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Microbial production of 3-hydroxypropionic acid

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(54) **Title**: MICROBIAL PRODUCTION OF 3-HYDROXYPROPIONIC ACID

(57) **Abstract**: A yeast cell havinga reduced level of activity of NAD dependent glyceraldehyde-3-phosphate dehydrogenase (GAP-DH) has at least one exogenous gene encoding NADP dependent GAPDH and/or has up-regulation of at least one endogenous gene expressing NADP dependent GAPDH, wherein combined expression of the enzymes NADP dependent GAPDH, PDC, ALD, ACS, ACC* and MCR in said host cell increases metabolic flux towards 3-HP via malonyl-CoA compared to an otherwise similar yeast cell lacking said genetic modification.

Microbial production of 3-hydroxypropionic acid

Technical Field

The present invention relates to a recombinant yeast cells and their use in the production of 3-hydroxypropionic acid (3-HP).

Background Art

For more than a century, fossil fuels have been the primary feedstock for the chemical industries. However, new discoveries of fossil fuel deposits are diminishing whilst demand for fossil fuel based chemicals are ever increasing, and soon the supply of fossil fuels will be outweighed by the demand. In an attempt to address this issue a large amount of effort has gone into developing novel biotechnological strategies for producing chemical feedstock from renewable sources (e.g. sugars). In 2004 the Department of Energy in the USA identified a list of 12 target feedstock chemicals to be produced through biotechnological routes. 3-hydroxy propionic acid (3-HP) has been chosen as one of the 12 feedstock chemicals as it can serve as a platform for the development of a range of 3-carbon petrochemical intermediates, and in particular it can be dehydrated to form acrylic acid. More than 1 billion kilograms of acrylic acid are produced annually as it is the monomeric building block for polymeric acrylates which can be used in a wide range of consumer products, e.g. personal care products, adhesives, coatings and paints, and the annual total market size is over USD100 billion. One particularly important application of 3-HP is for the production of superabsorbent polymers (SAP), which constitute a significant part of baby diapers and incontinence products. It is evidently desirable to develop a more sustainable way of producing acrylic acid, hence this is why a significant amount of research continues towards the development of a biotechnological method of producing 3-HP, the acrylic acid precursor.

Conventional biological processes for producing 3-HP are performed by a complicated metabolic pathway. Therefore, it is difficult to control the process effectively, resulting in low production yield and productivity. For this reason it is necessary to design a 3-HP production pathway which controls the quantity of biochemical precursors in the cytosol such that the flux towards late stage biochemical intermediates in said 3-HP production pathway is favoured and alternative biological pathways are disfavoured.

EP 2505656 discloses a method of producing 3-HP using a malonic semialdehyde reducing pathway, wherein the process utilises an NADPH dependent malonyl-CoA reductase which may be derived from *C. aurantiacus* and an NADP/NADPH dependent GAPDH variant to resolve a redox imbalance within the metabolic process. The maximum reported yield of 3-HP was approximately 1.3 g/L.

Rathnasingh et al. (J. Biotechnol. 2012) discloses a method of producing 3-HP using *Escherichia coli* cells, wherein said cells overexpress MCR from *C. aurantiacus* and ACC in the malonyl-CoA pathway. The maximum reported yield of 3-HP was 2.14 mmol/L (0.19 g/L).

WO 2008/080124 discloses a method of producing butanol using modified yeast, wherein said method produces increased quantities of cytosolic acetyl-CoA by overexpressing PDC1 and ALD6 which may be derived from *S. cerevisiae* and ACS which may be derived from *S. entherica*. This method does not utilise the malonyl-CoA pathway.

WO 2007/024718 discloses a method of producing isoprenoid compounds using genetically modified host cells, wherein said cells are modified to produce increased levels of acetyl-CoA by increasing ALD and ACS activity. This method does not utilise the malonyl-CoA pathway.

In *S. cerevisiae*, acetyl-CoA carboxylase is tightly regulated at the transcriptional, translational and post-translational levels (Shirra, M.K. et al, 2001; Nielsen, J. 2009). At the level of the protein, Snf1 kinase is the major kinase which phosphorylates and inactivates ACC1 in vivo (Shirra, M.K. et al, 2001). WO 2012/017083 discloses a method of producing wax esters using modified yeast, wherein the quantity of cytosolic acetyl-CoA is increased through increasing the activity of ACC1 by mutating ACC1 at dephosphorylation sites such that it is no longer inactivated by Snf1.

Description of the Invention

The present invention relates to a recombinant yeast cell which produces high supernatant concentrations (up to 10 g/L) of 3-HP by increasing the flux towards cytosolic malonyl-CoA, which is reduced to 3-HP by malonyl-CoA reductase (MCR). The recombinant yeast cell overexpresses pyruvate decarboxylase (PDC), aldehyde dehydrogenase (ALD), Acetyl-CoA synthase (ACS), and a mutated Acetyl-CoA Carboxylase (ACC*) (the enzyme is mutated at two dephosphorylation sites to prevent inactivation by Snf1) which increases the conversion of pyruvate to malonyl-CoA. MCR derived from *Chloroflexus aurantiacus* reduces malonyl-CoA to 3-HP using NADPH as a cofactor. In order to resolve the resulting redox imbalance within the metabolic process the endogenous NAD dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is replaced with with an NADP dependent GAPDH variant.

None of the above cited art discloses the specific combination of the features of the herein described invention. Furthermore, the recombinant yeast of present invention produces 3-HP in substantially greater yields than the cited art.

In a first aspect the present invention relates to a yeast cell for use in producing 3-hydroxypropionic acid (3-HP), wherein said yeast cell incorporates genetic modification such that said cell expresses the enzymes:

- Pyruvate decarboxylase (PDC)
- Aldehyde dehydrogenase (ALD)
- Acetyl-CoA synthase (ACS)
- Acetyl-CoA carboxylase (ACC*) mutated in at least one dephosphorylation site to prevent inactivation by Snf1
- Malonyl-CoA reductase (MCR),

said cell has a reduced level of activity of NAD dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH), suitably by virtue of deletion, attenuation, disruption, down-regulation, or mutation of one or more genes expressing NAD dependent GAPDH and has at least one exogenous gene encoding NADP dependent GAPDH and/or has up-regulation of at least one endogenous gene expressing NADP dependent GAPDH. It has been found that combined expression of the enzymes NADP dependent GAPDH, PDC, ALD, ACS, ACC* and MCR in said host cell increases metabolic flux towards 3-HP via malonyl-CoA compared to an otherwise similar yeast cell lacking said genetic modification.

In a preferred embodiment of the invention the recombinant yeast strain comprises one or more exogenous nucleic acid molecules encoding at least one of PDC, ALD, ACS, ACC* and/or MCR. Preferably, said nucleic acid molecule is expressed from multiple integrations of said nucleic acid molecule in the host cell genome. Such a multiply integrated nucleic acid molecule may encode, for example, MCR.

The nucleic acid molecule encoding PDC may be derived from Saccharomyces cerevisiae. 'Derived from' is used herein to specify the species from which the original genetic material encoding the specified enzyme originated.

The nucleic acid molecule encoding ALD may be derived from *Saccharomyces cerevisiae*.

The nucleic acid molecule encoding ACS may be derived from *Salmonella enterica*.

The ACC* enzyme may be mutated in at least two dephosphorylation positions in the enzyme. In a preferred embodiment, the ACC* enzyme is mutated at amino acid positions Ser659 and Ser1157. Suitably Ser659 and Ser1157 are replaced by amino acids comprising side chains which are incapable of being phosphorylated, preferably Ala, Val, Leu, Ile, Pro, Phe, Trp and/or Met. The nucleic acid molecule encoding the non-mutated version of the ACC* enzyme may be derived from *Saccharomyces cerevisiae*.

The nucleic acid molecule encoding MCR may be derived from *Chloroflexus aurantiacus*.

