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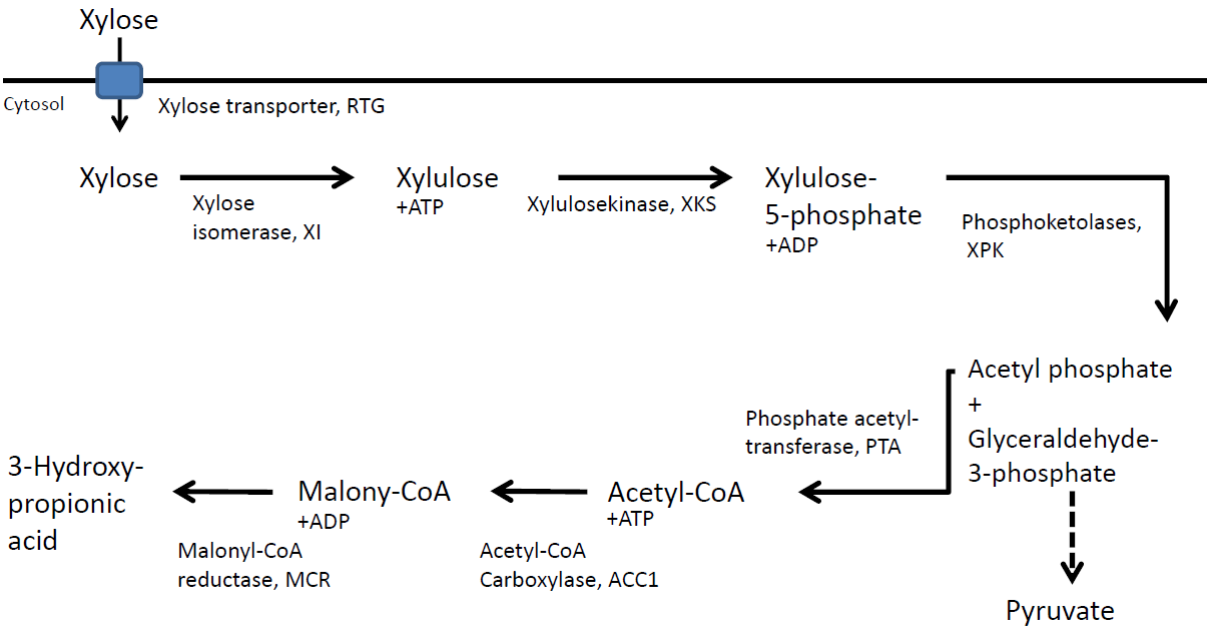

A platform for Acetyl-CoA synthesis using xylose as feedstock in Saccharomyces cerevisiae

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Foreword

I would like to thank my supervisor Yun Chen, Director at the Systems and Synthetic Biology group at Chalmers University of Technology, for his help and guidance in this project. Further this project would not have happen without Jens Nilsen and the whole of the Sysbio department, especially Alexandra Bergman and Zongjie Dai. Finally I would like to thank Jeroen Koendjbiharie for sharing his material and thoughts.

Contents

Abbreviations used

3-Hydroxypropionic acid (3-HP)
5-Fluoroorotic acid (5-FOA)
Acetate kinase (ACK)
Acetatealdehyde dehydrogenase (ALD)
Acetyl Co-enzyme A (Acetyl-CoA)
Acetyl Co-enzyme A carboxylase 1 (ACC1)
Acetyl phosphate (AcP)
Acetyl-CoA synthetase (ACS)
Alcohol dehydrogenase (ADH)
Aldolase reductase (AR)
Carbon capture and storage (CCS)
Co-enzyme A (CoA-SH)
Cyllamyces aberensis (Cy)
Fructose-6-phosphate (F6P)
Glyceraldehyde 3-phosphate (G3P)
Glycerol-1-phosphatase (GPP1/GPP2)
Malonyl- Co-enzyme A reductase (MCR)
Pentose phosphate pathway (PP-pathway)
Phosphate-acetyl transferase (PTA)
Phosphoketolase (XPK)
Piromyces sp. (Pi)
Pyruvate decarboxylase (PDC)
Pyruvate dehydrogenase (PDH)
Xylitol dehydrogenase (XDH)
Xyloluse-5-phosphate (X5P)
Xylose isomerase (XI)
Xylulose kinase (XKS)

Abstract

Global rise in temperature and diminishing oil reserves has stimulated a market of alternative replacements to traditional petroleum based products. An alternative is the use of a bio refinery capable of converting biomass to the normally petroleum based products. The baker yeast *Saccharomyces cerevisiae* is an attractive cell factory as its existing large-scale infrastructures for bioethanol production. However, it cannot utilize xylose, an otherwise unusable part of the plant biomass, which represents of utmost importance in the bio-refinery development. To also have a strain capable to produce a wide range of products, it could be used as a platform to base a bio refinery upon. Therefore, the aim of this study is to generate platform strains capable of forming acetyl-CoA, an intermediate metabolite in many of the cells metabolic reactions and also for many other industrially relevant bio-chemicals. With this goal in mind, the metabolism of *S. cerevisiae* was engineered. The genes encoding an isomerase-based xylose assimilation pathway (RTG, XI, XKS), and a phosphoketolase pathway (XPK, PTA), were cloned into the yeast strain CEN.PK113-5D to enable the yeast to take up and convert xylose into acetyl-CoA. The functionality of this synthetic pathway were evaluated for the production of 3-hydroxypropionic acid via introduction of ACC1** and MCR genes into the engineered strains. By characterisation of all the engineered strains on glucose growth we found increase of acetate production in strains with the phosphoketolase pathway expressed, indicating the *in vivo* activity of this pathway. However, expression of the xylose assimilation pathway through genome integration did not render the strains able to grow on xylose, suggesting the low efficiency of the assembled xylose assimilation pathway. To overcome this adaptive laboratory evolution is recommended.

Key words: *Saccharomyces cerevisiae,* xylose utilization, acetyl-CoA platform, metabolic engineering.

1. Introduction

To prevent global warming the need to phase out petroleum based products from today's society is becoming more and more apparent. This is an effect which according to IPCC fifth Assessment report from 2013 (1) is extremely likely caused by the release of greenhouse gases as a consequence of human activity. The two prime reasons are first the effect carbon dioxide has on the climate and the second is that the crude oil will eventually run out, although not before its depletion has caused irreparable change to the climate. Since carbon dioxide has a half-life of up to 1 000 years in the atmosphere either the emission has to completely cease or through carbon capture and storage (CCS) the CO_2 needs to be pumped back into the bedrock, to stop the change in climate. The development and funding of CCS are diminishing, although it could reduce the cost of stabilizing the CO_2 emissions at 350 ppm by 50 %. If used in combination with a bio energy production to create a negative flux of CO_2 it could be 80 % (2). To cease emitting CO_2, viable and market competitive alternatives have to be developed.

One alternative would be to replace petroleum based fuels, chemicals and materials and instead base it on biomass, by converting it with the help of a microorganism. Currently the majority of bio-based products, e.g. ethanol, use grains or cereals as feedstock as the process of releasing the sugars form the carbohydrate chains is fairly efficient. But this usage is in direct competition with food production and if used in high quantity will lead to an increased price of food. This has led to the increased focus on the use of cellulose as feed for the microorganism. Wood and other cellulose materials such as straw consist of a mixture of cellulose, hemicellulose and lignin. Cellulose is a polysaccharide of glucose, hemicellulose consist of a mixture of hexose and pentose and lignin is a polymer of different aromatic hydrocarbons (3). One of the predominantly industrial used microorganisms *Saccharomyces cerevisiae*, can only grow on the glucose in the cellulose and hemicellulose, assuming the biomass has previously been hydrolysed. This leaves a large part of the available energy in the biomaterial unused. Other microorganisms, i.e. certain fungus and bacteria, can metabolize the pentose in hemicellulose but lack many of the industrially interesting properties of *S. cerevisiae*, such as its robustness and tolerance towards harsh fermentation conditions. These properties are especially important if wood and other lignocellulose materials are to be used as feed, when a number of different inhibitors are released from the lignin when hydrolysed (4). In addition the accumulated knowledge of *S. cerevisiae* cellular biology and the available molecular biological techniques have made the preferred strategy not to increase the robustness of other microorganism but to use metabolic engineering on *S. cerevisiae* to make it capable of utilizing other energy sources.

1.1 Xylose utilization

Xylose can be the most abundant sugar in hemicellulose which constitutes of between 30 to 50 % of the total biomass in wood. The other types of sugars in hemicellulose are glucose, mannose, galactose, arabinose and rhamnose. As a result xylose, in turn, can be 35 % of the total biomass, depending on the type (3), making it an important start point to alter *S. cerevisiae* metabolism.

