_U1_fw	EcRutE_U1_rv	gDNA of *E. coli* SEQ ID NO20
EcYdfG<-	3-hydroxypropanoate dehydrogenase from *Escherichia coli*	EcYdfG_U1_fw	EcYdfG_U1_rv	gDNA of *E. coli* SEQ ID NO22
PpBAPAT<-	Beta-alanine-pyruvate aminotransferase from *Pseudomonas putida* KT2440	pE1_fw	pE1_rv	pE1-PpBAPAT
BcBAPAT<-	Uncharacterized aminotransferase *yhxA* from *Bacillus cereus* AH1272	GeneArt_1U_fw	GeneArt_1U_rv	GeneArt plasmid with synthetic gene sequence SEQ ID NO2.
ScGabT<-	Gamma-aminobutyrate transaminase *uga1* from *S. cerevisiae*	ScGabT_U1_fw	ScGabT_U1_rv	gDNA of *S. cerevisiae CEN.PK113-7D* SEQ ID NO24
CaGabT<-	Gamma-aminobutyrate transaminase from *Clostridium acetobutylicum* ATCC 824 (as control)	pE1_fw	pE1_rv	pE1-CaGabT
ScPTEF1<-	Promoter of *tef1* gene from *S. cerevisiae*	PTEF1_fw	PTEF1_rv	gDNA of *S. cerevisiae*

				CEN.PK113-7D SEQ ID NO25
EcPanD<-	Aspartate 1-decarboxylase *panD* from *E. coli*	EcPAND_U1_fw	EcPAND_U1_rv	gBLOCK from Integrated DNA Technologies SEQ ID NO50
CgPanD<-	Aspartate 1-decarboxylase *panD* from *C. glutamicum*	CgPAND_U1_fw	CgPAND_U1_rv	gBLOCK from Integrated DNA Technologies SEQ ID NO51
ScGAD1<-	Glutamate decarboxylase *gad1* from *S. cerevisiae*	ScGAD1_U1_fw	ScGAD1_U1_rv	gDNA of *S. cerevisiae CEN.PK113-7D* SEQ ID NO52
EcGAD1<-	Glutamate decarboxylase *gad1* from *E. coli*	EcGAD1_U1_fw	EcGAD1_U1_rv	gDNA of *E. coli* SEQ ID NO53
RnGAD1<-	Glutamate decarboxylase *gad1* from *R. norvegicus*	RnGAD1_U1_fw	RnGAD1_U1_rv	GeneArt plasmid with synthetic gene sequence SEQ ID NO54
MsHPDH->	3-hydroxypropanoate dehydrogenase from *Metallosphaera sedula*	pE2_fw	pE2_rv	pE2-MsHPDH
EcYdfG->	3-hydroxypropanoate dehydrogenase from *Escherichia coli*	EcYdfG_U2_fw	EcYdfG_U2_rv	gDNA of *E. coli* SEQ ID NO22
<-ScPTEF1-ScPPGK1->	Fused promoters of *tef1* and *pgk1* genes from *S. cerevisiae*	PTEF1_fw	PPGK1_rv	plasmid pSP-GM1 SEQ ID NO66
TcPanD<-	Aspartate 1-decarboxylase from *T. castaneum*	TcPAND_U1_fw	TcPAND_U1_rv	GeneArt plasmid with synthetic gene sequence SEQ ID 69
ScPYC1<-	Pyruvate carboxylase *PYC1* from *S. cerevisiae*	ScPYC1_U1_fw	ScPYC1_U1_rv	gDNA of *S. cerevisiae CEN.PK113-7D* SEQ ID 78
ScPYC2->	Pyruvate carboxylase *PYC2* from *S. cerevisiae*	ScPYC2_U2_fw	ScPYC2_U2_rv	gDNA of *S. cerevisiae CEN.PK113-7D* SEQ ID 79

ScAAT2<-	Aspartate aminotransferase *AAT2* from *S. cerevisiae*	ScAAT2_U1_fw	ScAAT2_U1_rv	gDNA of *S. cerevisiae CEN.PK113-7D* SEQ ID 80

Table 4. Expression plasmids

Plasmid name	Parent plasmid	Selection marker	Cloned fragment (-s)	Promoter	Terminator
pPaHIBADH	pESC-HIS-USER	SpHIS5	PaHIBADH<-	ScPTEF1<-	ScTADH1
pCaHIBADH	pESC-HIS-USER	SpHIS5	CaHIBADH<-	ScPTEF1<-	ScTADH1
pBcHIBADH	pESC-HIS-USER	SpHIS5	BcHIBADH<-	ScPTEF1<-	ScTADH1
pPpHIBADH	pESC-HIS-USER	SpHIS5	PpHIBADH<-	ScPTEF1<-	ScTADH1
pMsHPDH	pESC-HIS-USER	SpHIS5	MsHPDH<-	ScPTEF1<-	ScTADH1
pStMSR	pESC-HIS-USER	SpHIS5	StMSR<-	ScPTEF1<-	ScTADH1
pEcRutE	pESC-HIS-USER	SpHIS5	EcRutE<-	ScPTEF1<-	ScTADH1
pEcYdfG	pESC-HIS-USER	SpHIS5	EcYdfG<-	ScPTEF1<-	ScTADH1
pPpBAPAT	pESC-URA-USER	KlURA3	PpBAPAT<-	ScPTEF1<-	ScTADH1
pBcBAPAT	pESC-URA-USER	KlURA3	BcBAPAT<-	ScPTEF1<-	ScTADH1
pScGabT	pESC-LEU-USER	KlLEU2	ScGabT<-	ScPTEF1<-	ScTADH1
pCaGabT	pESC-LEU-USER	KlLEU2	CaGabT<-	ScPTEF1<-	ScTADH1
pESC-URA-BcBAPAT-MsHDPH	pESC-URA-USER	KlURA3	BcBAPAT<-, MsHPDH->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1
pESC-URA-BcBAPAT-EcYdfG	pESC-URA-USER	KlURA3	BcBAPAT<-, EcYdfG->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1
pESC-HIS-EcPanD	pESC-HIS-USER	SpHIS5	EcPanD<-	ScPTEF1<-	ScTADH1
pESC-HIS-CgPanD	pESC-HIS-USER	SpHIS5	CgPanD<-	ScPTEF1<-	ScTADH1
pESC-HIS-TcPanD	pESC-HIS-USER	SpHIS5	TcPanD<-	ScPTEF1<-	ScTADH1
pESC-HIS-ScGAD1	pESC-HIS-USER	SpHIS5	ScGAD1<-	ScPTEF1<-	ScTADH1
pESC-HIS-EcGAD1	pESC-HIS-USER	SpHIS5	EcGAD1<-	ScPTEF1<-	ScTADH1
pESC-HIS-RnGAD1	pESC-HIS-USER	SpHIS5	RnGAD1<-	ScPTEF1<-	ScTADH1
pXI-1-LoxP-KlLEU2-PYC1<-PTEF1-PPGK1->PYC2	pXI-1-LoxP-KlLEU2 (SEQ ID NO 87)	KlLEU2	ScPYC1<-ScPYC2->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1
pX-2-LoxP-KlURA3-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-2-LoxP-KlURA3 (SEQ ID NO 86)	KlURA3	BcBAPAT<-, EcYdfG->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1
pTY-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pTY*	KlURA3-tagged	BcBAPAT<-, EcYdfG->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1
pTY-TcPanD<-PTEF1	pTY	KlURA3-tagged	TcPanD<-	ScPTEF1<-	ScTADH1
pX-4-LoxP-SpHIS5-TcPanD<-PTEF1	pX-4-LoxP-SpHIS5 (SEQ ID NO 89)	SpHIS5	TcPanD<-	ScPTEF1<-	ScTADH1
pX-4-LoxP-SpHIS5-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHIS5	SpHIS5	BcBAPAT<, EcYdfG->	<-ScPTEF1-ScPPGK1->	ScTADH1, ScTCYC1