The nucleic acid molecule encoding NADP dependent GAPDH may be derived from *Clostridium acetobutylicum*, *Kluyveromyces lactis* or *Bacillus subtilis*.

In another aspect, the present invention relates to a method for producing 3-HP, said method comprising culturing yeast cells as described herein under conditions such that 3-HP is produced.

In a preferred embodiment of the invention, the yeast cells are cultured on a medium comprising at least one carbon substrate, wherein said carbon substrate may be glucose or galactose.

Preferably, culturing said yeast cells on a medium comprising at least one carbon substrate produces a supernatant concentration of at least 5 g/L 3-HP, more preferably said yeast cells produce a supernatant concentration of at least 6 g/L 3-HP, more preferably said yeast cells produce a supernatant concentration of at least 7 g/L 3-HP, more preferably said yeast cells produce a supernatant concentration of at least 8 g/L 3-HP, and most preferably said yeast cells produce a supernatant concentration of at least 9 g/L 3-HP.

In another preferred embodiment of the invention, said method further comprises isolating 3-HP produced by said recombinant yeast strain.

Figures

Figure 1: Bar chart comparing yeast cells transformed with ACC comprising zero, one or two mutations at dephosphorylation sites.

Figure 2: Bar chart comparing the supernatant concentration of 3-HP produced by yeast cells transformed with a multicopy vector, a single integrative vector and a multiple integrative vector.

Figure 3: Bar chart comparing the supernatant concentration of 3-HP produced by yeast cells overexpressing ACS, ALD6 and/or PDC1 in both DELFT and FIT media.

Figure 4: Bar chart comparing the supernatant concentration of 3-HP produced by yeast cells with increased pool of available NADPH and those with no increased pool of available NADPH.

Figure 5: Bar chart demonstrating the effect of replacing the coding sequence of endogenous NAD dependent GAPDH with NADP dependent GAPDH on supernatant concentration of 3-HP in both DELFT and FIT media.

Figure 6: Bar chart comparing the supernatant concentration of 3-HP produced by yeast cells with improved NADPH supply in DELFT and FIT media.

Figure 7: Graph showing the supernatant concentration of 3-HP, glucose, glycerol, acetate and ethanol vs time in an N and C limited fed batch fermentation of highest producing yeast strain ST687

Figure 8: Graph showing the supernatant concentration of 3-HP, glucose, glycerol, acetate and ethanol vs time in a C limited fed batch fermentation of the highest producing yeast strain ST687

Examples

Table 1. Oligonucleotide sequences

Oligo name	**Oligo sequence 5'--> 3'**	**Seq ID NO**
ACC1m_fw	CGTGCGAUTCATTTCAAAGTCTTCAACAATTT	Seq ID NO 11
ACC1m_rv	AGTGCAGGUAAAACAATGAGCGAAGAAAGCTTA	Seq ID NO 12
CaMCR_fw_NEW	ATCTGTCAUAAAACAATGAGTGGTACAGGTAG	Seq ID NO 13
CaMCR_rv_NEW	CACGCGAUTCAGACTGTAATGGCTCTACCTC	Seq ID NO 14
PTEF1_fw	ACCTGCACUTTGTAATTAAAACTTAG	Seq ID NO 15

PPGK1_rv	ATGACAGAUTTGTTTTATATTTGTTG	Seq ID NO 16
ACC1-WT-UP	ATTTGCGGCCGCTTTAGTTTCTACCATGAGCGAAG	Seq ID NO 17
ACC1-WT-DOWN	GGCGAGCTCGCAAGGTTTATTTCAAAGTCTT	Seq ID NO 18
F-1-DOWNz	CATATGACAAATCTGAAACAGCAACAGCCCTGTTCATACC	Seq ID NO 19
F-2-UP	GGTATGAACAGGGCTGTTGCTGTTTCAGATTTGTCATATG	Seq ID NO 20
F-3-DOWN	ATGGCAATCAAAAGACCACCATCAGCTAGTTGACGCAGTA	Seq ID NO 21
F-4-UP	TACTGCGTCAACTAGCTGATGGTGGTCTTTTGATTGCCAT	Seq ID NO 22

ACSse_U1_fw	AGTGCAGGUAAAACAATGTCACAAACACAC	Seq ID NO 23
ACSse_U1_rv	CGTGCGAUTCATGATGGCATAGCAATAG	Seq ID NO 24
ald6_U2_fw	ATCTGTCAUAAAACAATGACTAAGCTACACTTTGACAC	Seq ID NO 25
ald6_U2_rv	CACGCGAUTCACAACTTAATTCTGACAGCTTTTAC	Seq ID NO 26
pdc1_U1longer_fw	AGTGCAGGUAAAACAATGTCTGAAATTACTTTGGGTAAATATTTG	Seq ID NO 27
pdc1_U1longer_rv	CGTGCGAUTCATTGCTTAGCGTTGGTAGCAGCAGTC	Seq ID NO 28
PTEF1_rv	CACGCGAUGCACACACCATAGCTTC	Seq ID NO 29

Table 2. Primers and templates used to generate gene fragments for USER cloning and yeast transformation by PCR

Fragment name	Gene	Fw_primer	Rv_primer	Template DNA
ACC**<-	ACC1 $^{Ser659Ala,\ Ser1157Ala}$ from *S. cerevisiae*	ACC1m_fw	ACC1m_rv	pAD
->CaMCR	Malonyl-CoA reductase from *Chloroflexus aurantiacus*	CaMCR_fw_NEW	CaMCR_rv_NEW	pYC6
<-ScPTEF1-ScPPGK1->	Fused promoters of tef1 and pgk1 genes from *S. cerevisiae*	PTEF1_fw	PPGK1_rv	plasmid pSP-GM1
ACC-pTEFpPGK-CaMCR	ACC1**<-ScPTEF1-ScPPGK1->CaMCR	ACC1m_fw	CaMCR_rv_NEW	P298
ACSse<-	Acetyl-CoA synthetase from *Salmonella enterica*	ACSse_U1_fw	ACSse_U1_rv	P324
ScALD6->	Acetaldehyde dehydrogenase 6 from *S. cerevisiae*	ald6_U2_fw	ald6_U2_rv	*S. cerevisiae* gDNA

ScPDC1<-	Pyruvate decarboxylase isozyme 1 from *S. cerevisiae*	pdc1_U1longer_fw	pdc1_U1longer_rv	*S. cerevisiae* gDNA
<-ScPTEF1	TEF1 promoter from *S. cerevisiae*	PTEF1_fw	PTEF1_rv	*S. cerevisiae* gDNA

Table 3. Plasmids

Plasmid name	**Parent plasmid**	**Selection marker**	**Gene 1**	**Promoter**	**Gene 2**
P298	p054 pESC-URA-USER	URA3	ACC1**<- (Seq ID NO 1)	<-ScPTEF1-ScPPGK1-> (Seq ID NO 3)	->CaMCR (Seq ID NO 2)
P343	P0255 pX-2-loxP-KlURA3	KlURA3	ACC1**<- (Seq ID NO 1)	<-ScPTEF1-ScPPGK1-> (Seq ID NO 3)	->CaMCR (Seq ID NO 2)
P376	P322	KlURA3	ACC1**<-	<-ScPTEF1-ScPPGK1->	->CaMCR

			(Seq ID NO 1)	(Seq ID NO 3)	(Seq ID NO 2)
P474	P376	KlURA3	ACC1**<- (Seq ID NO 1)	<-ScPTEF1-ScPPGK1-> (Seq ID NO 3)	->CaMCR (Seq ID NO 2)
P380	p257 (pX-3-KlLEU2)	KlLEU2	ACSse<- (Seq ID NO 4)	<-ScPTEF1-ScPPGK1-> (Seq ID NO 3)	ScALD6-> (Seq ID NO 5)
P382	p258 (pX-4-LoxP-SpHiS5)	SpHIS5	ScPDC1<- (Seq ID NO 6)	<-ScPTEF1 (Seq ID NO 7)	