S. cerevisiae as mentioned cannot utilize xylose which is why research has been conducted to identify enzymes that can convert xylose into a metabolite *S. cerevisiae* can utilize. As a result two major enzymatic pathways have been identified and implemented into *S. cerevisiae.* The first one implemented is termed the oxidoreductase pathway and consists of the enzymes aldose reductase (AR), which converts xylose into xylitol and xylitol dehydrogenase (XDH)

which converts the xylitol to xylulose. The xylulose can then be converted to xylulose-5 phosphate (X5P) by the enzyme xylulose kinase (XKS) at the cost of one ATP. X5P is an intermediate metabolite in the PP-pathway. The conversion rate of *S. cerevisiae* native XKS is low, which is why a heterologous XKS can be included which converts the xylulose into X5P (5). The most commonly used versions of the AR and XDH are dependent on the cofactors NADPH and NAD^+ respectively (6). AR can use the cofactor NADH as well but has a higher specificity for NADPH. For example the AR from *Pichia stipitis* has a higher specificity for NADPH than for NADH, the K_m value equals to 3.2 µmol/l for NADPH and 40 µmol/l for NADH (5). This leads to an imbalance between the cofactors which limit the utilization and causes xylitol to accumulate (7). The other pathway, named the isomerase pathway, is independent of cofactors and originates mainly form bacteria but can be found in certain species of yeast. Unlike the oxidoreductase pathway it consists of the one enzyme, xylose isomerase (XI) which directly converts xylose to xylulose. The isomerase pathway gets its name from that enzyme

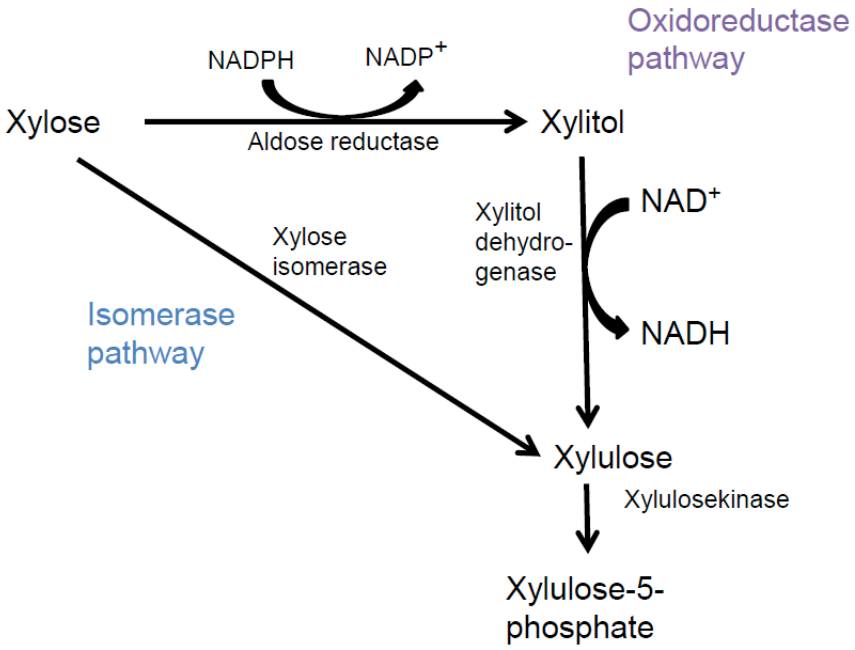

Figure 1 Outline of the oxidoreductase and isomerase pathways for the cellular conversion of xylose into xylulose. The oxidoreductase pathway consists of the enzymes aldolase reductase and xylitol dehydrogenase which converts xylose into xylitol and then the xylitol into xylulose at the cost of one NADPH and NAD^+. Xylose isomerase can directly convert the xylose into xylulose and is the sole contributor in the isomerase pathway. A common contributor to the oxidoreductase and isomerase pathways is the enzyme xylulosekinase which is native to S. cerevisiae *and converts the xylulose into the pentose phosphate pathway intermediate xylulose-5-phosphate.*

The growth rate of *S. cerevisiae* strains expressing the isomerase pathway has been found to be lower than the oxidoreductase but the ethanol yield on glucose were higher (8). The theoretical yield in the oxidoreductrase pathway is limited by the cofactor balance while the isomerase pathway is limited by the enzyme kinetics of xi. The potential to improve the isomerase pathway is large, partly as the pathway has been implemented later in *S. cerevisiae* and because of its simplicity (5). Recent unpublished findings from this group, have found two potential candidates of heterologous XI which showed high activity in *S. cerevisiae*. Originating form *Piromyces sp.* (Pi) and *Cyllamyces aberensis* (Cy), they both displayed a high activity when cloned into *S. cerevisiae*.

Regardless of which utilization pathway is implemented, *S. cerevisiae* has an inherent low intake of extracellular xylose. Logically *S. cerevisiae* has no dedicated xylose transport

proteins but xylose can be transported into the cell via hexose transport proteins, namely hxt7p, hxt5p, hxt4p, hxt2p, hxt1p and gal2p (9-11). These have a low activity on xylose illustrated in a strain expressing the oxidoreductase pathway, with a high activity of AR, that the flux control coefficient for the native transport was found to be 0.5 (12). To increase the intake rate of xylose heterologous xylose transporters have been expressed in yeast. The xylose transporter RTG form *Scheffersomyces stipites* allowed a growth rate of 0.119 h^{-1}, the highest of 46 cloned transporter and compared with *S. cerevisiae* native growth rate 0.033 h^{-1} on xylose, an increase by 360 % (13).

The majority of research published on xylose utilization has focused on producing ethanol. As focus on developing a second generation of biofuel has then driven the research forward. Additionally the described enzymes here would direct the carbon flux through the pentose phosphate pathway (PP-pathway) through its intermediate metabolite X5P resulting in slow and inefficient energy production. Therefore it has to be complemented and if a fully formed bio-refinery is built other products are desired which are based on other metabolites than ethanol. One of the most important intermediary metabolites to fulfil this goal is acetyl Co-enzyme A (Acetyl-CoA).

1.2 Acetyl Co-enzyme A synthesis

Accetyl-CoA is an intermediate metabolite in many of the cellular metabolic pathways. It consists of an acetic acid connected with a thioester bond to a Co-enzyme A (CoA-SH). It is involved in a wide variety of metabolic reactions where it can transfer the two carbon acetyl group to another metabolite. Cytosolic *de novo* synthesis occurs in *S. cerevisiae* from pyruvate produced in the glycolysis via acetaldehyde and acetate.

The acetyl-CoA supply in *S. cerevisiae* is compartmentalized in different parts of the cell, the cytosol, mitochondria, peroxisome and the nucleus. The main source of acetyl-CoA is derived from pyruvate, the end metabolite in the glycolysis. In the peroxisome it can also be derived form β-oxidation of fatty acids and in the mitochondria by conversion of pyruvate. In the cytosol acetaldehyde and CO_2 is formed from pyruvate by the enzyme pyruvate decarboxylase (PDC). The acetaldehyde can be turned, and usually is when growth is not limited by glucose, into ethanol but acetate can also be formed by the enzyme acetaldehyde dehydrogenase (ALD). The acetate can then either stay in the cytosol or be transported into the peroxisome or nucleus, where the enzyme acetyl-CoA synthetase (ACS) converts it into acetyl-CoA. The main mitochondrial supply is formed from pyruvate, which is converted into acetyl-CoA by the enzyme complex pyruvate dehydrogenase (PDH) (14). In addition to its central role in the energy production in the TCA-cycle acetyl-CoA is used as a building block in many industrially interesting products, as it is used in many of the drains from the central energy metabolism for the biosynthesis of other metabolites. As a result it has a wide range of compounds that are derived from it.

This is the reason why acetyl-CoA is an interesting metabolite in building a bio-refinery. Because what needs to be considered when developing bio-based production of a chemical is mainly the titter, rate and yield and by simply developing the immediate pathway to the desired product is sometimes not enough. By also considering the intermediate metabolites in the development, i.e. the building blocks of the product, the overall yield can be increased. With the cellular metabolism optimized towards formation of universal precursors, these cell factory platforms can then be used by ”plug-and-play” for the production of different types of related products. By having a strain that can effectively produce acetyl-CoA it can be used as a platform to build upon. As the focus of ethanol production and second generation of

biofuels is to replace petroleum based energy, the goal with this study is to create a yeast platform strain that can be used to replace a wide range of petroleum based products. The cell use acetyl-CoA, for example, in fatty acid biosynthesis which starts from a simple fatty acid which is then elongated in steps by malonyl-CoA derived from acetyl-CoA via conversion by acetyl-CoA carboxylase (ACC1). Other industrially interesting products derived from acetyl-CoA are isoprenoids, polyketides, poly-hydroxyalkanoates and 1-butanol, see Table 1 for a summary.

Table 1 A list of industrial intresting products dreived from acetyl-CoA and their us.

Products derived from Acetyl-CoA	**Industrial use**
Fatty acids and lipids	Dietary supplements, pharmaceuticals and biodiesels
Isoprenoids	Flavours and fragrances, biodiesels, antimalarial and anticancer drugs, antibiotics, rubber, dietary supplements, food ingredients and vitamins
Polyketides	Antibiotics, anticancer drugs and immune suppressors
Polyhydroxyalkanoates	Plastics
1-butanol	Biofuels

Although the enzymes described in the previous sections present a way for yeast to utilize xylose, it requires all the carbon flux to go through the PP-pathway and the down part of the glycolysis. As xylose is not native to this host, it has been shown the efficiency is not as high as on glucose. It is not necessary to have high flux to go through acetyl-CoA node when introduction of xylose assimilation pathway. Therefore, other enzymes or enzymatic pathways are needed to convert the X5P further more directly into acetyl-CoA.

1.3 The phosphoketolase pathway

X5P is as mentioned an intermediate metabolite in the PP-pathway in yeast and other eukaryotes. In other organisms, i.e. certain bacteria and filamentous fungi, the X5P does not go through the same PP-pathway but are catalysed by other enzymes. One of these is the enzyme is called phosphoketolase that can catalyse the reaction of X5P to acetyl phosphate (AcP) and glyceraldehyde 3-phosphate (G3P), namely XPK. The same enzyme is also active on fructose-6-phosphate, then termed FPK, which results in AcP and erytrose-4-phosphate (E4P) formation. First characterised in the lactic acid bacteria *Bifidobacterium lactis* (15), XPK is thought to work together with acetate kinase (ACK) and acetyl-CoA synthase (ACS) to form acetyl-CoA in alternate version of the glycolysis when the cell consumes xylose (14). The enzyme phosphate-acetyl transferase (PTA) can also catalyse the conversion of AcP but together with CoA-SH converts the AcP to acetyl-CoA and phosphate (16). The direct conversion to acetyl-CoA has the advantage over ACK and ACS as no ATP is consumed.