pXII-1-LoxP-KlLEU2-AAT2<-PTEF1	pXII-1-LoxP-KlLEU2 (SEQ ID NO 88)	KlLEU2	ScAAT2<-	ScPTEF1<-	ScTADH1

*pTY, a vector designed for multiple chromosomal integration by targeting TY repeat regions. The vector contains the same USER cloning cassette as the rest of the parent plasmids listed in Table 4.

Table 5. Strains and 3HP titers in cultivation with β-alanine addition

Parent strain	Plasmid with URA3 marker	Plasmid with HIS3 marker	Plasmid with LEU2 marker	3HP, mg/L
CEN.PK113-11C (ura-his-)	pPpBAPAT	pPaHIBADH	-	-10±2
CEN.PK113-11C (ura-his-)	pPpBAPAT	pCaHIBADH	-	-16±3
CEN.PK113-11C (ura-his-)	pPpBAPAT	pBcHIBADH	-	-11±5
CEN.PK113-11C (ura-his-)	pPpBAPAT	pPpHIBADH	-	-10±1
CEN.PK113-11C (ura-his-)	pPpBAPAT	pMsHPDH	-	-12±6
CEN.PK113-11C (ura-his-)	pPpBAPAT	pStMSR	-	-4±4
CEN.PK113-11C (ura-his-)	pPpBAPAT	pEcRutE	-	-6±5
CEN.PK113-11C (ura-his-)	pPpBAPAT	pEcYdfG	-	-14±2
CEN.PK113-11C (ura-his-)	pBcBAPAT	pPaHIBADH	-	474±15
CEN.PK113-11C (ura-his-)	pBcBAPAT	pCaHIBADH	-	489±73
CEN.PK113-11C (ura-his-)	pBcBAPAT	pBcHIBADH	-	434±29
CEN.PK113-11C (ura-his-)	pBcBAPAT	pPpHIBADH	-	496±14
CEN.PK113-11C (ura-his-)	pBcBAPAT	pMsHPDH	-	1,852±103
CEN.PK113-11C (ura-his-)	pBcBAPAT	pStMSR	-	1,445±40
CEN.PK113-11C (ura-his-)	pBcBAPAT	pEcRutE	-	394±8
CEN.PK113-11C (ura-his-)	pBcBAPAT	pEcYdfG	-	2,145±89
CEN.PK102-5B.URA3 (his-leu-)	-	pPaHIBADH	pCaGabT	-7±4
CEN.PK102-5B.URA3 (his-leu-)	-	pCaHIBADH	pCaGabT	-1±5
CEN.PK102-5B.URA3 (his-leu-)	-	pBcHIBADH	pCaGabT	19±20
CEN.PK102-5B.URA3 (his-leu-)	-	pPpHIBADH	pCaGabT	-9±0
CEN.PK102-5B.URA3 (his-leu-)	-	pMsHPDH	pCaGabT	-9±4
CEN.PK102-5B.URA3 (his-leu-)	-	pStMSR	pCaGabT	-5±4
CEN.PK102-5B.URA3 (his-leu-)	-	pEcRutE	pCaGabT	6±2
CEN.PK102-5B.URA3 (his-leu-)	-	pEcYdfG	pCaGabT	-10±2
CEN.PK102-5B.URA3 (his-leu-)	-	pPaHIBADH	pScGabT	233±17
CEN.PK102-5B.URA3 (his-leu-)	-	pCaHIBADH	pScGabT	205±29
CEN.PK102-5B.URA3 (his-leu-)	-	pBcHIBADH	pScGabT	191±19

CEN.PK102-5B.URA3 (his-leu-)	-	pPpHIBADH	pScGabT	202±11
CEN.PK102-5B.URA3 (his-leu-)	-	pMsHPDH	pScGabT	493±23
CEN.PK102-5B.URA3 (his-leu-)	-	pStMSR	pScGabT	435±23
CEN.PK102-5B.URA3 (his-leu-)	-	pEcRutE	pScGabT	170±11
CEN.PK102-5B.URA3 (his-leu-)	-	pEcYdfG	pScGabT	457±18

Table 6. Strains and 3HP titers in cultivation with L-aspartate addition

Parent strain	Plasmid with URA3 marker	Plasmid with HIS3 marker	3HP, mg/L
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-MsHDPH	pESC-HIS-EcPanD	-1±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-MsHDPH	pESC-HIS-CgPanD	79±8
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-MsHDPH	pESC-HIS-ScGAD1	0±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-MsHDPH	pESC-HIS-EcGAD1	0±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-MsHDPH	pESC-HIS-RnGAD1	-1±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-EcPanD	-1±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-CgPanD	269±53
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-ScGAD1	0±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-EcGAD1	0±0
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-RnGAD1	0±1
CEN.PK113-5D (ura-)	pESC-URA-BcBAPAT-EcYdfG	-	1±0
CEN.PK113-7D (WT)	-	-	0±0

Table 7. Strains and 3HP titers in cultivation on glucose as the sole carbon source

Parent strain	Plasmid with URA3 marker	Plasmid with HIS3 marker	3HP, mg/L on Delft medium	3HP, mg/L on FIT medium
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-TcPanD	605±18	1638±19
CEN.PK113-11C (ura-his-)	pESC-URA-BcBAPAT-EcYdfG	pESC-HIS-CgPanD	214±23	826±33

Table 8. Yeast strains with chromosomally integrated genes for 3HP biosynthesis

Final strain	Parent strain	Plasmid with URA3 marker	Plasmid with HIS3 marker	Plasmid with LEU2 marker
SCE-R2-180	CEN.PK102-5B (ura-his-leu-)	pX-2-LoxP-KlURA3-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1
SCE-R2-182	CEN.PK102-5B (ura-his-leu-)	pTY-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1
SCE-R2-184	CEN.PK102-5B (ura-his-leu-)	pTY-TcPanD<-PTEF1	pX-4-LoxP-SpHiS5-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1