Table 4. Yeast strains

Strain	Parent strain	Genotype
CEN.PK102-5B		mata,ura3,his3,leu2
CEN.PK102-5D		mata, ura3
tdh3-null	CEN.PK102-5B	mata,ura3,his3,leu2,tdh3::LoxP
tdh1::CaGAPDH,tdh3	tdh3-null	mata,ura3,his3,leu2,tdh1::CaGAPDH-LoxP,tdh3::LoxP

tdh2::CaGAPDH,tdh3	tdh3-null	mata,ura3,his3,leu2,tdh2::CaGAPDH-LoxP,tdh3::LoxP
tdh1+2::CaGAPDH,tdh3	tdh2::CaGAPDH,tdh3	mata,ura3,his3,leu2,tdh1::CaGAPDH-LoxP,tdh2::CaGAPDH-LoxP,tdh3::LoxP
tdh3::CaGAPDH	CEN.PK102-5B	mata,ura3,his3,leu2,tdh3::CaGAPDH-LoxP
tdh1+3::CaGAPDH	tdh3::CaGAPDH	mata,ura3,his3,leu2,tdh1::CaGAPDH-LoxP,tdh3::CaGAPDH-LoxP
tdh2+3::CaGAPDH	tdh3::CaGAPDH	mata,ura3,his3,leu2,tdh2::CaGAPDH-LoxP,tdh3::CaGAPDH-LoxP
tdh1+2+3::CaGAPDH	tdh2+3::CaGAPDH	mata,ura3,his3,leu2,tdh1::CaGAPDH-LoxP,tdh2::CaGAPDH-LoxP,tdh3::CaGAPDH-LoxP

Example 1. Cloning of over-expression targets into expression plasmids.

All plasmids listed in table 3 were generated by USER cloning using PCR generated gene fragments, which were amplified according to table 2. The typical USER reaction was as follows: 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 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.

Example 2. ACC1 Engineering acetyl-CoA carboxylase for improving the production of 3-hydroxypropionic acid**

Ser659 and Ser1157 of ACC1 were identified as two putative phosphorylation sites according to the phosphorylation recognition motif (Hyd-X-Arg-XX-Ser-XXX-Hyd) for yeast Snf1 (Dale, S. et al, 1995). One of which, Ser1157 was verified by a phosphoproteome study (Ficarro, S. et al, 2002). Ser659 has not been reported through experimental data so far. Therefore, we have constructed mutated ACC1 with either one or two assumed phosphorylation sites.

The endogenous ACC1 gene (wild-type) encoding acetyl-CoA carboxylase was amplified from genomic DNA of CEN.PK.113-5D by PCR with Phusion high-fidelity polymerase. The primers are listed in Table 1. The single mutatation ACC1^{Ser1157Ala} and double mutation ACC1$^{Ser659Ala, Ser1157Ala}$ were introduced by oligonucleotide primers. Three versions of ACC1 were digested with NotI and SacI, and then ligated into the corresponding sites of pSP-GM2 (Chen et al., 2012), resulting in plasmid pAW (containing wild-type ACC1), pAS (containing single mutated ACC1) and pAD (containing double mutated ACC1), respectively.

For re-constructing the pathway for 3-HP production, the gene CaMCR encoding malonyl-CoA reductase from *Chloroflexus aurantiacus* was codon optimized for expression in yeast and synthesized by GenScript (Piscataway, NJ, USA). CaMCR was cloned into pIYC04 (Chen et al., 2013) using the BamHI and XhoI cloning sites downstream of the TEF1 promoter, resulting in plasmid pYC6. To evaluate the effect of engineered ACC1 on 3-HP production, plasmids combinations pSP-GM2 / pYC6, pAW / pYC6, pAS / pYC6 and pAD / pYC6 were transformed into CEN.PK 113-11C to construct yeast recombinant strain HPY15 to HPY18, respectively.

For the cultivation of yeast recombinant strains, 20 ml cultures in 100 ml unbaffled cotton-stopped flasks were inoculated with an amount of pre-culture that resulted in a final optical density of 0.02 at 600 nm (OD600). The strains were grown at 30 °C with 180 r.p.m. orbital shaking in defined minimal medium with 20 g l^{-1} glucose as described before (Chen et al., 2013). Samples were taken periodically to measure the cell mass, concentration of 3-HP, residual glucose and other metabolites.

The results are shown in figure 1. Overexpression of wild-type ACC1 HPY16 increased 3-HP production by 60%, compared to the reference strain HPY15. Overexpression of mutated ACC1 by blocking the phosphorylation sites, HPY17 and HPY18, further enhanced the production of 3-HP. Double mutated ACC1 $^{Ser659Ala, Ser1157Ala}$ HPY18 gave the highest improvement, around three-fold, relevant to that of the reference strain.

Example 3. Production of 3HP in *S. cerevisiae* by over-expression of CaMCR and ACC1 from multiple integration plasmids**

CEN.PK102-5D was transformed with either episomal multicopy plasmid p298, or single integrative plasmid p343, or multiple integration plasmid p376. All three plasmids tested harboured ACC1** and CaMCR. Four single transformants for each plasmid tested were inoculated in 0.5 ml SC ura- 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 (Delft medium described in WO 2011/147818) in a 96-deep well plate and 0.5 ml FIT Fed-batch-media (M2P labs). 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 a spectrophotometer (BioTek).

The culture broth was spun down and the supernatant analyzed for 3-hydroxypropionic acid concentration using enzymatic assay, which was performed 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 a multichannel pipette. The start absorbance at 340 nm was measured and the plate was sealed and incubated at 30°C for 1.5 hours. After incubation the 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 each sample was calculated from the standard curve.

Expression of ACC1** and CaMCR from the multiple integration plasmid p376 led to a 5 times improvement of 3HP production in the best clone, when compared to a *S. cerevisiae* strain bearing a single integrative vector with the same genes (figure 2).

Example 4. Improving 3HP production in *S. cerevisiae* by increasing the precursor supply towards Acetyl-CoA

Strains harbouring either p380-ALD6-ACS or p380-ALD6-ACS in combination with p382-PDC1 were transformed with p474-CaMCR-ACC1**. A minimum of 6 clones were picked, fermented and tested for 3HP production by enzymatic assay as in example 2 (figure 3). The best producer of strains having p380-ALD6-ACS in combination with p474-CaMCR-ACC1** gave up to 1.5 fold higher 3HP titer than the wild type (WT) strain with p474-CaMCR-ACC1**, and strains with all three plasmids combined gave up to 2.5 times more than the WT background in both DELFT and FIT media.

Example 5. Effect of Increasing the pool of available NADPH on the production of 3-hydroxypropionic acid

The effect of increasing NADPH supply on the production of 3-hydroxypropionic acid was tested. The gapN gene from

Streptococcus mutants, which encodes non-phosphorylating NADP-dependent glyceraldehyde-3-phosphate dehydrogenase, was codon optimized and synthesized by GeneScript (Piscataway, NJ, USA). The gene gapN was cloned into pIYC04 (Chen et al., 2013) using the restriction sites NotI and SacI, resulting in plasmid pJC2. Plasmids pJC2 and pYC1 were transformed into CEN.PK 113-11C, forming the recombinant yeast strain HPY09. It was found that the over-expression of gapN alone resulted in a final titer of 122 mg l^{-1} 3-HP, which is a 30% improvement compared to the reference strain (figure 4).