The additional production of G3P from the usage of XPK splits the carbon flux towards acetyl-CoA. An apparent downside, but it theoretically allows the AcP flux to be entirely directed to acetyl-CoA and any potential end product. The G3P could then act as a substitute of carbon flux otherwise formed in the glycolysis and supply the cell with the required energy and carbon derived biomass. Furthermore in strains where XPK and PTA have been implemented cellular accumulation of acetate occurred (17). An effect that is thought to be the result of dephosphorylation of AcP by the two versions of the native yeast enzyme glycerol-1-phosphatase, Gpp1 and Gpp2 (17). Gpp1 and Gpp2 have previously thought to only have

glycerol-1-phosphatase activity. The deletion of one or both genes decreased the acetate concentration in the cells.

Unpublished results have identified versions were the XPK and PTA enzyme with high *in vitro* activity. The previous study identified two versions of the phosphokeotlase gene one originating from *Leuconostoc mesenteroides* with specificity for X5P (XPK) and one with specificity for fructose-6-phosphate (F6P) from *Clostridium acetobutylicum* (FPK) to have high activity. A higher activity version of the PTA was also identified originating from *Salmonella enterica*. With these enzymes cloned into yeast in addition to the ones detailed in Section 1.1 a complete metabolic pathway can be created starting from xylose to acetyl-CoA. This pathway is visually represented in Figure 2 with additional enzymes are added which allow the formation of acetyl-CoA to be detected, because of the difficulty in detection of cellular compartmentalized acetyl-CoA. 3-Hydroxypropionic acid (3-HP) is a metabolite derived from acetyl-CoA via malonyl-CoA by the two enzymes acetyl Co-enzyme A carboxylase 1 (acc1) and malonyl- Co-enzyme A reductase (MCR), both reactions located in the cytosol. 3-HP in itself is a useful precursor in plastic formation (18) and as a result efficient ways to produce it has been developed in *S. cerevisiae* (19). Utilizing a mutated version of *S. cerevisiae* ACC1 which removed posttranslational regulation (20) and an MCR from *Chloroflexus aurantiacus* (19) to produce 3-HP from Acetyl-CoA it can be used for evaluation of the synthetic pathway for the acetyl-CoA production in the cell.

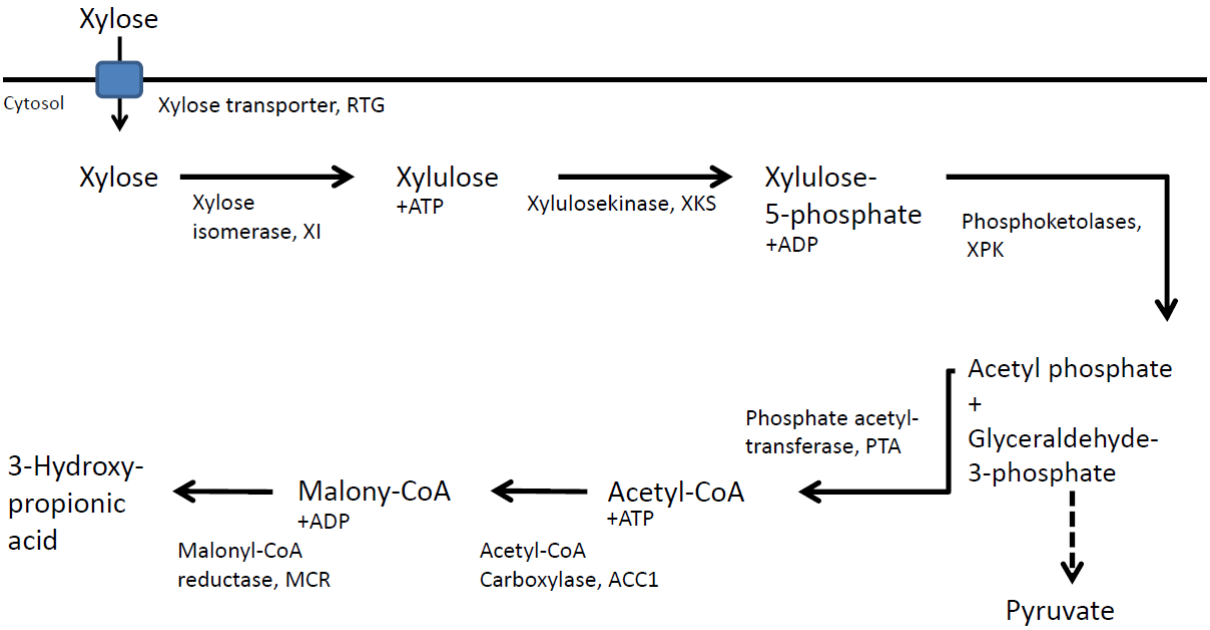

Figure 2 Metabolic pathway outlining a convention rout form xylulose via Acetyl-CoA to 3-HP. The transport protein and enzymes responsible for each reaction of each metabolite are written under each arrow.

1.4 Aim of the study

To build an acetyl-CoA based platform in *S. cerevisiae* capable of utilizing xylose, the aim of the study was to implement a new metabolic pathway in yeast intended to enable production and detection of acetyl-CoA using xylose as the substrate. This will be achieved by expressing the genes RTG, XKS, XI, XPK, PTA, ACC1 and MCR1 under strong consecutive promoters by either genome integration or a multi-copy plasmid. The detailed characterization of obtained strains will be performed of the production of 3-HP will be measured to be used as an estimation of the efficiency of acetyl-CoA synthesis.

2. Material and methods

2.1 Strain construction

The metabolic pathway constitution of the seven genes (RTG, XKS, XI, XPK, PTA, ACC1** and MCR) was distributed on three different cassettes. Two versions of the cassette 1 and 2 were used, 1.1, 1.2, 2.1 and 2.2. The different versions of cassette 1 differed in the XI gene, 1.1 used a XI origination from *Cyllamyces aberensis* (Cy) and 1.2 from *Piromyces sp.* (Pi). However, in common between the two versions were the RTG and XKS from *Scheffersomyces stipites*. Cassette 2 used a version of PTA from *Salmonella enterica* and 2.1 a XPK from *Leuconostoc mesenteroides* and 2.2 a FPK from *Clostridium acetobutylicum*. Cassette 3 contained a mutated version of *S. cerevisiae* ACC1 (ACC1**) which removed posttranslational regulation (20) and a MCR from *Chloroflexus aurantiacus* (19). Each gene was put under control of a strong constitutive promoter and with a yeast terminator. The cassettes contained the same selection marker, the URA3 gene. Cassette 1 and 3 contained a sequence at each end of to allow genomic integration and cassette 2 was on a plasmid. A detailed outline of the design of the cassettes can be seen in Figure 3.

Each gene, promoter, terminator and integration site was amplified using the primers listed in appendix Table 1 using Phusion polymerase according to manufacturer's specifications. These fragments were then fused into longer fragments with long overlapping regions to allow for homologous recombination, see Figure 3. Achieved in a two-step fusion PCR reaction, in the first reaction no primers were added and the DNA fragments were mixed in a molar ratio according to a Pascal triangle (i.e. 1:1, 1:2:1, 1:3:3:1 and so on). This first round only last for 15 cycles as the fragments is not amplified only fused as a result of the lack of primers. The product of the first round in then used as template in a second round with primers to amplify the DNA fragments. All fusion PCRs were performed using PrimeSTAR DNA Polymerase. The resulting PCR product was purified from the PCR mixture by a GeneJET PCR purification kit or if gel separation was required GeneJET Gel extraction kit using the centrifugation method (protocol A). One consistent exception with the protocol was that the DNA was eluted using MQ-water instead of elution buffer. Cassette 1.1 and 1.2 were constructed in this study and cassette 2.1, 2.2 (appendix Figure 9) and cassette 3 were constructed previously in this lab.

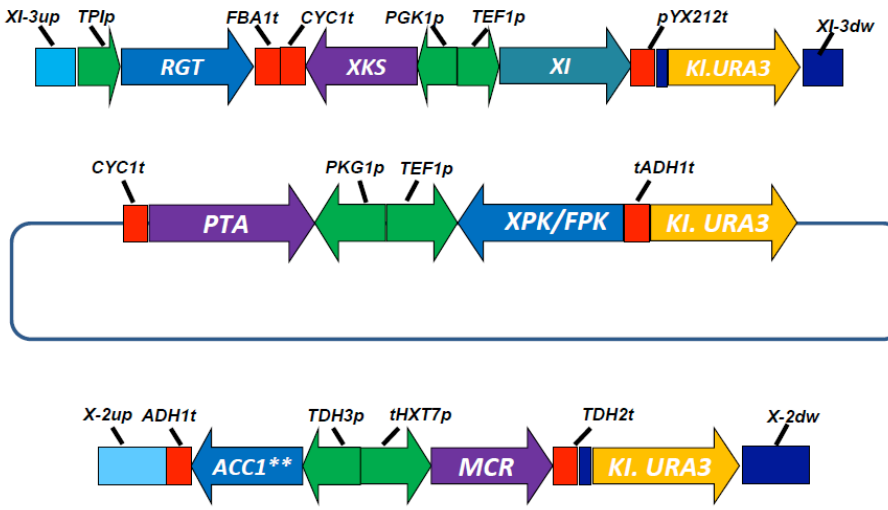

Figure 3 The design of the three used cassettes. Top describes the composition of cassette 1, middle cassette 2 and bottom cassette 3. Green arrows are promoters, red blocks are terminators, blue blocks are the integration sites and larger arrows are genes.