SCE-R2-188	CEN.PK113-11C (ura-his-)	pX-2-LoxP-KlURA3-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	-
SCE-R2-190	CEN.PK113-11C (ura-his-)	pTY-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	-
SCE-R2-192	CEN.PK113-11C (ura-his-)	pTY-TcPanD<-PTEF1	pX-4-LoxP-SpHiS5-BcBAPAT<-PTEF1-PPGK1->EcYdfG	-
SCE-R2-196	ST738 (PYC1^,PYC2^,ura-his-leu-)	pX-2-LoxP-KlURA3-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1
SCE-R2-198	ST738 (PYC1^,PYC2^,ura-his-leu-)	pTY-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1
SCE-R2-200	ST738 (PYC1^,PYC2^,ura-his-leu-)	pTY-TcPanD<-PTEF1	pX-4-LoxP-SpHiS5-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pXII-1-LoxP-KlLEU2-AAT2<-PTEF1
SCE-R2-204	ST724 (PYC1^,PYC2^,ura-his-)	pX-2-LoxP-KlURA3-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	-
SCE-R2-206	ST724 (PYC1^,PYC2^,ura-his-)	pTY-BcBAPAT<-PTEF1-PPGK1->EcYdfG	pX-4-LoxP-SpHiS5-TcPanD<-PTEF1	-
SCE-R2-208	ST724 (PYC1^,PYC2^,ura-his-)	pTY-TcPanD<-PTEF1	pX-4-LoxP-SpHiS5-BcBAPAT<-PTEF1-PPGK1->EcYdfG	-

Results obtained in the following Examples are in part given in the accompanying drawings, in which:

Figure 1 shows a metabolic pathway leading from pyruvate to 3-HP via aspartate and beta-alanine and malonic semialdehyde.

Figure 2 shows NMR results obtained in Example 2.

Figure 3 shows the **i**nfluence of integrating multiple copies of genes and of overexpression of precursor supply genes on 3HP titer. The concentration of 3HP in the culture broth was determined by HPLC method and is given in g L^{-1}. ↑ - single copy of gene is integrated into the genome, ↑↑ - multiple copies of gene are integrated into the genome (Example 6).

Figure 4 shows growth and metabolite concentrations in glucose-limited fed-batch cultivation of SCE-R2-200 at pH5. Representative graph of one cultivation out of three (Example 7).

As illustrated in Figure 1, apartate can be converted to beta-alanine by the enzyme PanD, aspartate 1-decarboxylase. β-alanine is convertible to malonic semialdehyde by either BAPAT or GabT, and malonic semialdehyde is convertible to 3-HP by HIBADH/HPDH. The present invention uses the route via BAPAT.

Example 1. Cloning of heterologous beta-alanine-pyruvate aminotransferase, 3-hydroxyisobutyrate dehydrogenase, and 3-hydroxypropanoate dehydrogenase and overexpession of heterologous and native gamma-aminobutyrate transaminase in *S. cerevisiae*

Genes encoding a putative *B. cereus* aminotransferase yhxA (SEQ ID NO1), *Pseudomonas putida* beta-alanine-pyruvate aminotransferase (SEQ ID NO3), *P. aeruginosa* 3-hydroxybutyrate dehydrogenase (SEQ ID NO5), *Candida albicans* 3-hydroxybutyrate dehydrogenase (SEQ ID NO7), *P. putida* 3-hydroxybutyrate dehydrogenase (SEQ ID NO9), *Bacillus cereus* 3-hydroxybutyrate dehydrogenase (SEQ ID NO11), *Metallosphaera sedula* 3-hydroxypropanoate dehydrogenase (SEQ ID NO13), *Sulfolobus tokadaii* 3-hydroxypropanoate dehydrogenase (SEQ ID NO15), and *Clostridium acetobutylicum* gamma-aminobutyrate transaminase (SEQ ID NO17) were synthesized by GeneArt (Life Technologies) in versions codon-optimized for yeast *S. cerevisiae* (corresponding SEQ ID NO2, SEQ ID NO4, SEQ ID NO6, SEQ ID NO8, SEQ ID NO10, SEQ ID NO12, SEQ ID NO14, SEQ ID NO16, SEQ ID NO18).

The ordered gene constructs had a general structure: GGTACC*AAAACA***ATG**NN…NN**TGA**GTCGAC (SEQ ID NO67), where GGTACC is a *Kpn*I restriction site, AAAACA is the Kozak sequence, ATG is the start codon, NN…NN represents the protein coding sequence

without start and stop codons, TGA is the stop codon, GTCGAC is a *Sal*I restriction site.

The synthetic genes were excised from the plasmids using *Kpn*I and *Sal*I, gel-purified and ligated into plasmid pE1 (SEQ ID 81) or pE2 (SEQ ID82), which were digested with the same enzyme pair. The resulting ligation mix was transformed into chemically competent *E. coli* DH5alpha using heat shock and the cells were selected on Luria-Bertani (LB) agar medium with 100 µg/ml amplicillin.

The clones with correct inserts were identified by colony PCR, inoculated in liquid LB medium with 100 µg/ml ampicillin and the plasmids were isolated (Table 2). The resulting plasmids were confirmed by sequencing.

The gene fragments carrying the genes and correct overhangs for USER-cloning were generated by PCR amplification using primers and templates as indicated in Table 3. The PCR mix contained: 28 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 5 µl 2mM dNTP, 1 µl Phusion® polymerase, 2.5 µl forward primer at 10 µM concentration, 2.5 µl reverse primer at 10 µM concentration, and 1 µl DNA template. The cycling program was: 95°C for 2 min, 30 cycles of [95°C for 10 sec, 50°C for 20 sec, 68°C for 2 min], 68°C for 5 min, pause at 10°C. The gene fragments were resolved on 1% agarose gel containing SYBR®-SAFE (Invitrogen) and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The promoter fragments were also generated by PCR followed by gene purification (Table 3). The terminators were already present on the expression plasmids.

The parent plasmids pESC-Ura-USER (SEQ ID NO 85), pESC-His-USER (SEQ ID NO 83) and pESC-Leu-USER (SEQ ID NO 84) were linearized with FastDigest® AsiSI (Fermentas) for 1 hour at 37°C and nicked with Nb.BsmI for 1 hour at 37°C. The

resulting linearized nicked DNA was purified from the solution and eluted in 5mM Tris buffer, pH 8.0.

The expression plasmids were created by USER-cloning using the following protocol. 1 µl of linearized and nicked parent plasmid was mixed with 1 µl of promoter fragment, 2 µl of gene fragment, 0.5 µl Taq polymerase buffer, 0.5 µl USER enzyme (NEB). The mix was incubated at 37°C for 25 min, at 25°C for 25 min and transformed into chemically competent *E. coli* DH5alpha. The clones with correct inserts were identified by colony PCR and the plasmids were isolated from overnight *E. coli* cultures and confirmed by sequencing. The expression plasmids are listed in Table 4.

The expression plasmids were transformed into *S. cerevisiae* cells using the lithium acetate transformation protocol. The cells were selected on synthetic complete (SC) agar medium without uracil, histidine and leucine. The resulting strains are listed in Table 5.