Example 6. Construction of strain with improved NADPH supply

An elevated level of NADPH was achieved by overexpression of an NADP dependent glyceraldehyde-3-phosphate dehydrogenase gene from either *Clostridium acetobutylicum*, CaGAPDH (Seq ID NO 08), *Kluyveromyces lactis*, KlGAPDH (Seq ID NO 9), or *Bacillus subtilis*, BsGapB (Seq ID NO 10). The NADP dependent GAPDH was expressed in yeast strains, where one, two or three of the endogenous NAD dependent glyceraldehyde-3-phosphate dehydrogenase genes TDH1-3 were deleted and/or exchanged with the CDS of GAPDH. By exchanging the CDS we aimed to ensure that the introduced GAPDH had the same expression profile as the endogenous NAD dependent GAPDH. Additionally, any potential futile cycling between the endogenous GAPDH and the introduced GAPDH was avoided by removing or lowering the level of endogenous GAPDH activity. Eight different combinations were made according to Table 4. Each of those eight strains and a WT strain were all transformed with p380-ALD6-ACS in combination with p382-PDC1 and p474-CaMCR-ACC1**. A minimum of 12 clones for each strain were tested for 3HP production as in example 2 (figure 5). In both media tested, there was a significant increase in 3HP yield for the strains where TDH3 was replaced with CaGAPDH. However, there was no further effect when this exchange was combined with any of the other CDS exchanges. The three best producers for the WT and the tdh3::CaGAPDH strains were analyzed further by HPLC (figure 6). The 'WT' strains gave 1.46 ±0.14 g/l and 3.31 ±0.34 g/l 3HP in DELFT and FIT, respectively. The 'tdh3::CaGAPDH' strains gave 2.01 ±0.01 g/l and 5.10 ±0.22 g/l 3HP in DELFT and FIT, respectively. Furthermore, the ratio between 3HP formed and glycerol formed was higher for the tdh3::CaGAPDH strains in both media tested.

The best producer among the tdh3::CaGAPDH strains was named ST687 and was used in future fermentation experiments.

Example 7. Fermentations of high producing strain (ST687)

Strain ST687 was fermented under two different fermentation regimes; 1, N and C limited fed batch, and 2, C limited fed batch.

	N and C limited fed batch	**C limited fed batch**
Parameter	Value	Value
Reactor number	A1, A3, B2	A1, A2, A3
Organism	S. cerevisiae	S. cerevisiae
Strain	ST687	ST687

Batch medium	Mix per reactor: 20 ml $(NH_4)_2SO_4$ (100 g/L) , 25 ml KH_2PO_4 (120 g/L) , 10 ml $MgSO_4$, $7H_2O$ (50 g/L), 1 ml trace metals, 0.2 ml antifoam, add water to 500 ml. Separatelly autoclave 110 g dextrose in 500 ml water, add 100 ml of this glucose solution to reactor after autoclavation. Also add 1 ml vitamins.	Mix per reactor: 75 ml $(NH_4)_2SO_4$ (100 g/L) , 25 ml KH_2PO_4 (120 g/L) , 10 ml $MgSO_4$, $7H_2O$ (50 g/L), 2 ml trace metals, 0.2 ml antifoam, add water to 500 ml. Separatelly autoclave 110 g dextrose in 500 ml water, add 40 ml of this glucose solution to reactor after autoclavation. Also add 1 ml vitamins.
Feed medium	Mix per feed bottle: 0.5 L of 200 g/L glucose solution. Add about 100 ml (20 g) before inoculation to start the batch phase, then add the rest during the fed-batch phase.	Mix per feed bottle: 225 ml $(NH_4)_2SO_4$ (100 g/L) , 75 ml KH_2PO_4 (120 g/L) , 30 ml $MgSO_4$, $7H_2O$ (50 g/L), 6 ml trace metals, 0.3 ml antifoam. This will make a total of 336 ml. Add the remaining glucose solution (160 ml) to the feed bottle after autoclavation. Also add 3 ml vitamins.
Temperature	30°C	30°C
pH	5	5
pH control	with 2M NaOH	with 2M NaOH
DO	not controlled	controlled at >20% by stirring speed and aeration

Working volume	batch with 0.5L, then fill up to 1 L during fed-batch	batch with 0.5L, then fill up to 1 L during fed-batch
Agitation	800 rpm	800 rpm (variable in the fed-batch phase)
Aeration	1 vvm (1 L/min)	1 vvm (1 L/min) (variable in the fed-batch phase)
Aeration gas	Air	Air
Fermentation lengh	70 hours	120 hours
Sampling frequency	2-3 times a day	2 times a day

The inoculum was prepared as follows. A stock tube of ST/687 was inoculated into 50 ml SC-ura-his-leu and grown overnight at 30°C. 400 ml fresh medium is added and divided into 3 flasks, 150 ml in each and grown overnight at 30°C. The cultures from overnight shake flasks is combined to obtain a total of about 450 ml, which then is poured into 6x50ml Falcon tubes. Tubes are spun 4,000xg for 2 min and supernatant is discarded. The rest of the overnight culture is added to the 6 tubes (about 25 ml/tube), resuspended, and pooled into 2 tubes into one to end up with 3 tubes. Inoculate 1 tube per reactor.

Each sample was analyzed by HPLC as in example 4. The results are summarized in table below and in figures 7 and 8.

	N and C limited	C limited
Titers (3-HP)	9.5 g/L	9.83 ± 0.43 g/L
Prod. Rate in fed batch phase	0.20 g/L/h	0.09 ± 0.01 g/L/h
Specific yield, g/g DW	1.01 g/g DW	0.69 ± 0.05 g/g DW
Overall yield, % C-mol/C-mol glucose	18 %	13 ± 1 %

Both fermentations involving the best 3-HP producing yeast strain ST687 produced supernatant concentrations of >9 g/L 3-HP. This is a significant increase over the supernatant concentrations disclosed in the prior art.

References

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Nielsen, J. Systems biology of lipid metabolism: From yeast to human. FEBS Letters **583**, 3905-3913 (2009).

Rathnasingh, C., Raj, S. M., Lee, Y., Catherine, C., Ashok, S., Park, S., J. Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant Escherichia coli strains. J. Biotechnol., 633-640 (2012).

Shirra, M.K. et al. Inhibition of Acetyl Coenzyme A Carboxylase Activity Restores Expression of the INO1 Gene in a snf1 Mutant Strain of Saccharomyces cerevisiae. Mol. Cell. Biol. **21**, 5710-5722 (2001).

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 invention may be summarised according to the following clauses:

1. A yeast cell for use in producing 3-hydroxypropionic acid (3-HP), wherein said yeast cell incorporates genetic modification such that said cell expresses the enzymes:

- Pyruvate decarboxylase (PDC)
- Aldehyde dehydrogenase (ALD)
- Acetyl-CoA synthase (ACS)
- Acetyl-CoA carboxylase (ACC*) mutated in at least one dephosphorylation site to prevent inactivation by Snf1
- Malonyl-CoA reductase (MCR),

said cell has a reduced level of activity of NAD dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by virtue of deletion, attenuation, disruption, down-regulation, or mutation of one or more genes expressing NAD dependent GAPDH and has at least one exogenous gene encoding NADP dependent

GAPDH and/or has up-regulation of at least one endogenous gene expressing NADP dependent GAPDH, and

wherein combined expression of the enzymes NADP dependent GAPDH, PDC, ALD, ACS, ACC* and MCR in said host cell increases metabolic flux towards 3-HP via malonyl-CoA compared to an otherwise similar yeast cell lacking said genetic modification.

2. A yeast cell as defined in clause 1, comprising one or more exogenous nucleic acid molecules encoding at least one of PDC, ALD, ACS, ACC* and/or MCR.

3. A yeast cell as defined in clause 2, wherein a said nucleic acid molecule is expressed from multiple integrations of said nucleic acid molecule in the host cell genome.

4. A yeast cell as defined in clause 2, wherein a nucleic acid molecule encoding PDC is derived from *Saccharomyces cerevisiae*.

5. A yeast cell as defined in clause 2, wherein the nucleic acid molecule encoding ALD is derived from *Saccharomyces cerevisiae*.