The three different cassettes were transformed into the *S. cerevisiae* strain CEN.PK113-5D (MATa SUC2 MAL2-8c ura3-52), see Figure 4 for the general strategy. Using LiAc/ss carrier DNA/Peg method (21) 15 different strains were created, see Table 2. Each cassette was transformed separately and the URA3 gene was looped out by growth on 5-Fluoroorotic acid (5-FOA) plates after the transformation of cassette 1 and 3. 5-FOA forms together with uracil the toxic compound 5-fluorouracil causing cell death (22). The loop out was aided by the addition a 60 bp flanking region upstream of the URA3 gene identical to part of the integration site. Thus the flanking regions facilitate homologous recombination upon negative selection pressure on the URA3 gene. The loop out was confirmed by lack of growth on uracil deficient media and diagnostic PCR. Cassette 1.1, 1.2 and cassette 3 were integrated on two sites on chromosome X and XI separated by essential genes to prevent any unwanted homologues recombination (23). The notations used for the integration sites are consistent with the article. The XPK/FPK and PTA genes were not genomically integrated but constructed on a plasmid, thus the URA3 marker gene was not looped out.

Table 2 List of created strains using the yeast strain CEN.PK113-5D as background. The list details which type of cassette and which genes are cloned in each strain.

Strain	*Cassettes*	Genes	Origin
OEÖ 1	*Cas 1.1*	RTG, XKS and XI Cy,	This study
OEÖ 2	*Cas 1.2*	RTG, XKS and XI Pi,	This study
OEÖ 3	*Cas 3*	ACC1** and MCR	This lab
OEÖ 4	*Cas 1.1+cas 2.1*	RTG, XKS, XI Cy, XPK and PTA	This study
OEÖ 5	*Cas 1.1+cas 2.2*	RTG, XKS, XI Cy, FPK and PTA	This study
OEÖ 6	*Cas 1.2+cas 2.1*	RTG, XKS, XI Pi, XPK and PTA	This study
OEÖ 7	*Cas 1.2+cas 2.2*	RTG, XKS, XI Pi, FPK and PTA	This study
OEÖ 8	*Cas 1.1+cas 3*	RTG, XKS, XI Cy, ACC1** and MCR	This study
OEÖ 9	*Cas 1.2+cas 3*	RTG, XKS, XI Pi, ACC1** and MCR	This study
OEÖ 10	*Cas 2.1+cas 3*	XPK, PTA, ACC1** and MCR	This study
OEÖ 11	*Cas 2.2+cas 3*	FPK, PTA, ACC1** and MCR	This study
OEÖ 12	*Cas 1.1+cas 2.2+cas 3*	RTG, XKS, XI Cy, FPK, PTA, ACC1** and MCR	This study
OEÖ 13	*Cas 1.2+cas 2.1+cas 3*	RTG, XKS, XI Pi, XPK, PTA, ACC1** and MCR	This study
OEÖ 14	*Cas 1.1+cas 2.1+cas 3*	RTG, XKS, XI Cy, XPK, PTA, ACC1** and MCR	This study
OEÖ 15	*Cas 1.2+cas 2.2+cas 3*	RTG, XKS, XI Pi, FPK, PTA, ACC1** and MCR	This study

The transformations were confirmed by PCR with two different methods to extract the DNA depending on whether the cassette was genomic integration or on a plasmid. For genomically integrated DNA, i.e. cassette 1 and 3, an overnight culture were centrifuged and the pellet resuspended in 100 µl of 0.2 M LiAc, 1 % SDS (w/v) solution and incubated at 70 °C for 5 minutes. 300 µl of 96 % ethanol was added to the suspension and centrifuged for 3 min at 15 000xg. The supernatant was removed and the pellet was washed in 70 % ethanol, centrifuged again and supernatant removed. To separate the genomic DNA from the cellular debris the pellet was resuspended in 100 µl of MQ water and centrifuge for 20 seconds at top speed and 1 µl of the supernatant was used as template in a PCR (24).

For the plasmids containing the different versions of cassette 2 colonies were picked and suspended in 20 µl 20 mM NaOH and incubated at 99 °C for 15 minutes. The cellular debris was spun down by centrifuging the PCR tube for 2 min in a mintabletop centrifuge. 1 µl of the supernatant was the used as template in a PCR reaction.

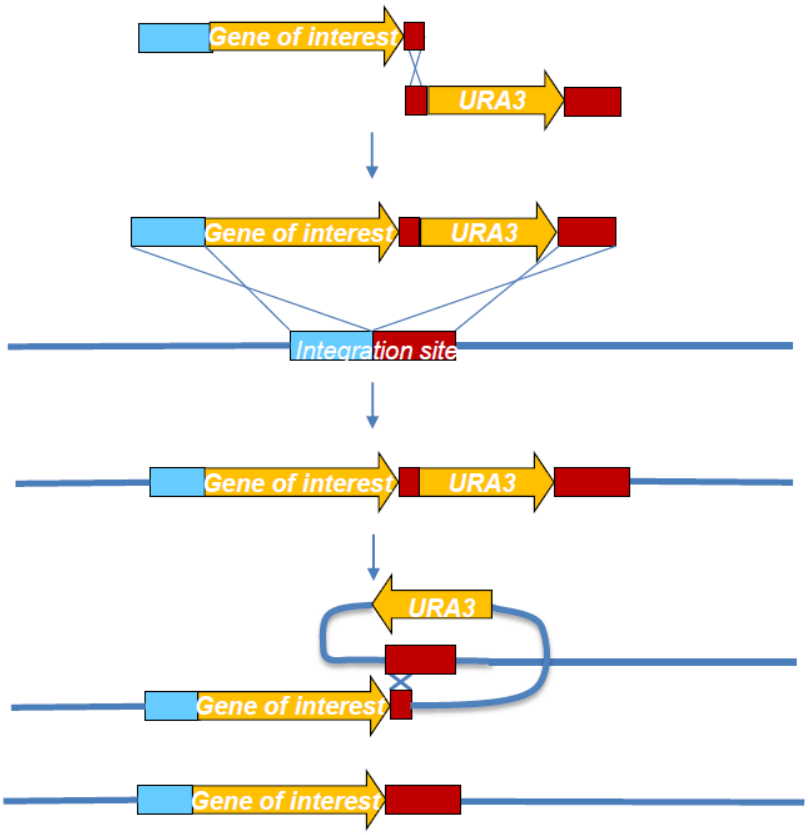

Figure 4 General strategy to genomically integrate one or several genes of interest into a yeast chromosome by homologous recombination. First, two or more linear DNA fragment are created containing the two flanking region homologues to the desired integration site (blue and red), the gene(s) of interest (yellow) and an URA3 selection marker with approx. 60 bp repeated sequence of the downstream integration site. The transformed fragment will by S. cerevisiae *native mechanism for homologous recombination integrate the cassette inside the integration site. The later parts describes the loop out proses of the URA3 marker gene upon negative selection by 5-FOA.*

2.2 Media and growth conditions

In common between all *S. cerevisiae* cultures was an incubation temperature of 30 °C and the liquid cultures were carried out in Erlenmeyer flasks while shaken at 200 rounds per minute (rpm). Cultures in preparation for transformation were carried out in 100 ml flasks and in preparation for growth rate and HPLC sample in 250 ml flask, all with 50 ml liquid media and either YPD or synthetic minimal medium (Delft) respectively. Table 3 lists the composition of the different media used. If solid media for plate were desired 20 g/l of agar-agar was added to the media.

Table 3 List detailing the composition of the different media used in for growing the yeast strains.

Media type	Concentrations
YPD	*Yeast extract 10 g/l, peptone from meat 20 g/l, glucose 20 g/l.*
SC-URA	*YNB without amino acids 6.9 g/l, CSM -URA drop-out 0.77 g/l, glucose 20 g/l.*
Delft	*$(NH_4)_2SO_4$ 7.5 g/l, KH_2PO_4 14.4 g/l, $MgSO_4 \cdot 7H_2O$ 0.5 g/l, metal solution 1 ml/l, vitamin 1 ml/l, 20 g/l sugar.*
5-FOA	*YNB w/o aa, 6.9 g/l, CSM -Ura drop-out 0.77 g/l, Uracil mg 50 mg/l, 5-FOA 750mg/l, Glucose 20 g/l.*

2.3 Growth rate determination

To determine the growth rate (μ) of the constructed strains on both glucose and xylose as carbon source the strains were inoculated in 50 ml Delft synthetic media from a pre-culture to an optical density at 600 nm (OD_{600}) of 0.05 and 0.1 respectively. For the glucose cultivation the OD_{600} were measured once every third hour 10 hours after inoculation, for the xylose the OD_{600} was measured once each 24 hours after observed growth. The exponential growth rate is the determined by plotting the natural logarithm of the OD measurements in the exponential phase against time were the slope is the growth rate.