Example 2. Production of 3-hydroxypropionic acid in *S. cerevisiae* cultivated on β-alanine

At least four independent yeast transformants were streak-purified on SC ura-his-leu- agar plates. Four single colonies originating from independent transformants were inoculated in 0.5 ml SC ura-his-leu- in a 96-deep well microtiter plate with air-penetrable lid (EnzyScreen). The plates were incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µl of the overnight cultures were used to inoculate 0.5 ml minimal mineral (Delft) medium with 10 g/L β-alanine in a 96-deep well plate.

The composition of the of Delft medium was as following: 7.5 g $(NH_4)_2SO_4$, 14.4 g KH_2PO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 22 g dextrose, 2 mL trace metals solution, and 1 mL vitamins. pH of the medium was adjusted to 6. The trace metals solution contained per

liter: 4.5 g $CaCl_2 \cdot 2H_2O$, 4.5 g $ZnSO_4 \cdot 7H_2O$, 3 g $FeSO_4 \cdot 7H_2O$, 1 g H_3BO_3, 1 g $MnCl_2 \cdot 4H_2O$, 0.4 g $Na_2MoO_4 \cdot 2H_2O$, 0.3 g $CoCl_2 \cdot 6H_2O$, 0.1 g $CuSO_4 \cdot 5H_2O$, 0.1 g KI, 15 g EDTA. The trace metals solution was prepared by dissolving all the components except EDTA in 900 mL ultra-pure water at pH 6 followed by gentle heating and addition of EDTA. Finally the trace metal solution pH was adjusted to 4, and the solution volume was adjusted to 1 L and autoclaved (121°C in 20 min). Trace metals solution was stored at +4°C. The vitamins solution contained per liter: 50 mg biotin, 200 mg p-aminobenzoic acid, 1 g nicotinic acid, 1 g Ca-pantotenate, 1 g pyridoxine-HCl, 1 g thiamine-HCl, 25 g myo-inositol. Biotin was dissolved in 20 mL 0.1 M NaOH and 900 mL water is added. pH was adjusted to 6.5 with HCl and the rest of the vitamins was added. pH was re-adjusted to 6.5 just before and after adding m-inositol. The final volume of the vitamin solution was adjusted to 1 l and sterile-filtered before storage at +4°C.

Fermentation was carried out for 72 hours at the same conditions as above.

At the end of the cultivation the OD_{600} was measured. 10 µl of the sample was mixed with 190 µl water and absorbance was measured at 600 nm wave length in spectrophotometer (BioTek).

The culture broth was spun down and the supernatant analyzed for 3-hydroxypropionic acid concentration using enzymatic assay (Table 5). No 3HP production was obtained when *P. putida* beta-alanine-pyruvate aminotransferase or *C. acetobutylicum* gamma-aminobutyrate transaminase were used in combination with 3-hydroxybutyrate dehydrogenase or 3-hydroxypropanoate dehydrogenase. However 3HP production from beta-alanine was observed when putative *B. cereus* aminotransferase YhxA or *S. cerevisiae* gamma-aminobutyrate transaminase were combined with 3-hydroxybutyrate

dehydrogenase or 3-hydroxypropanoate dehydrogenase (Table 5: strains 133-147). The best enzyme combination under the conditions tested was strain 147 expressing *B. cereus* aminotransferase YhxA and *E. coli* 3-hydroxypropanoate dehydrogenase YdfG, where 2,145±89 mg/L 3HP was obtained.

Enzymatic assay was carried out as follows. 20 µl of standards (3HP at concentrations from 0.03 to 1 g/L in Delft medium) and samples were added to a 96-well flat bottom transparent plate (Greiner). 180 µl of mix (14.8 ml water, 2 ml buffer (1 mM Tris, 25 mM $MgCl_2$, pH 8.8), 1 ml NADP+ solution (50 mg/ml), and 0.2 ml purified YdfG enzyme in PBS buffer (1500 µg/ml)) was added per well using multichannel pipet. The start absorbance at 340 nm was measured, the plate was sealed and incubated at 30°C for 1.5 hours. After that the end absorbance at 340 nm was measured again. The difference between the end and the start values corrected for the background were in linear correlation with 3HP concentrations. The concentration of 3HP in the samples was calculated from the standard curve.

The identity of 3-hydroxypropionic acid in the best sample was confirmed by NMR analysis (Figure 2). The concentration measured by NMR correlated well with the value found by enzymatic assay.

Example 3. Cloning of aspartate-1-decarboxylase or glutamate decarboxylase in *S. cerevisiae*

Genes encoding *E. coli* aspartate 1-decarboxylase (SEQ ID NO50) and *C. glutamicum* aspartate 1-decarboxylase (SEQ ID NO52) were synthesized as gBLOCKs by Integrated DNA Technologies (in versions codon-optimized for yeast *S. cerevisiae* corresponding SEQ ID NO51 and SEQ ID NO53).

Gene encoding glutamate decarboxylase from *Rattus norvegicus* (SEQ ID NO58) was synthesized by GeneArt (Life

Technologies) in version codon-optimized for yeast *S. cerevisiae* (SEQ ID NO59).

The ordered gene constructs had a general structure: GGTACC*AAAACA***ATG**NN…NN**TGA**GTCGAC (SEQ ID NO67), where GGTACC is a *KpnI* restriction site, AAAACA is the Kozak sequence, ATG is the start codon, NN…NN represents the protein coding sequence without start and stop codons, TGA is the stop codon, GTCGAC is a *SalI* restriction site.

The gene fragments carrying the genes and correct overhangs for USER-cloning were generated by PCR amplification using primers and templates as indicated in Table 3. The PCR mix contained: 28 µl water, 10 µl high fidelity Phusion® polymerase buffer (5x), 5 µl 2mM dNTP, 1 µl Phusion® polymerase, 2.5 µl forward primer at 10 µM concentration, 2.5 µl reverse primer at 10 µM concentration, and 1 µl DNA template. The cycling program was: 95°C for 2 min, 30 cycles of [95°C for 10 sec, 50°C for 20 sec, 68°C for 2 min], 68°C for 5 min, pause at 10°C. The gene fragments were resolved on 1% agarose gel containing SYBR®-SAFE (Invitrogen) and purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel). The promoter fragments were also generated by PCR followed by gene purification (Table 3). The terminators were already present on the expression plasmids.

The parent plasmids pESC-Ura-USER, pESC-His-USER and pESC-Leu-USER were linearized with FastDigest® AsiSI (Fermentas) for 1 hour at 37°C and nicked with Nb.BsmI for 1 hour at 37°C. The resulting linearized nicked DNA was purified from the solution and eluted in 5mM Tris buffer, pH 8.0.

The expression plasmids were created by USER-cloning using the following protocol. 1 µl of linearized and nicked parent plasmid was mixed with 1 µl of promoter fragment, 2 µl

of gene fragment, 0.5 µl Taq polymerase buffer, 0.5 µl USER enzyme (NEB). The mix was incubated at 37°C for 25 min, at 25°C for 25 min and transformed into chemically competent *E. coli* DH5alpha. The clones with correct inserts were identified by colony PCR and the plasmids were isolated from overnight *E. coli* cultures and confirmed by sequencing. The expression plasmids are listed in Table 4.