6. A yeast cell as defined in clause 2, wherein a nucleic acid molecule encoding ACS is derived from *Salmonella entherica*.

7. A yeast cell as defined in clause 2, wherein a ACC* enzyme is mutated in at least two dephosphorylation positions in the enzyme.

8. A yeast cell as defined in clause 7, wherein the ACC* enzyme is mutated at amino acid positions Ser659 and Ser1157, wherein Ser659 and Ser1157 are replaced by amino acids

comprising side chains which are incapable of being phosphorylated.

9. A yeast cell as defined in clause 8, wherein said amino acids comprising side chains which are incapable of being phosphorylated are Ala, Val, Leu, Ile, Pro, Phe, Trp, Met.

10. A yeast cell as defined in clause 8, wherein the nucleic acid molecule encoding the non-mutated version of the ACC* enzyme is derived from *Saccharomyces cerevisiae*.

11. A yeast cell as defined in clause 2, wherein a nucleic acid molecule encoding MCR is derived from *Chloroflexus aurantiacus*.

12. A yeast cell as defined in clause 1, wherein a nucleic acid molecule encoding NADP dependent GAPDH is derived from *Clostridium acetobutylicum*, *Kluyveromyces lactis* or *Bacillus subtilis*.

13. A method for producing 3-HP, said method comprising culturing yeast cells as claimed in any of the preceding claims under conditions such that 3-HP is produced.

14. A method as defined in clause 13, wherein said yeast cells are cultured on a medium comprising at least one carbon substrate.

15. A method as defined in clause 14, wherein said carbon substrate is glucose, xylose, arabinose, or galactose.

16. A method as defined in clause 13, wherein said yeast cells produce a supernatant concentration of at least 5 g/L 3-HP.

17. A method as defined in clause 13, wherein said yeast cells produce a supernatant concentration of at least 6 g/L 3-HP.

18. A method as defined in clause 13, wherein said yeast cells produce a supernatant concentration of at least 7 g/L 3-HP.

19. A method as defined in clause 13, wherein said yeast cells produce a supernatant concentration of at least 8 g/L 3-HP.

20. A method as defined in clause 13, wherein said yeast cells produce a supernatant concentration of at least 9 g/L 3-HP. n

21. A method as defined in clause 13, wherein said method further comprises isolating 3-HP produced by said yeast cells.

CLAIMS

1. A yeast cell for use in producing 3-hydroxypropionic acid (3-HP), wherein said yeast cell incorporates genetic modification such that said cell expresses the enzymes:

- Pyruvate decarboxylase (PDC)
- Aldehyde dehydrogenase (ALD)
- Acetyl-CoA synthase (ACS)
- Acetyl-CoA carboxylase (ACC*) mutated in at least one dephosphorylation site to prevent inactivation by Snf1
- Malonyl-CoA reductase (MCR),

and has a reduced level of activity of NAD dependent glyceraldehyde-3-phosphate dehydrogenase (GAPDH) by virtue of deletion, attenuation, disruption, down-regulation, or mutation of one or more genes expressing NAD dependent GAPDH and has at least one exogenous gene encoding NADP dependent GAPDH and/or has up-regulation of at least one endogenous gene expressing NADP dependent GAPDH, and

wherein combined expression of the enzymes NADP dependent GAPDH, PDC, ALD, ACS, ACC* and MCR in said host cell increases metabolic flux towards 3-HP via malonyl-CoA compared to an otherwise similar yeast cell lacking said genetic modification.

2. A yeast cell as claimed in claim 1, comprising one or more exogenous nucleic acid molecules encoding at least one of PDC, ALD, ACS, ACC* and/or MCR.

3. A yeast cell as claimed in claim 2, wherein a said nucleic acid molecule is expressed from multiple integrations of said nucleic acid molecule in the host cell genome.

4. A yeast cell as claimed in claim 2, wherein a nucleic acid molecule encoding PDC is derived from *Saccharomyces cerevisiae*.

5. A yeast cell as claimed in claim 2, wherein the nucleic acid molecule encoding ALD is derived from *Saccharomyces cerevisiae*.

6. A yeast cell as claimed in claim 2, wherein a nucleic acid molecule encoding ACS is derived from *Salmonella entherica*.

7. A yeast cell as claimed in claim 2, wherein a ACC* enzyme is mutated in at least two dephosphorylation positions in the enzyme.

8. A yeast cell as claimed in claim 7, wherein the ACC* enzyme is mutated at amino acid positions Ser659 and Ser1157, wherein Ser659 and Ser1157 are replaced by amino acids comprising side chains which are incapable of being phosphorylated.

9. A yeast cell as claimed in claim 8, wherein the nucleic acid molecule encoding the non-mutated version of the ACC* enzyme is derived from *Saccharomyces cerevisiae*.

10. A yeast cell as claimed in claim 2, wherein a nucleic acid molecule encoding MCR is derived from *Chloroflexus aurantiacus*.

11. A yeast cell as claimed in claim 1, wherein a nucleic acid molecule encoding NADP dependent GAPDH is derived from *Clostridium acetobutylicum*, *Kluyveromyces lactis* or *Bacillus subtilis*.

12. A method for producing 3-HP, said method comprising culturing yeast cells as claimed in any of the preceding claims under conditions such that 3-HP is produced.

13. A method as claimed in claim 13, wherein said yeast cells are cultured on a medium comprising at least one carbon substrate.

14. A method as claimed in claim 13, wherein said yeast cells produce a supernatant concentration of at least 5 g/L 3-HP.

15. A method as claimed in claim 13, wherein said method further comprises isolating 3-HP produced by said yeast cells.

Figure 1.

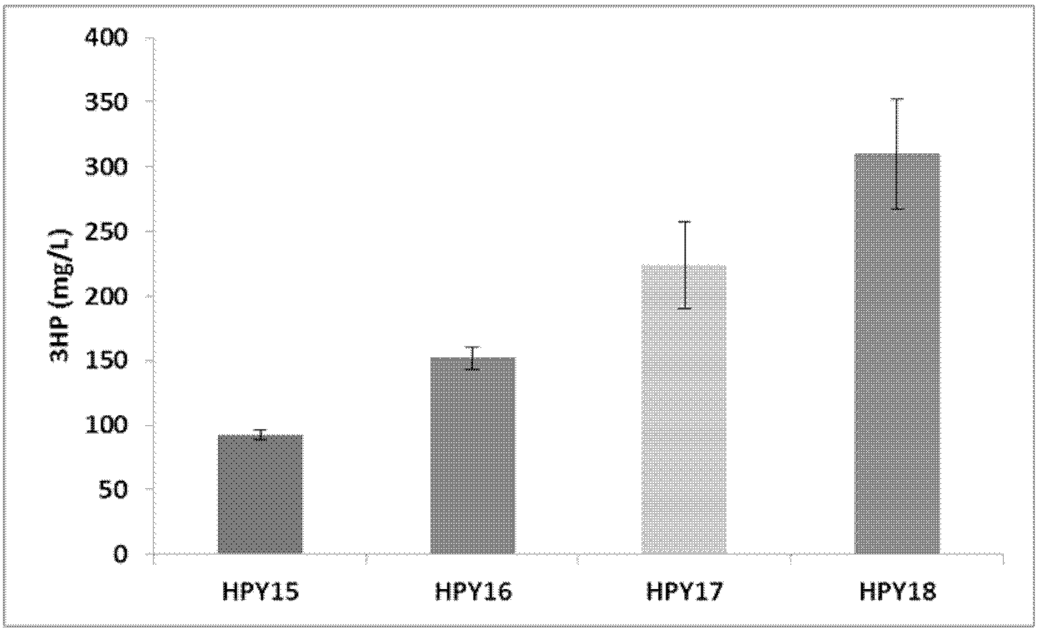

3HP (mg/L)
400
350
300
250
200
150
100
50
0
HPY15
HPY16
HPY17
HPY18

Figure 2.