2.4 Analytical methods

Constructed yeast strains were inoculated in 50 ml Delft synthetic media using glucose as carbon source. To strains lacking the ura3 gene, i.e. strain 1, 2, 3, 7 and 8, 100 mg/l uracil was added to the medium. The start OD_{600} was approximate 0.05 and 0.1 for the glucose and xylose cultivations respectively. Aliquots of the cellular cultures were taken at regular intervals, as a minimum one each 24 hours after inoculation. The OD_{600} was measured and the aliquots were centrifuged at 14 000xg for 7 min and 500 µl of the supernatant were removed to be stored in -18 C. The samples were analysed using an Dionex UltiMate 3000 HPLC system (Dionex Softron GmbH, Germering, Germany) with an Aminex HPX-87H Column (BioRad, California) using 0.5 M H_2SO_4 as eluent. The HPLC used a flow rate of 0.5 ml/min and temperature of 65 °C during the analysis. The detection relied on both UV and RI. The contents of the standard used for the analysis are listed in Table 4 in there undiluted state. This standard was then diluted in the eluent to form six different dilutions, one 100 %, one 75 %, one 50 %, one 25 %, one 12.5 % and one 6.25 % dilution.

Table 4 List of the concentration of chemicals used in the standards in the HPLC. Note that only glucose or xylose was used in one experiment and never in combination.

Compound	Concentration (g/l)
Glucose	*20*
Xylose	*20*
Pyruvate	*0.5*
Succinate	*0.5*
Glycerol	*1*
Acetate	*2*
Ethanol	*15*
3-HP	*1*

2.5 Determination of optical density to dry weight ratio

A culture of a CEN.PK113-5D strain was grown in Delft media with glucose as carbon source to an approximate OD_{600} of 1. 5 ml of the culture were dried on pre-dried and pre-weighed 0.45 µm nitrocellulose filters and washed with distilled water. The difference in weight of the filters before and after filtration was used to calculate the dry weight to OD_{600} ratio.

3. Results

With the aim to create a new way for yeast to metabolize xylose and turn it into acetyl-CoA the different genes were cloned into the yeast strain CEN.PK113-5D. To examine whether the strains can utilize xylose and produce 3-HP the constructed strains were grown on glucose and xylose separately. The growth and extracellular metabolites were monitored during shake flask cultivation

3.1 Strain design and creation

With the aim to clone the seven genes RTG, XKS, XI, XPK, PTA, ACC1 and MCR, the strategy was to distribute them on three different cassettes. Cassette 1 contained the genes RTG, XKS and XI, cassette 2 contained XPK and PTA and cassette 3 contained ACC1 and MCR. To attempt to not limit the flux of metabolic pathway by the individual protein concentration, each individual gene was put under control of a strong constitutive yeast promoter.

The reasoning behind the division was that it is easier to construct the cassettes by PCR amplification and to have as high transformation efficiency as possible and reduce the number of transformations needed, as they are time consuming. By having all genes on one cassette it would have been almost 30 kbp. The chance that all fragments are integrated at the corrected order, place and without fault was estimated to be minute. On the other side of the spectrum the time needed to integrate seven different genes into *S. cerevisiae* genome and then removing the selection marker, would exceed the time limit of the project. But not to make the division arbitrary each cassette was designed for a purpose. What was desired was to have a cassette with approximate 10-15 kbp, as to limit the required fragments in each transformation to 3-5. This entails having two or three genes together not to exceed the limit. But also to divide the genes in a way they would allow as many conclusions as possible to be made. As the two version genes XI and XPK/FPK were aimed to be examined, to have them on two different integration cassettes would be beneficial in order to easily construct a strain with the different versions. As then RTG is a xylose transport protein producing the XI without sufficient intracellular concentration of xylose would not be efficient. The product of XI, i.e. xylulose, only has a low native conversion rate to X5P the addition of RTG and XKS to the same cassette as the XI appeared natural. Then by having PTA gene together with one of the two versions of the XPK/FPK gene the pathway to acetyl-CoA would be complete by two cassettes. As the ACC1 gene is 7 kbp long combining it with the XPK/FPK and PTA genes was impractical and therefore combined with the MCR gene on a separate cassette.

With the design primers the individual DNA fragments were amplified using Phusion DNA polymerase and confirmed with separation by gel electrophoresis (results not shown). The approximate 30 bp overhang created by the primers between each fragment allowed the fusion PCR to happen where several DNA fragments were fused together. Again this was confirmed with gel electrophoresis (result not shown).

Additionally to the division of the genes on different cassettes, how they were combined could give answers to different questions. The main question to answer is how different strains combination of XI and XPK genes gives the highest production of acetyl-CoA and consequently 3-HP. But also to examine the individual contribution of each cassette to the final result and whether the pathway works as intended. As only strain OEÖ 12-15 have the entire cloned pathway they are used to evaluate how well the pathway produced 3-HP and the difference in gene combination. OEÖ 3 is used as control for the 3-HP production on glucose as it only contains cassette 3 and can be used as a references strain. Strain 1 and 2 with one each of the two versions of cassette 1 can be used to determine which version of the XPK results in the highest growth rate on glucose and compare how the rest of the cassettes affect the growth rate. They can also be used as a negative control for the production of 3-HP and any other potential by-product for the genes in cassette 1. With strain OEÖ 4-7 one would know the stress put on by cassette 1 and 2 and if they cause the formation of any by-product. With strain OEÖ 8 and 9 the contribution of cassette 2 to the formation of 3-HP would be known. Strain 10 and 11 can be used to evaluate the effect cassette 2 has on the production of 3-HP and the growth on xylose. If compared to cassette 3 one could see whether the addition of the XPK/FPK and PTA increase the yield of 3-HP as it should shuffle any X5P or F6P towards acetyl-CoA.

To confirm the transformation of each cassette either the genomic or plasmid DNA was extracted from the yeast cells. Using the DNA as template in a PCR reaction with either DreamTaq or Phusion DNA polymerase several fragments were amplified form each cassette. In Figure 5 amplified fragments of the genes RTG, PTA and MCR can be seen from the different strains as well with extracted genomic DNA form CEN.PK113-5D and plasmid containing the respective gene as negative and positive control respectively. The sequences of the used primers are listed in Table 11.

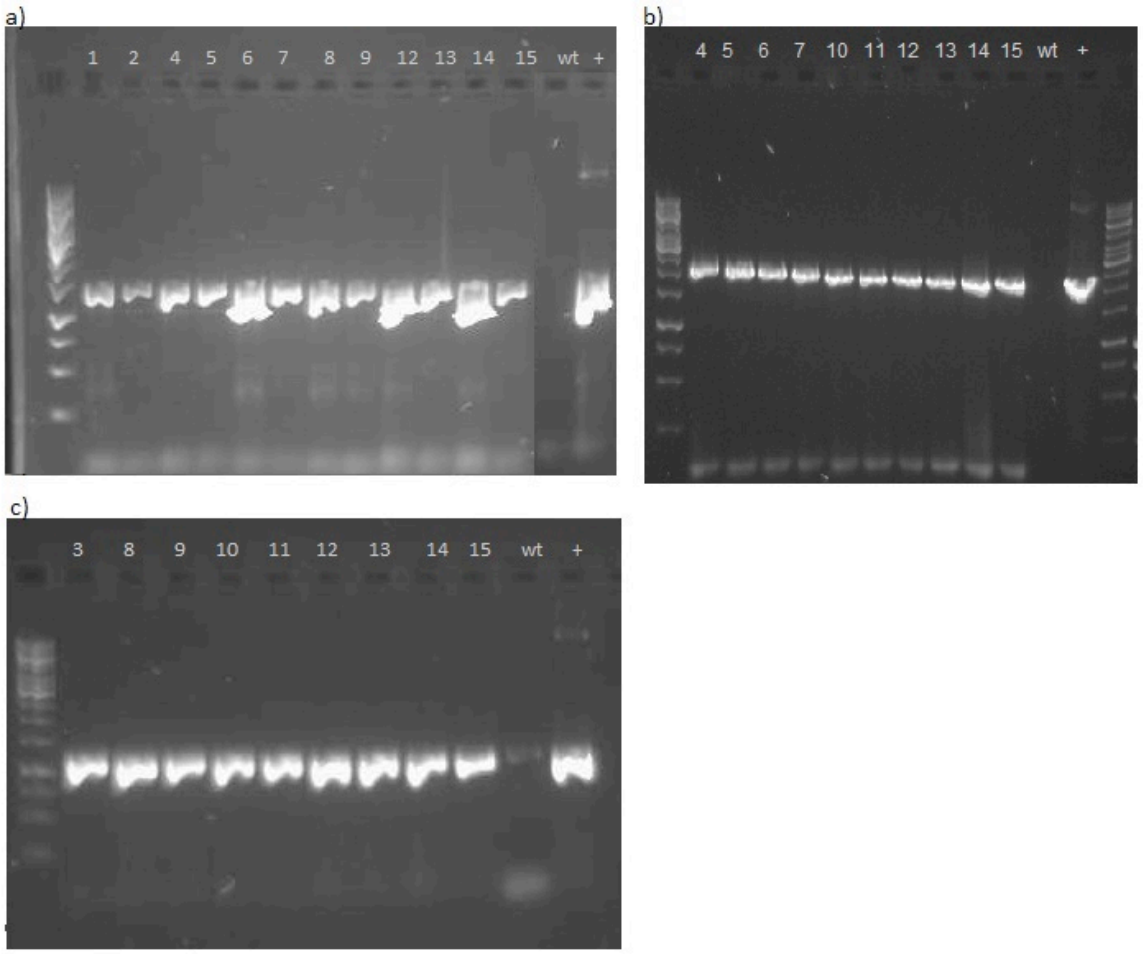

Figure 5 Diagnostic PCR of purified genome and plasmid DNA separated on a 1 % agarose gel. a) Pcr fragment of TPIp-RTG b) PTA and c) part of the MCR gene. The number depicted which strain DNA that was used as template, e.g. 1 is OEÖ 1, wt is CEN.PK113-5D and + is a plasmid containing one of the genes. The ladder use in each gel is a Thermo Scientific GeneRuler 1 kb DNA Ladder, ready-to-use.