The expression plasmids were transformed into *S. cerevisiae* cells using the lithium acetate transformation protocol. The cells were selected on synthetic complete (SC) agar medium without uracil, histidine and leucine. The resulting strains are listed in Table 6.

Example 4. Production of 3-hydroxypropionate in *S. cerevisiae* cultivated on L-aspartate

At least four independent yeast transformants were streak-purified on SC ura-his-leu- agar plates. Four single colonies originating from independent transformants were inoculated in 0.5 ml SC ura-his-leu- in a 96-deep well microtiter plate with air-penetrable lid (EnzyScreen). The plates were incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µl of the overnight cultures were used to inoculate 0.5 ml Delft medium with 10 g/L L-aspartate in a 96-deep well plate. Fermentation was carried out for 72 hours at the same conditions as above.

The culture broth was spun down and the supernatant analyzed for 3-hydroxypropionic acid concentration using enzymatic assay as described in Example 2 (Table 6).

3HP production from L-aspartate was observed only when aspartate 1-decarboxylase from *C. glutamicum* was expressed in combination with enzymes converting beta-alanine into 3HP (putative *B. cereus* aminoransferase YhxA and *E. coli* 3-hydroxypropanoate dehydrogenase YdfG or *Metallosphaera sedula*

3-hydroxypropanoate dehydrogenase). The best combination was aspartate 1-decarboxylase from *C. glutamicum*, putative *B. cereus* aminoransferase YhxA and *E. coli* 3-hydroxypropanoate dehydrogenase YdfG, which resulted in 269±53 mg/L 3HP.

In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof.

Example 5. Expression of aspartate-1-decarboxylase from red flour beetle in *S. cerevisiae* and production of 3HP from glucose

The gene encoding *Tribolium castaneum* aspartate 1-decarboxylase TcPanD (SEQ ID 68) was synthesized in version codon-optimized for *S. cerevisiae* (SEQ ID 69) by GeneArt (LifeTech Sciences).

The TcPanD gene was amplified using PCR in order to generate USER-cloning compatible overhangs as described in Example 1 using primers TcPanD_U1_fw and Tc_PanD_rv (Table 3). The resulting DNA fragment TcPanD<- was cloned into expression plasmid pESC-HIS-USER along with *TEF1* promoter to result in plasmid pESC-HIS-TcPanD (Table 4). Correct insertion of TcPanD gene and the promoter was confirmed by sequencing.

The plasmids were transformed into *S. cerevisiae* strain using the lithium acetate transformation protocol; the resulting strains are shown in Table 7.

At least three independent yeast transformants were inoculated in 0.5 ml SC ura-his-leu- in a 96-deep well microtiter plate with air-penetrable lid (EnzyScreen). The plates were incubated at 30°C with 250 rpm agitation at 5 cm orbit cast overnight. 50 µl of the overnight cultures were used to inoculate 0.5 ml minimal mineral (Delft) medium or 0.5 ml Feed-in-time medium (FIT) for *S. cerevisiae* (M2P Labs, Germany) in 96-deep well plates.

Fermentation was carried out for 72 hours at the same conditions as inoculum preparation. The culture broth was spun down and the supernatant was analyzed for 3-hydroxypropionic acid concentration using HPLC (Table 7).

HPLC analysis was performed on Dionex UltiMate 3000 system (Thermo Fisher Scientific) with Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA) operating at 60°C. The injection volume was 20 µl. The mobile phase was 1 mM H_2SO_4 at a flow rate of 0.6 ml/min. 3HP was detected on DAD-3000 Diode Array Detector (Dionex) using the read at 210 nm. The calibration curve was made using 3-hydroxypropionic acid purchased from TCI. The identity of the 3-hydroxypropionic acid was additionally verified by comparison of the spectrum with the standard.

Aspartate 1-decarboxylase from *T. castaneum* resulted in almost 3-fold higher 3HP titer on Delft and 2-fold higher 3HP titer on FIT medium than aspartate 1-decarboxylase from *C. glutamicum*. Thus we have confirmed that if the strain capable of producing 3HP from β-alanine is supplemented with aspartate 1-decarboxylase enzyme from *C. glutamicum* or better from *T. castaneum* then it can produce 3HP directly from glucose.

Example 6. Improvement of 3HP production by overexpression of precursor

Once the biosynthesis of 3HP from glucose via beta-alanine has been established in yeast, the next goal was to improve the expression of the biosynthetic genes and to increase the flux towards L-aspartate. As this would require stable simultaneous overexpression of several genes, we used EasyClone integrative vectors for yeast. We tested the effect of overexpressing native cytoplasmic aspartate aminotransferase Aat2p, pyruvate carboxylases Pyc1p and Pyc2p and of the combination thereof. We also investigated the effect of multiple chromosomal integration of the key biosynthetic genes leading from aspartate to 3HP.

The genes encoding aspartate aminotransferase AAT2 and pyruvate carboxylases PYC1 and PYC2 were amplified from gDNA of *S. cerevisiae* CEN.PK113-7D using primers as in Table 3 and PCR conditions as in Example 1. The resulting DNA fragments were purified and cloned into EasyClone expression vectors as described in Example 1 (see Table 4).

Strain ST724 (PYC1^,PYC2^,ura-his-) was created by transforming *S. cerevisiae* CEN.PK102-5B (ura-his-leu-) with plasmid pXI-1-LoxP-KlLEU2-PYC1<-PTEF1-PPGK1->PYC2, selecting the transformants on SC drop-out medium without leucine and confirming the correct integration of the plasmid by PCR on genomic DNA of the transformant. Strain ST724 was used to create strain ST738 (PYC1^,PYC2^,ura-his-leu-) by looping out the KlLEU2 selection marker using LoxP-Cre-mediated recombination.

The yeast strains were transformed with expression plasmids according to Table 8 and transformants were selected on SC drop-out medium without uracil, histidine and leucine.

The strains were cultivated and 3HP concentrations were analyzed as described in Example 5. The results are shown in Figure 3.

Increasing copy number of *BcBAPAT/EcYdfG* or of *TcPanD* lead to improvement of 3HP titer for all the four background strains tested (reference, overexpressing *AAT2*, overexpressing *PYC1*&*PYC2* and overexpressing *AAT2*&*PYC1*&*PYC2*). The effect of multiple integrations of *TcPanD* was larger than that of multiple copies of *BcBAPAT/EcYdfG*.

The increased precursor supply (via overexpression of *PYC1/PYC2* and/or *AAT2*) had a positive effect on 3HP production in strains with multiple copies of *TcPanD* or *BcBAPAT/EcYdfG* genes, but not in the strains that had only single copies of the latter genes. The positive effect of overexpressing pyruvate carboxylase genes was only observed on feed-in-time medium, which simulates fed-batch conditions. The highest titers were obtained for the strain SCE-R2-200 (AAT2↑PYC1↑PYC2↑BcBAPAT↑EcYdfG↑TcPanD↑↑): 1.27±0.28 g/L and 8.51±1.05 g/L on mineral and feed-in-time media correspondingly.