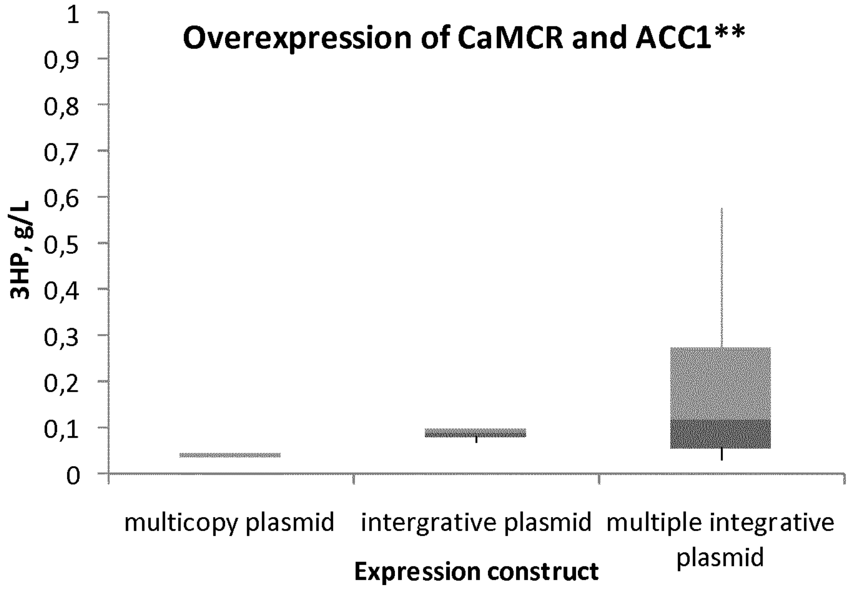

Overexpression of CaMCR and ACC1**
3HP, g/L
1
0,9
0,8
0,7
0,6
0,5
0,4
0,3
0,2
0,1
0
multicopy plasmid
intergrative plasmid
multiple integrative plasmid
Expression construct

Figure 3.

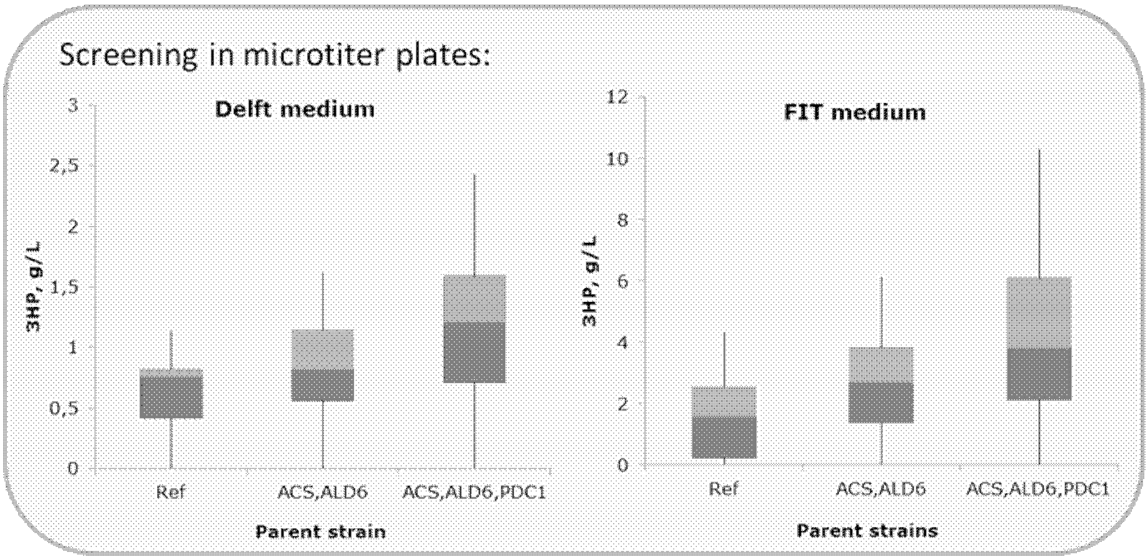

Screening in microtiter plates:
Delft medium
3HP, g/L
3
2,5
2
1,5
1
0,5
0
Ref
ACS,ALD6
ACS,ALD6,PDC1
Parent strain
FIT medium
3HP, g/L
12
10
8
6
4
2
0
Ref
ACS,ALD6
ACS,ALD6,PDC1
Parent strains

Figure 4.

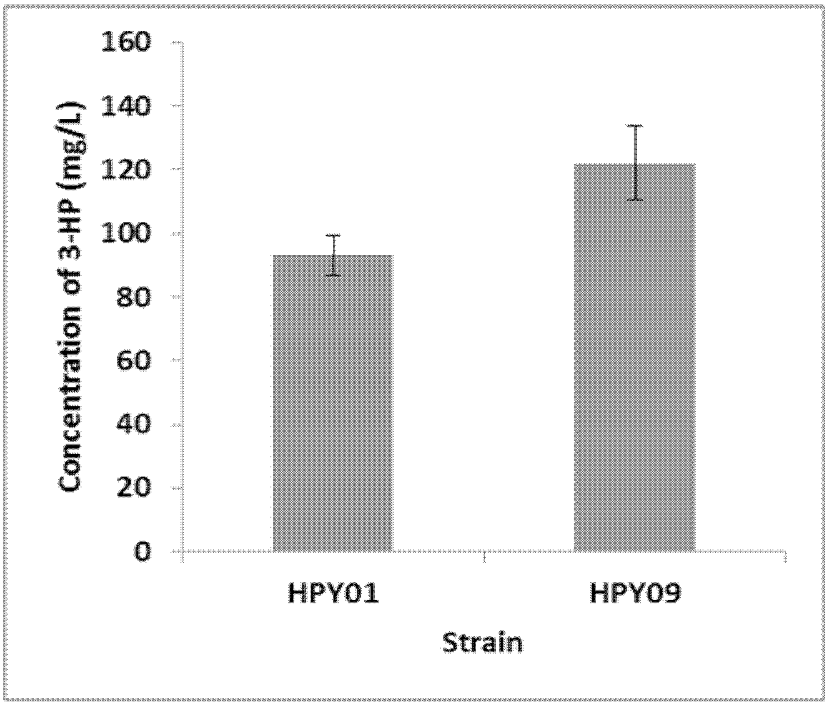

Concentration of 3-HP (mg/L)
160
140
120
100
80
60
40
20
0
HPY01
HPY09
Strain

Figure 5.