3.2 Strain characterisation

To estimate the dry weight to OD_{600} ratio a culture of CEN.PK113-5D was grown in Delft synthetic media with glucose as carbon source to an OD_{600} of 0.969, determined by nine replicates. In eight replicates the dry weight of 1 l of the cell culture was determined to be 1.255 g. The variance between samples was determined by a Student's t-test to be within 95 % significance. Both results give a DW to OD ratio of 1.29 g DW/l/OD_{600}. In Table 5 the measured values of the OD_{600} and DW are listed.

Table 5 Measured values of the optical density at 600 nm (OD_{600}) of a culture of CEN.PK113-5D grown in 50 ml Delft media with glucose as the carbon source. The calculated dry weigh (DW) of 1 l of the same culture is listed in eight replicates as well as the average and standard divination of both the OD_{600} and DW measurements.

Replicate	OD_{600}	DW (g/l)
1	0.935	1.102
2	1.25	1.016
3	0.965	1.056
4	1.01	2.072
5	0.985	0.964
6	0.83	1.212
7	0.87	1.078
8	0.97	1.542
9	0.91	
Average	0.969444	1.255
Standard divination	0.1197248	0,3757
Ratio	1.29 g DW/l/OD_{600}	

To examine the metabolic strain and the general fitness of the created strains the growth rate of each strain was determined, the individual values are listed in Table 6. The strains were grown on Delft media with glucose or xylose as carbon source. When the strains are grown on glucose what can be observed is that the strains with all cassettes 2 and 3 have growth rate on the lower end. However the extremes are strains OEÖ 1-2 and OEÖ 11 with the highest and lowest growth rate respectively. But with a total average of 0.26 the standard divination of all the growth rates lie in a 95 % significance with each other, as determined by a student t-test. None of the strains displayed growth on xylose after continued observation of liquid cell cultures for 18 days.

Table 6 The determined growth rate of each constructed strain based on glucose or xylose as carbon source.

Strain	Growth rate, μ (h^{-1})	
	Glucose	Xylose
OEÖ 1	0.4282	0
OEÖ 2	0.4018	0
OEÖ 3	0.3036	
OEÖ 4	0.2447	0
OEÖ 5	0.2500	0
OEÖ 6	0.2774	0
OEÖ 7	0.2476	0
OEÖ 8	0.2194	0
OEÖ 9	0.3216	0
OEÖ 10	0.2262	
OEÖ 11	0.1839	
OEÖ 12	0.2289	0
OEÖ 13	0.2332	0
OEÖ 14	0.2353	0
OEÖ 15	0.2404	0

To further characterise the constructed strains, the level of remaining sugar, organic acids and 3-HP were measured using a HPLC. The same cultures were used for this as in the determination of the growth rate. The measured values of glucose, glycerol, ethanol, dry weight, acetate and 3-HP over time are shown, for each strain in Figure 6. The DW was calculated by multiplying the measured $OD_{600,}$ at the same time point, with the previously determined DW/OD_{600} ratio. To start from the first time point, what can be observed is that the starting values of glucose, ethanol and glycerol are different from what is listed in Table 3. The reduction of glucose could perhaps be caused by human error when adding the glucose to the media but when one considers that there are also ethanol and glycerol in the media, which were not added, the conclusion would be that it was contaminated when stored in preparation for the HPLC measurement. If the contamination happened after or before inoculation could be answered by if and when any observable growth was visible in the media. This was visible two days after the HPLC measurement suggesting the contamination only affected the measurement of the starting values and not the strain cultivations. To further support this theory all the strain that were expected to produce 3-HP did, whilst those that were not expected to did not produce any 3-HP. Therefore if the media were contaminated it did not influence the 3-HP production. However as a consequence the starting concentrations of cannot be used.

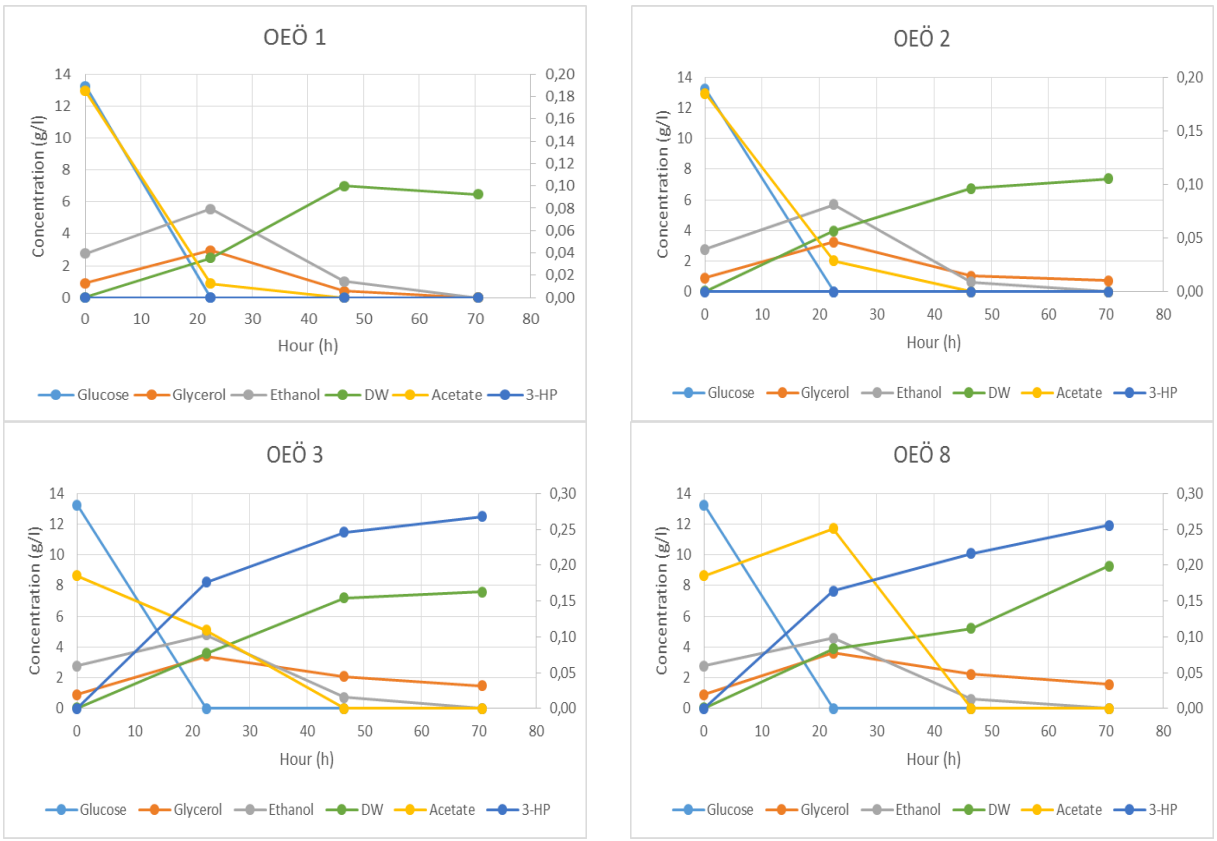

Figure 6 The different figures display the measured concentration in g/l of glucose, glycerol, ethanol, biomass, acetate and 3-HP at three different time points, 22.5, 46.5 and 70.5 hours for the constructed strains. Note the different scale on the acetate and 3-HP concentration compared to the other chemicals.

The 3-HP is exported from the cell to the culture, and can then be detected using a HPLC from the supernatant, as an indication of the acetyl-CoA formation in the cell. These values

and the total biomass content are listed in Table 7. As expected strains OEÖ 1-2 and 4-7 do not produce any 3-HP since they do not have the ACC1 and MCR genes. OEÖ 3 which only have the cloned ACC1 and MCR genes can be used as a reference for the production of 3-HP on glucose. But it did not produce the highest amount of 3-HP, which OEÖ 9 did, but if one looks at the ratio between 3-HP production and biomass OEÖ 3 do have a higher production. What can be seen is the addition of cassette 2, i.e. XPK/FPK and PTA, has a negative influence on the 3-HP production as strain 10-15 has approximate 20 % lower production. But only in terms of the titter of 3-HP if one considers the yield of 3-HP on biomass the difference is less. The yield of 3-HP on biomass in OEÖ 3 is still the largest but for strain 8 and 9 the yield is more comparable to the other strains. What is also observable was the decrease in yield, which is lower in strain 12 and 15 compared to strain 13 and 14. The first pair contains the FPK version of the phosphoketolase and the second pair the XPK version. This difference is on the other hand not displayed between strain OEÖ 10 and 11 which both have respectively the XPK and FPK genes.

Table 7 3-HP production and biomass content in g/l after 70.5 h based on two replicates on growth on glucose.

Strain	3-HP (g/l)	Biomass (g/l)	3-HP/DW
OEÖ 1	0	6.48±0.59	0
OEÖ 2	0	7.38±0.17	0
OEÖ 3	0.268±0.001	7.59±0.37	0.0353
OEÖ 4	0	7.30±0.96	0
OEÖ 5	0	6.84±1.22	0
OEÖ 6	0	6.63±0.79	0
OEÖ 7	0	7.64±0.23	0
OEÖ 8	0.256±0.001	9.26±0.20	0.0276
OEÖ 9	0.281±0.015	8.90±0.14	0.0316
OEÖ 10	0.218 ±0.007	8.05±0.14	0.0271
OEÖ 11	0.198±0.009	7.59±0.25	0.0261
OEÖ 12	0.213±0.013	7.46±0.59	0.0285
OEÖ 13	0.216±0.011	8.26±0.085	0.0261
OEÖ 14	0.203±0.004	7.69±0.51	0.0265
OEÖ 15	0.202±0.001	7.10±0.37	0.0285

From the available data it could not be said whether the addition of cassette 2 had any additional effects on, for example, any by-product formation, such as acetate as previously described, as the data of the consumption glucose and formation of organic acids are not comparable. As mentioned, the starting values of glucose in the media were unreliable but the maximum production of ethanol and acetate are also unknown. Since the different strains has also had different growth rates, the time point where the glucose growth phase ended were not in common between the strains. Therefore the measured maximum production of ethanol and acetate were incomparable, as they will be consumed when the glucose consumption is over.