Example 7. Production of 3HP by yeast in fed-batch cultivation at pH5

The best isolate of strain SCE-R2-200 described above was cultivated in aerobic fed-batch cultivation with glucose-limited feed at pH5 in triplicates.

SCE-R2-200 glycerol stock (0.3 ml) was inoculated in 150 ml Delft medium in 500-ml baffled shake flask and propagated at 30°C with 250 rpm agitation for about 24 hours. The culture was concentrated down to 50 ml by centrifugation at 4,000xg for 2 min and used to inoculate 0.5 L medium in 1L-Sartorius reactor. The final medium in the reactors contained per liter: 15 g $(NH_4)_2SO_4$, 6 g KH_2PO_4, 1 g $MgSO_4 \cdot 7H_2O$, 4 ml trace

metals solution, 2 ml vitamins solution, 0.4 ml antifoam A (Sigma-Aldrich), and 44 g dextrose. Dextrose was autoclaved separately, vitamins solution was sterile filtered and added to the medium after autoclavation. The trace metal and vitamins solutions are the same as described in Example 2. The agitation rate was 800 rpm, the temperature was 30°C, aeration was 1 L min^{-1} air and pH was maintained at 5.0 by automatic addition of 2N NaOH. Carbon dioxide concentration in the off-gas was monitored by acoustic gas analyzer (model number 1311, Bruël & Kjær). Once the glucose was exhausted, which was observed from decline in CO_2 production and was also confirmed by residual glucose detection using glucose strips Glucose MQuant™ (Merck Millipore), the feed was started at 5 g h^{-1}. The feed contained per liter: 45 g $(NH_4)_2SO_4$, 18 g KH_2PO_4, 3 g $MgSO_4 \cdot 7H_2O$, 12 ml trace metals solution, 6 ml vitamins solution, 0.6 ml antifoam A, and 176 g dextrose. Dextrose was autoclaved separately, vitamins solution was sterile filtered and added to the feed after autoclavation.

24 hours after the feed start the feed rate was ramped up to 10 g h^{-1} and 48 hours after the feed start it was further increased to 15 g h^{-1}. The reactors were sampled twice a day to measure biomass dry weight and metabolites. For metabolites analysis the sample was immediately centrifuged and the supernatant was stored at -20°C until HPLC analysis. HPLC analysis of glucose, succinate, acetate, 3HP, glycerol, ethanol, and pyruvate was carried out at described in Example 5. Glucose, glycerol and ethanol were detected using RI-101 Refractive Index Detector (Dionex). 3HP, pyruvate, succinate and acetate were detected with DAD-3000 Diode Array Detector at 210 nm (Dionex).

The strain produced 3-hydroxypropionic acid at 13.7±0.3 g•L-1 titer, 14±0% C-mol•C-mol-1 glucose yield and 0.24±0.0

g•L-1•h-1 productivity. No significant amounts of by-products as acetate, ethanol or glycerol were detected at the end of the fermentation. Results are shown in Figure 4.

In this specification, unless expressly otherwise indicated, the word 'or' is used in the sense of an operator that returns a true value when either or both of the stated conditions is met, as opposed to the operator 'exclusive or' which requires that only one of the conditions is met. The word 'comprising' is used in the sense of 'including' rather than in to mean 'consisting of'. All prior teachings acknowledged above are hereby incorporated by reference. No acknowledgement of any prior published document herein should be taken to be an admission or representation that the teaching thereof was common general knowledge in Australia or elsewhere at the date hereof. The content of the sequence listing filed herewith forms part of the description of the invention.

CLAIMS

1. A genetically modified yeast cell comprising an active fermentation pathway producing 3-HP, wherein the cell comprises and expresses an exogenous gene coding for the production of an enzyme having at least 80% identity with SEQ ID NO: 1 and catalysing a transamination reaction between beta-alanine and pyruvate to produce malonate semialdehyde.

2. A genetically modified yeast cell as claimed in claim 1, wherein said enzyme is the aminotransferase YhxA from *Bacillus cereus* AH1272.

3. A genetically modified yeast cell as claimed in claim 1 or claim 2, expressing a 3-hydroxyisobutyrate dehydrogenase (HIBADH).

4. A genetically modified yeast cell as claimed in claim 3, wherein said HIBADH is from *Pseudomonas aeruginosa*, *P. putida*, *Bacillus cereus*, or *Candida albicans*.

5. A genetically modified yeast cell as claimed in any preceding claim, expressing a 3-hydroxypropanoate dehydrogenase (3-HPDH).

6. A genetically modified yeast cell as claimed in claim 5, wherein said 3-HPDH is from *Metallosphaera sedula*, *Sulfolobus tokadaii* or *E. coli*.

7. A genetically modified yeast cell as claimed in any preceding claim expressing an exogenous gene producing

an aspartate-1-decarboxylase (EC 4.1.1.11) and/or expressing an exogenous gene expressing a glutamate decarboxylase (EC 4.1.1.15).

8. A genetically modified yeast cell as claimed in claim 7 expressing aspartate-1-decarboxylase from *Corynebacterium glutamicum* (SEQ ID NO52) or an enzyme having aspartate-1-decarboxylase activity which is at least 80% homologous with SEQ ID NO52.

9. A genetically modified yeast cell as claimed in claim 7 expressing aspartate-1-decarboxylase from *Tribolium castaneum* (SEQ ID NO68) or an enzyme having aspartate-1-decarboxylase activity which is at least 80% homologous with SEQ ID NO68.

10. A genetically modified yeast cell as claimed in claim 7 with increased activity of pyruvate carboxylase and/or aspartate transaminase.

11. A genetically modified yeast cell as claimed in claim 10 overexpressing native pyruvate carboxylase PYC1 or PYC2 and/or native aspartate aminotransferase AAT2.

12. A genetically modified yeast cell as claimed in any preceding claim, wherein the yeast is *S. cerevisiae*.

13. A method for the production of 3HP comprising culturing a yeast cell as claimed in any preceding claim, and recovering 3HP from the culture.

14. A method as claimed in claim 13, comprising supplying said culture with beta-alanine and/or L-aspartate.

15. A method as claimed in claim 13 or claim 14, wherein at least 100 mg of 3HP per litre of culture medium is produced or is recovered from said culture medium.