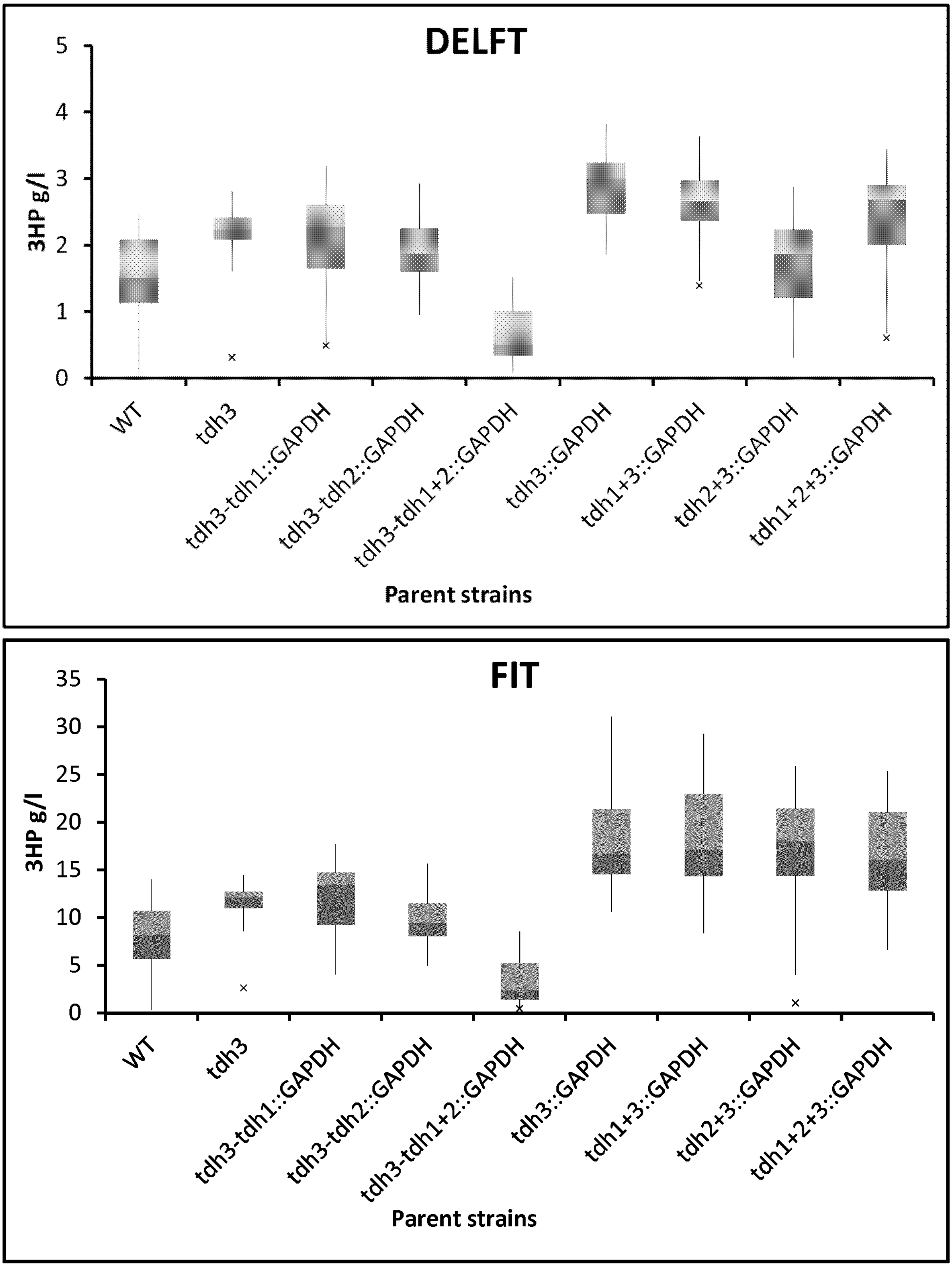

DELFT
3HP g/l
5
4
3
2
1
0
WT
tdh3
tdh3-tdh1::GAPDH
tdh3-tdh2::GAPDH
tdh3-tdh1+2::GAPDH
tdh3::GAPDH
tdh1+3::GAPDH
tdh2+3::GAPDH
tdh1+2+3::GAPDH
Parent strains
FIT
3HP g/l
35
30
25
20
15
10
5
0
WT
tdh3
tdh3-tdh1::GAPDH
tdh3-tdh2::GAPDH
tdh3-tdh1+2::GAPDH
tdh3::GAPDH
tdh1+3::GAPDH
tdh2+3::GAPDH
tdh1+2+3::GAPDH
Parent strains

Figure 6.

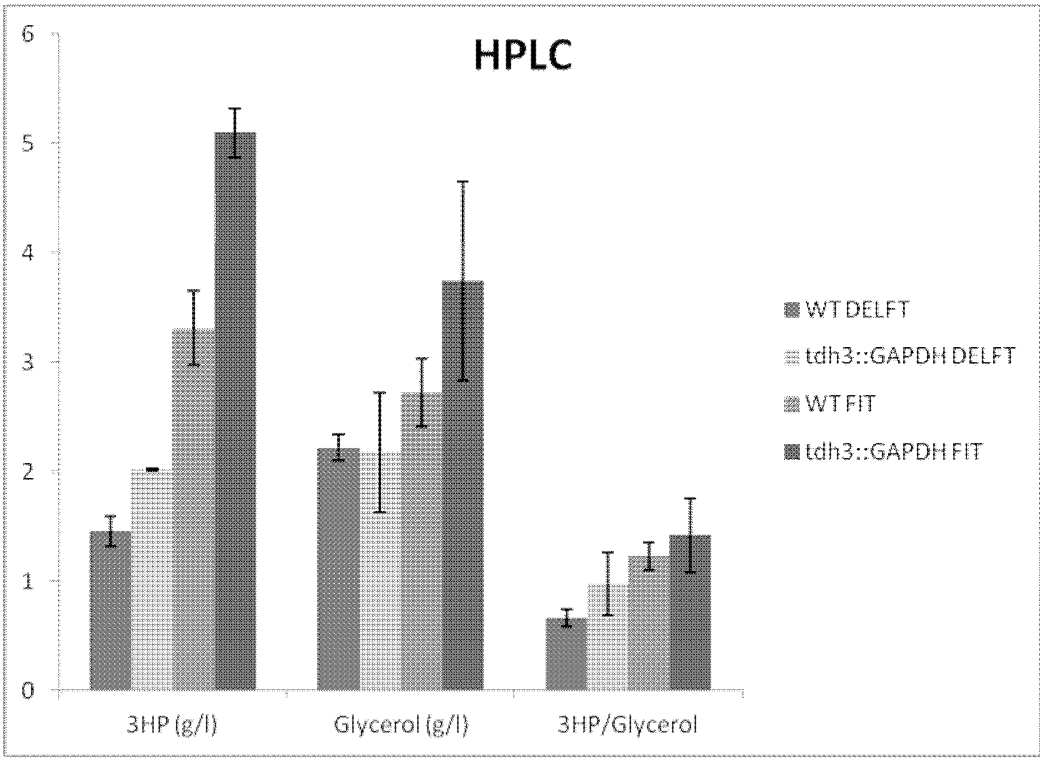

HPLC
6
5
4
3
2
1
0
3HP (g/l)
Glycerol (g/l)
3HP/Glycerol
WT DELFT
tdh3::GAPDH DELFT
WT FIT
tdh3::GAPDH FIT

Figure 7.