Therefore, to further examine and characterise the effect of cassette 2 on strain growth and 3-HP production, a new cell culture were prepared. Preformed the same way but samples for HPLC were taken once every third hour nine hours after inoculation for a duration of 15 hours. The result of that cell culture can be seen in Figure 7. As in the previous example strain OEÖ 3 produced more 3-HP and had a higher growth rate, but here the consumption of organic acids can be seen in more detail for strain OEÖ 3, 10 and 11. The production of

glycerol is significantly higher in strain OEÖ 3 as well and the production of acetate is lower. Because the different growth rates between strain OEÖ 10 and 11 the exact end of the glucose phase were not pinpointed. Assuming an exponential production of acetate and a linear consumption of acetate, after the end of the glucose phase, to extrapolate an approximate end of the glucose phase and therefore compare the acetate levels. Using this approximate maximum production of acetate it is 0.81 g/l at 27 h and 1.1 g/l at 28.7 h for strain OEÖ 10 and 11 respectively.

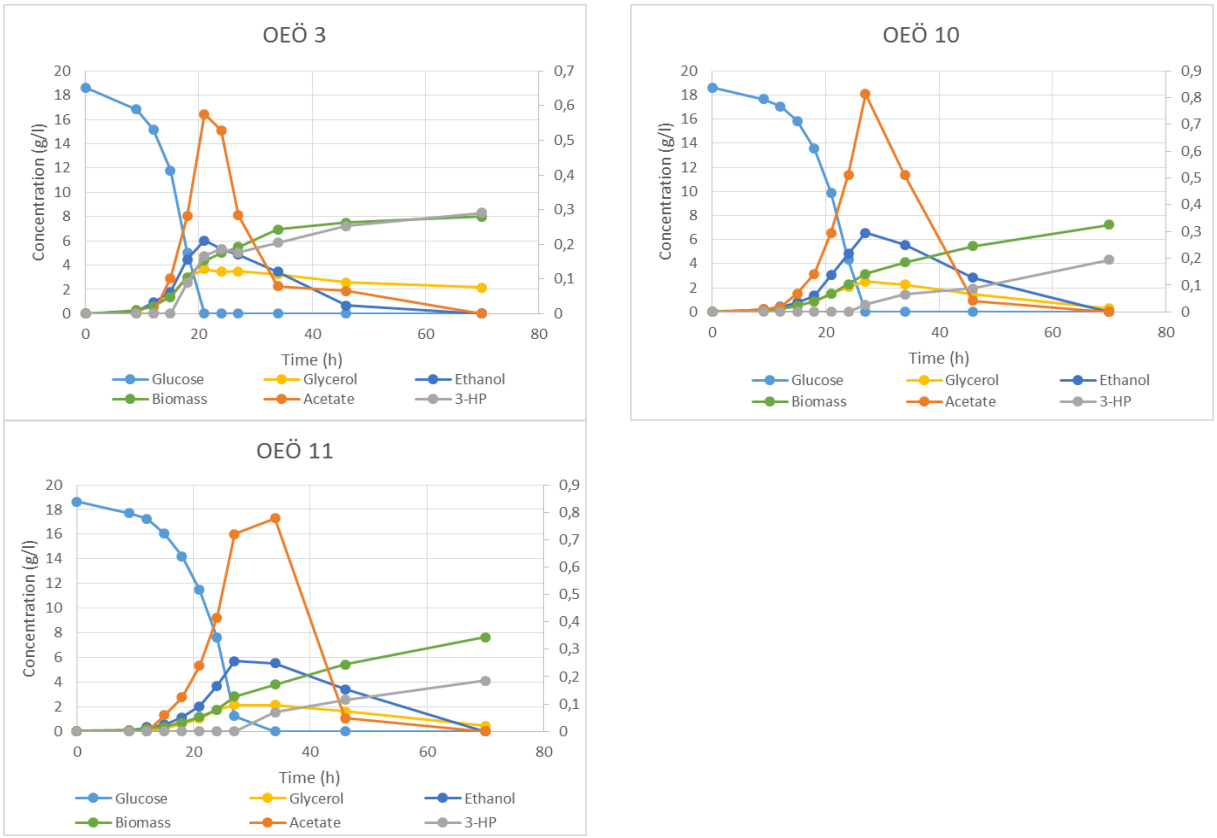

Figure 7 The different figures displays the measured concentration in g/l of glucose, glycerol, ethanol, biomass, acetate and 3-HP at ten different time points, 9, 12, 15, 18, 21, 24, 27, 34, 46 and 70 hours for the constructed strains. Note the different scale on the acetate and 3-HP concentration compared to the other chemicals.

4. Discussion

With the goal to create a strain that can utilize xylose to produce acetyl-CoA the genes RTG, XI, XKS, PK and PTA were chosen. The pathway was complemented by the genes ACC1** and MCR to convert the acetyl-CoA to 3-HP which can be detected using an HPLC. The seven genes were constructed on three different cassettes and successfully transformed into the *S. cerevisiae* strain CEN.PK113-5D. To examine the effect of the genes on the cellular growth they were cultivated in Delft synthetic minimal media using both glucose and xylose as carbon source.

What one could expect of the glucose cultivation is conformation of the difference in the *in vivo* specificity between the two phosphoketolases. As the substrate preference of the enzyme originating from *C. acetobutylicum* (cassette 2.2) were thought to be F6P the addition of the FPK gene should theoretically channel more of the carbon flux to the cytosolic acetyl-CoA pool. Since the growth on glucose will result in the formation of F6P from the glycolysis

which the FPK especially, but also the XPK to a smaller degree, could convert to AcP and if then the PTA is functioning it can convert to acetyl-CoA, Figure 8. Therefore one could imagine the production of 3-HP would also be increased. This was not the case as seen in Table 7 where the 3-HP titter of the strains with cassette 2 showed a decrease by approximately 20 %. As described previously it has been shown that the introduction of the phosphoketolase gene in *S. cerevisiae* increased the production of acetate. This could then explain the lack of 3-HP as depicted in Figure 6 and Figure 7 where some of the carbon flux is directed to acetate instead of acetyl-CoA. This acetate is not converted to acetyl-CoA but instead exported out of the cell. This is supported by the result depicted in Figure 7 which showed a clear increase in acetate production and a lowering of 3-HP production in strain OEÖ 10 and 11 compared to the control strain OEÖ 3. What could also be observed was a lowered production of glycerol in strain OEÖ 10 and 11. Glycerol is partly produced to maintain cytosolic redox balance in the cell as the formation of glycerol consumes NADH (25, 26). This is probably a side effect of the increased acetate production and the resulting decrease in acetyl-CoA, which is seen in the 3-HP production. This points to that PTA has very low efficiency and a future direction to work on, if this pathway is chosen. If the PTA would work efficiently in the cell it would increase the cytosolic supply and then also the 3-HP production. This is not the case, the main drain of acetyl-CoA is seemingly the formation of acetate from F6P for FPK and GPP1/GPP2. This explains both the decrease in acetyl-CoA and the lower growth rate.

From the measured data no clear difference in 3-HP or acetate production can be seen between strain OEÖ 10 and 11. The final 3-HP production of the two strains were in the first cultivation 0.22 g/l and 0.20 g/l and in the second culture both 0.19 g/l. In strain OEÖ 11 the maximum acetate concentration would be between the two measured points at 27 and 34 hours and therefore is unknown but it could be different form strain OEÖ 10 which has a higher measured acetate concentration. But if the extrapolated maximum production of acetate is used there is a different in acetate production. This suggests a difference in specificity between the two versions of the phosphoketolase. This supports *in vitro* findings of the previous unpublished result that the FPK from *Clostridium acetobutylicum* had a higher specificity against fructose-6-phosphate compared to the XPK from *Leuconostoc mesenteroides*.

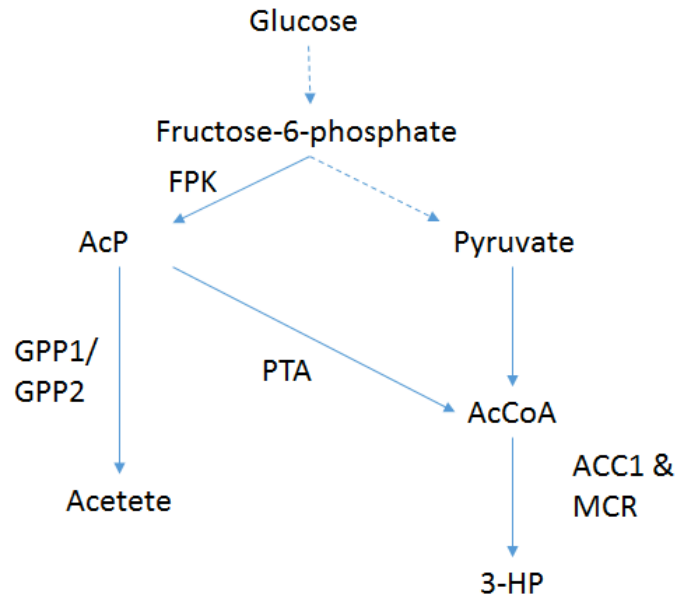

Figure 8 Illustration of the altering in yeast metabolism with the addition of the enzymes posphoketolase and phosphate acetyltransferase.