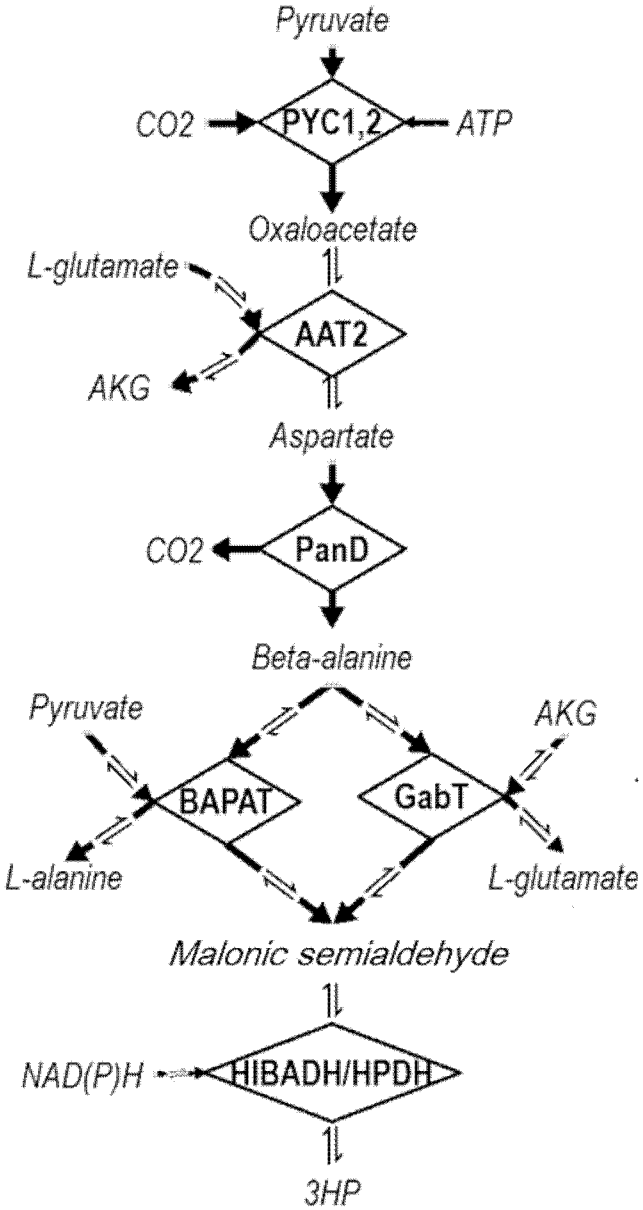

Pyruvate
CO2
PYC1,2
ATP
Oxaloacetate
L-glutamate
AAT2
AKG
Aspartate
CO2
PanD
Beta-alanine
Pyruvate
AKG
BAPAT
GabT
L-alanine
L-glutamate
Malonic semialdehyde
NAD(P)H
HIBADH/HPDH
3HP

Figure 1

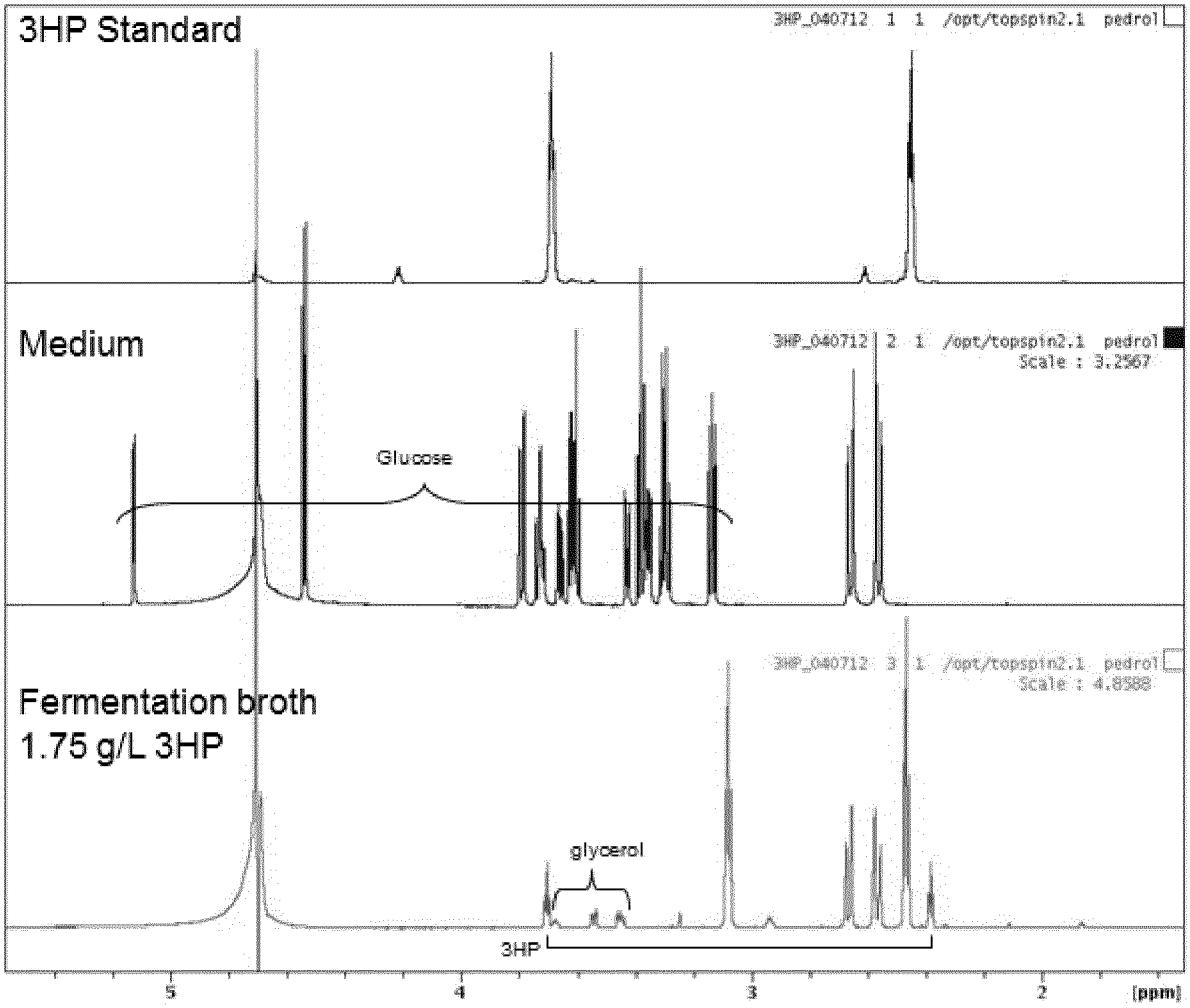

3HP Standard
3HP_040712 1 1 /opt/topspin2.1 pedrol
Medium
3HP_040712 2 1 /opt/topspin2.1 pedrol
Scale : 3.2567
Glucose
Fermentation broth
1.75 g/L 3HP
3HP_040712 3 1 /opt/topspin2.1 pedrol
Scale : 4.8588
glycerol
3HP
5
4
3
2
(ppm)