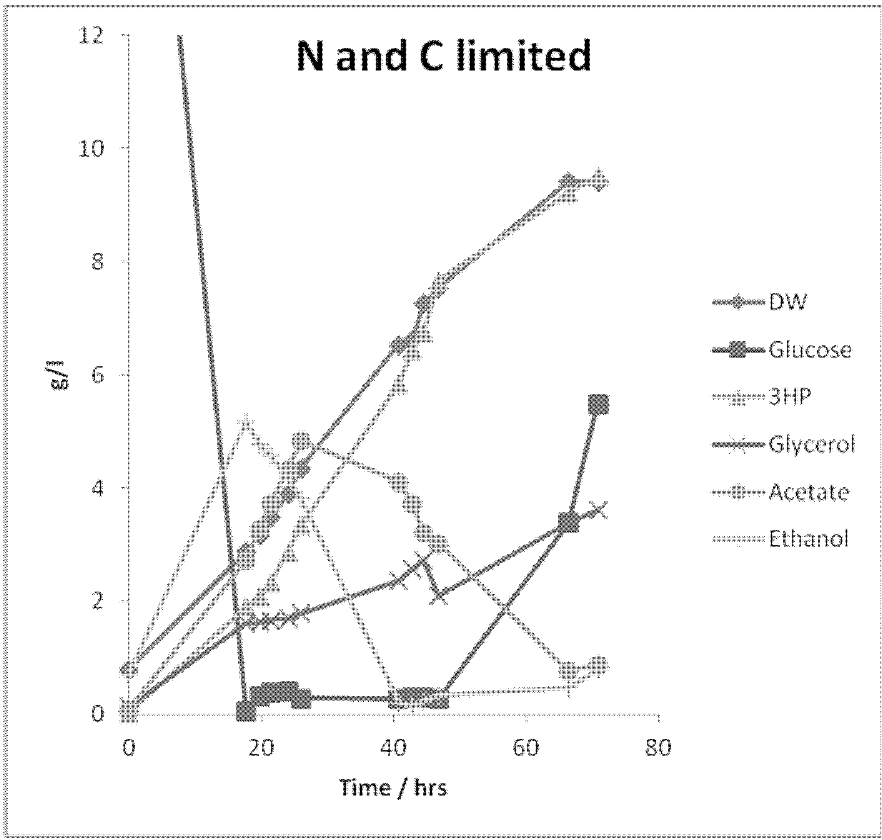
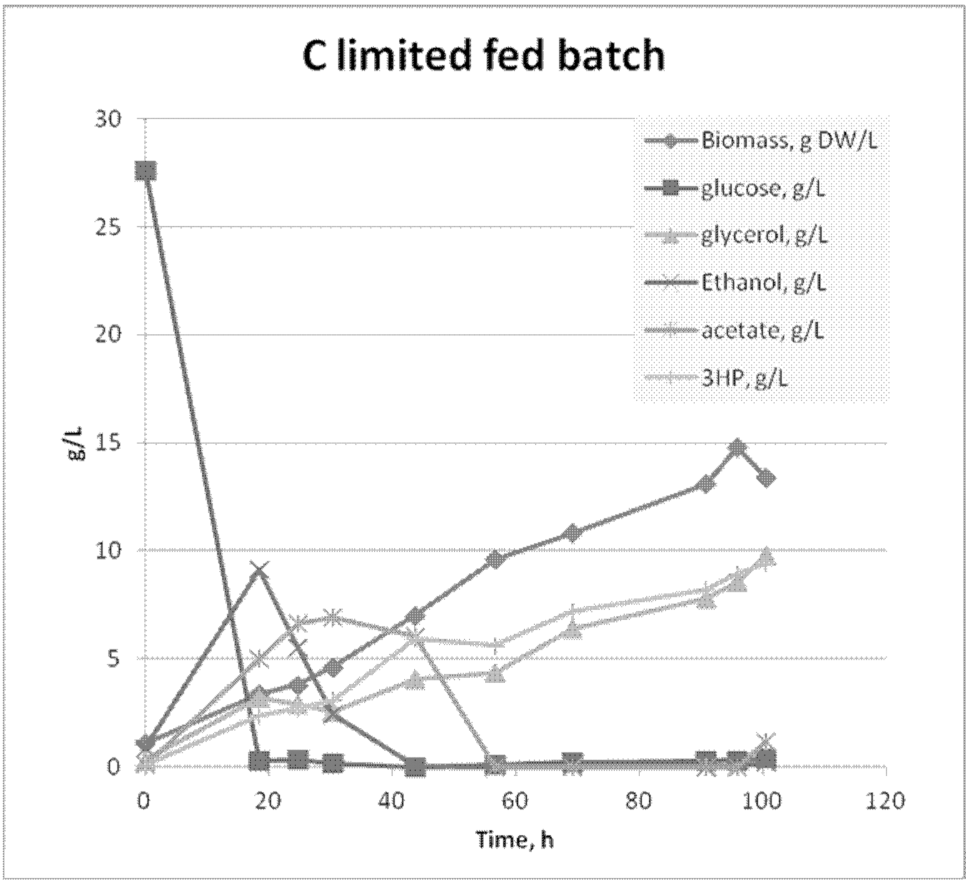

N and C limited
g/l
Time / hrs
0
2
4
6
8
10
12
0
20
40
60
80
DW
Glucose
3HP
Glycerol
Acetate
Ethanol

Figure 8.


C limited fed batch
30
25
20
15
10
5
0
g/L
0
20
40
60
80
100
120
Time, h
Biomass, g DW/L
glucose, g/L
glycerol, g/L
Ethanol, g/L
acetate, g/L
3HP, g/L

INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2014/062246

A. CLASSIFICATION OF SUBJECT MATTER
INV. C12N9/02 C12N9/00 C12P7/42 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)
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, BIOSIS, EMBASE, 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	WO 02/42418 A2 (CARGILL INC [US]; GOKARN RAVI R [US]; SELIFONOVA OLGA V [US]; JESSEN H) 30 May 2002 (2002-05-30) the whole document -----	1-15
Y	WO 2012/017083 A1 (NIELSEN JENS [SE]; SHI SHUOBO [SE]) 9 February 2012 (2012-02-09) the whole document -----	1-15
Y	US 2013/066035 A1 (BURGARD ANTHONY P [US] ET AL) 14 March 2013 (2013-03-14) the whole document -----	1-15
Y	WO 2008/028002 A1 (RICE UNIVERSITY [US]; SAN KA-YIU [US]; BENNETT GEORGE [US]; LIN HENRY) 6 March 2008 (2008-03-06) the whole document -----	1-15
	-/--	

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

[X] See patent family annex.

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"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
16 July 2014	11/08/2014

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	Roscoe, Richard

1

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

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2012/135481 A1 (JESSEN HOLLY [US] ET AL) 31 May 2012 (2012-05-31) the whole document -----	1-15
A	CHELLADURAI RATHNASINGH ET AL: "Production of 3-hydroxypropionic acid via malonyl-CoA pathway using recombinant Escherichia coli strains", JOURNAL OF BIOTECHNOLOGY, ELSEVIER SCIENCE PUBLISHERS, AMSTERDAM, NL , vol. 157, no. 4 20 February 2012 (2012-02-20), pages 633-640, XP002706395, ISSN: 0168-1656, DOI: 10.1016/J.JBIOTEC.2011.06.008 Retrieved from the Internet: URL:http:/www.sciencedirect.com/science/article/pii/S0168165611003075 [retrieved on 2011-06-23] the whole document -----	1-15
A	VERHO R ET AL: "IDENTIFICATION OF THE FIRST FUNGAL NADP-GADPH FROM KLUYVEROMYCES LACTIS", BIOCHEMISTRY, AMERICAN CHEMICAL SOCIETY, US, vol. 41, no. 46, 19 November 2002 (2002-11-19), pages 13833-13838, XP008015402, ISSN: 0006-2960, DOI: 10.1021/BI0265325 the whole document -----	1-15
A	WOODS ET AL: "Yeast SNF1 is functionally related to mammalian AMP-activated protein kinase and regulates acetyl-CoA carboxylase in vivo.", JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 269, no. 30, 1 July 1994 (1994-07-01) , pages 19509-19515, XP055068978, ISSN: 0021-9258 the whole document -----	1-15

1

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/EP2014/062246

Patent document cited in search report		Publication date	Patent family member(s)			Publication date
WO 0242418	A2	30-05-2002	AR	031480	A1	24-09-2003
			AR	046846	A2	28-12-2005
			AU	1981802	A	03-06-2002
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			CA	2429039	A1	30-05-2002
			CN	1556855	A	22-12-2004
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			MX	PA03004324	A	26-01-2004
			US	2004076982	A1	22-04-2004
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			US	2008076167	A1	27-03-2008
			US	2009053783	A1	26-02-2009
			US	2012077236	A1	29-03-2012
			US	2012244586	A1	27-09-2012
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			US	2013323808	A1	05-12-2013
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			WO	2013036764	A1	14-03-2013
WO 2008028002	A1	06-03-2008	US	2010009418	A1	14-01-2010
			WO	2008028002	A1	06-03-2008
US 2012135481	A1	31-05-2012	AU	2011336923	A1	30-05-2013
			CA	2818499	A1	07-06-2012
			CN	103502432	A	08-01-2014
			EP	2643450	A2	02-10-2013
			JP	2013542747	A	28-11-2013
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			US	2012135481	A1	31-05-2012
			US	2014065681	A1	06-03-2014
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