Figure 2

Cultivation medium	Mineral minimal medium (batch)			
Overexpression of biosynthetic genes/Overexpression of precursor supply	-	AAT2↑	PYC1↑ PYC2↑	AAT2↑ PYC1↑ PYC2↑
BcBAPAT↑ EcYdfG↑ TgPanD↑	0,4	0,3	0,5	0,4
BcBAPAT↑↑ EcYdfG↑↑ TgPanD↑	0,6	0,7	0,6	0,7
BcBAPAT↑ EcYdfG↑ **TgPanD↑↑**	1,0	**1,6**	1,0	1,3
Cultivation medium	**Feed-in-time medium (fed-batch)**			
Overexpression of biosynthetic genes/Overexpression of precursor supply	-	AAT2↑	PYC1↑ PYC2↑	AAT2↑ PYC1↑ PYC2↑
BcBAPAT↑ EcYdfG↑ TgPanD↑	1,9	1,9	2,8	2,0
BcBAPAT↑↑ EcYdfG↑↑ TgPanD↑	3,7	2,2	3,0	3,6
BcBAPAT↑ EcYdfG↑ **TgPanD↑↑**	5,6	5,7	7,0	**8,5**

Figure 3

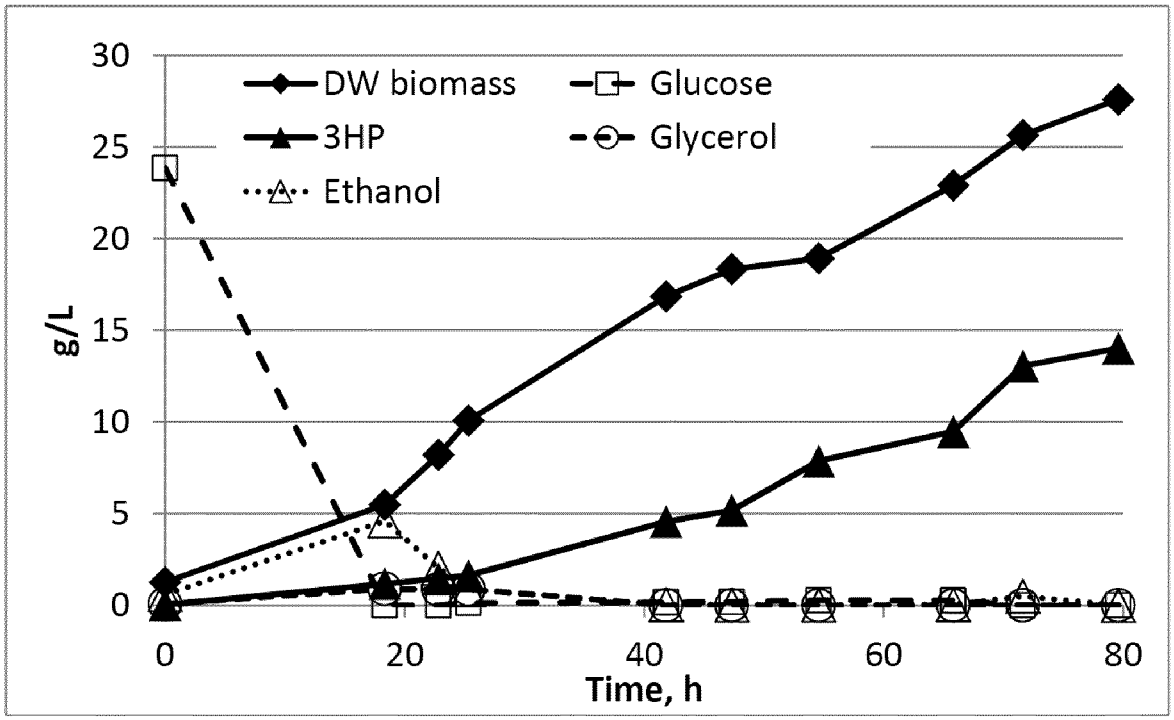


Figure 4

INTERNATIONAL SEARCH REPORT

International application No.
PCT/EP2013/071163

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application and necessary to the claimed invention, the international search was carried out on the basis of:

   a. (means)

   [ ] on paper

   [X] in electronic form

   b. (time)

   [X] in the international application as filed

   [ ] together with the international application in electronic form

   [ ] subsequently to this Authority for the purpose of search

2. [ ] In addition, in the case that more than one version or copy of a sequence listing and/or table relating thereto has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/071163

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N15/52 C12P7/52
ADD.

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
C12Y C12N C12P

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
EPO-Internal, Sequence Search, EMBASE, FSTA, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2012/135481 A1 (JESSEN HOLLY [US] ET AL) 31 May 2012 (2012-05-31) cited in the application page 1, paragraph 5-9 page 2, paragraphs 10,15,16 page 11, paragraphs 101,102 -----	1-8, 10-15
Y	WO 2005/118719 A2 (CARGILL INC [US]; LIAO HANS H [US]; GOKARN RAVI R [US]; GORT STEVEN JO) 15 December 2005 (2005-12-15) cited in the application page 2, lines 4-34 page 3, lines 10-15 page 6, line 24 - page 7, line 11 page 19, lines 1-34 page 22, lines 4-9 -----	1-8, 10-15
	-/--	

[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

* Special categories of cited documents :

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

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

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

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

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

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

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

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

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 November 2013	18/11/2013

Name and mailing address of the ISA/	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016	Bonello, Steve

1

Form PCT/ISA/210 (second sheet) (April 2005)

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2013/071163

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	DATABASE UniProt [Online] 16 June 2009 (2009-06-16), "SubName: Full=Uncharacterized aminotransferase yhxA;", XP002692141, retrieved from EBI accession no. UNIPROT:C2ZAL1 Database accession no. C2ZAL1 the whole document -----	1-8, 10-15
Y	WO 2010/031083 A2 (OPX BIOTECHNOLOGIES INC [US]; LYNCH MICHAEL D [US]; MERCOGLIANO CHRIST) 18 March 2010 (2010-03-18) claims 1,6,136, 152-154 page 2, lines 21-31 page 8, line 30 page 21, line 16 - page 22, line 24 -----	1-8, 10-15
A	US 6 852 517 B1 (SUTHERS PATRICK F [US] ET AL) 8 February 2005 (2005-02-08) column 2, line 60 - column 3, line 5 -----	1-15
A	DATABASE UniProt [Online] 2 October 2007 (2007-10-02), "SubName: Full=Aspartate 1-decarboxylase; EC=4.1.1.11;", XP002715871, retrieved from EBI accession no. UNIPROT:A7U8C7 Database accession no. A7U8C7 the whole document -----	9

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2013/071163

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2012135481 A1	31-05-2012	AU 2011336923 A1	30-05-2013
		CA 2818499 A1	07-06-2012
		EP 2643450 A2	02-10-2013
		US 2012135481 A1	31-05-2012
		WO 2012074818 A2	07-06-2012
WO 2005118719 A2	15-12-2005	AT 543873 T	15-02-2012
		BR PI0416546 A	09-01-2007
		DK 1706457 T3	21-05-2012
		EP 1706457 A2	04-10-2006
		US 2007107080 A1	10-05-2007
		US 2010136638 A1	03-06-2010
		US 2010267115 A1	21-10-2010
		WO 2005118719 A2	15-12-2005
WO 2010031083 A2	18-03-2010	EP 2326709 A2	01-06-2011
		US 2013189787 A1	25-07-2013
		WO 2010031083 A2	18-03-2010
US 6852517 B1	08-02-2005	NONE	