All tested strains did not grow on xylose, which could indicate that the expression level of the RTG or XI genes is too low and therefore the cell is unable to utilize the xylose. Considering the growth rate achieved by cloning the RTG gene in the reference material (13) also used a strong consecutive yeast promoter the here observed decrease of growth should be caused by too low expression level. In the unpublished result laying ground to the choice of the XI genes where it was also expressed with a strong consecutive promoter. What was different was that the genes were expressed on a plasmid which usually also entails a higher copy number compared to genomic integration. Therefore one could imagine that either number of the xylose transport or the catabolism proteins were insufficient. In that study the same gene variation of RTG, XI and XKS were tested and growth was observed after approximate 10 days. As the transformations were confirmed by colony PCR I therefore hypothesise that the expression caused by the single copy of the XI were unable to support cellular growth. The proposed pathway would also only supply half the energy from each mole of xylose compared glucose putting extra demand on a higher supply.

Increasing the copy number is not the optimal way to increase the growth rate, alternatively evolutionary engineering is an attractive way to increase both the yield and growth rate as many studies has shown before (27, 28). Continuously cultivating the strain with genomically integrated genes and selecting for high growth rate in a media that leads to an improved strain. Previously described results of integration of the xylose isomerase gene have shown low growth rates and xylose consumption (29). In another example where a cloned gene XI from *Piromyces sp.* displayed a specific growth rate of 0.005 h^{-1} which was increased after evolutionary engineering to 0.09 h−1, by 18-fold (30-32). To achieve an 18 fold increase might not be feasible but any improvement would be beneficial. Most of the genes were genomically integrated and evolutionary engineering could be performed on the strains after integrating cassette 2 in the genome.

5. Future aspects

What has been presented here is a way to produce acetyl-CoA from xylose as carbon source in yeast and aimed to be used as a platform for the production of many other products. As a consequence, when a cell is engineered for production of an acetyl-CoA derived product an increase in acetyl-CoA production will also amount to an increase in the final product. To increase the yield of acetyl-CoA per glucose different strategies have been developed to increase the synthesis. A main strategy is to avoid ethanol production as in *S. cerevisiae* normal metabolism with a high sugar influx leads to a shift in the energy metabolism where instead of respiration (ATP production through TCA-cycle and electron transport chain) it ferments (produce ethanol without oxygen as electron acceptor). Named the Crabtree effect it results in a much lower yield of ATP per glucose and yield of biomass per glucose, however it enable the cell to have a higher growth rate. Essentially for acetyl-CoA synthesis the carbon flux is shuffled to ethanol leaving less carbon for other processes. Additionally as the native process of forming cytosolic acetyl-CoA cost two ATP per acetate consumed it is beneficial to have as high ATP yield as possible. To increase the acetyl-CoA synthesis, it is therefore prudent to avoid the effect. It can be done by limiting the feed rate of the sugar and therefore also the growth rate. The strategy would therefore require long batch times and if the capital investment, cleaning and work cost of the production has a large effect on the economy of the production it would be a severe disadvantage with the strategy.

An alternative strategy would be to eliminate the ethanol production entirely. An essential enzyme in the formation of ethanol is pyruvate decarboxylase (PDC). Ethanol is formed from pyruvate derived from the glycolysis which is turned into acetyl aldehyde and CO_2 by PDC. The acetyl aldehyde is then converted to ethanol by alcohol dehydrogenase (ADH). There are several copies of the ADH making it difficult to remove this enzyme activity and eliminate the ethanol production in that way. PDC on the other hand, exist only in three copies in *S. cerevisiae* (PDC 1, 5 and 6) (33) which has made it possible to create a PDC deletion strain (34). The deletions has a side effect that a C_2 compound has to be added to the growth media as the acetyl aldehyde formed from PDC is the cellular source of cytosolic acetyl-CoA (35). The cytosolic acetyl-CoA as mentioned is essential for the cellular lipid metabolism thus building the cell membrane. The C_2 compounds that can be supplied are ethanol and acetate which can be formed from acetyl aldehyde in the reversible reactions that are deleted when PDC is removed. Therefore deletion of PDC cannot be used as a background for acetyl-CoA synthesis unless another source of cytosolic acetyl-CoA can be created, which the detailed metabolic pathway would do. Although the Crabtree effect is not present when *S. cerevisiae* grows on xylose which makes the PDC deletion redundant. However the final aim of the strain is to have it be able to grow on a lignocellulose biomaterial not pure xylose. In that situation the PDC deletion would be beneficial.

This strategy does not completely avoid the issue with a slow growth rate, which was the major limitation of the previously described PDC deletion strategy, in addition to also requiring a two carbon substrate in the growth media. But as the metabolic pathway outlined here would supply the cell with cytosolic acetyl-CoA the addition of C_2 compound would be unnecessary. Unfortunately the growth rate on xylose is even lower compared to the PDC deletion, strain further optimisation to the strain is needed before it would be a valid compensation. Therefore evolutionary engineering could also be performed on the PDC deletion background as well, especially as the growth rate on xylose and with the PDC deletion would probably decrease the growth even further. Additional the evolutionary engineering could be based on xylose and glucose in combination. Especially as the final goal

is to have a strain consume the biomass as a whole and the benefits gained on one of the carbon sources might be of less significance when the carbon sources are combined.

To go further with the aim to have a strain that can utilize hemicellulose, other wood and other biomasses contains other pentose then xylose. The second most abundant pentose is arabinose and similar pathways to the oxioreductase (36, 37) and isomerase pathway (38) have been identified that utilize arabinose. These have been successfully cloned into *S. cerevisiae* and both pathways result in the formation of X5P and could therefore easily be combined with the pathway constructed here.

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Appendix

Table 8 lists the pismires used to construct cassette 1.1 and 1.2. Table 9 describes how they were used to amplify each individual fragment. Table 10 shows the strategy to fuse the individual fragments and Table 11 lists the sequence of the primers used to confirm the transformation as seen in Figure 5. Figure 11 displays the full plasmid map for cassette 2.2. Cassette 2.1 has the same design with the exception that the FPK gene is replaced with the XPK gene.

Table 8 The primers used for the construction of cassette 1.1 and 1.2.

Name	Sequence
XKS-R1(CYC1t)	CATAACTAATTACATGACTCGAGGTCGACGGTATCTTAGTGCTTCAATTCTGATTCCATTTTAGC
XI-CY-F1(TEFp)	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGTAAAAGAATATTTCCCAGC
XI-CY R1(Pyx212t)	GGATACCCGGGTCGACGCGTAAGCTTGTGGGCCCTATCATTAATTAATCACATATACATGGCTAC
XI-Pi-F1(TEFp)	GAAAGCATAGCAATCTAATCTAAGTTTTAATTACAAAATGGCAAAAGAATACTTCCCT0043
XI-PI-R1(Pyx212t)	CGGATACCCGGGTCGACGCGTAAGCTTGTGGGCCCTATCATTAATTAATCATTGATACATTGCTACGATG
XI-3 up F	GTTACTTGCTCTATGCGTTTG
XI-3 up R (TPIp)	GGCATTATTCTAAGTAAGTTAAATATCCGGCGTCAATATCCTAAAAC
TPIp F (XI-3 up)	GTTTTCCGTTTTAGGATATTGACGCCGGATATTTAACTTACTTAGAATAATGCC
XKSmid R	TCTTCTGCTTCCAGTTCTGC
pYX212t R (XI-3 dw)	TGTACAAAACAGTTTAATAATGATCTGTATTGCTGGCTCAATCCACGTAATGCCGTAAACCACTAAATCG
URA3 F (XI-3 dw)	GAGCCAGCAATACAGATCATTATTAAACTGTTTTGTACATGATGTTAGTTTTAGCTTTGACATGATTAAG
URA3 R (XI-3 dw)	GATCTGTATTGCTGGCTCAATCCACGTAAATCGATAAGCTTGATATCGAATTCC
XI-3 dw F (URA3)	GCAGGAATTCGATATCAAGCTTATCGATTTACGTGGATTGAGCCAGC
XI-3dw R	TCCGGACCAGCAGATAATG

Table 9 Strategy for amplification the individual fragments in cassette 1.

Name	Primers	Product (size in bp)
FR 25	XI-3 F	X1-3 UP (585)
	XI-3 R (TPIp)	
FR 26	TPIp F (XI-3 up)	TPIp-RTG-FAB1t-CYC1t-tXKS (3350)
	XKSmid R	
FR 27	XKS R1 (CYC1t)	XKS-PGK1p-TEF1p-CY (4430)
	XI-CY-R1(Pyx212t)	
FR 28	XKS R1 (CYC1t)	XKS-PGK1p-TEF1p-PI (4430)
	XI-PI-R1(Pyx212t)	
FR 29	XI-CY-F1(TEFp)	CY-pXy212t (1970)
	pYX212t R (XI-3 dw)	
FR 30	XI-PI-F1(TEFp)	PI-pXy212t (1970)
	pYX212t R (XI-3 dw)	
Fr 31	URA3 F (XI-3 dw)	URA3 (1330)
	URA3 R (XI-3 dw)	
FR 32	XI-3 dw F (URA3)	XI-2 dw (558)
	XI-3 dw R	

Table 10 Strategy for the fusion PCR of cassette 1.1 and 1.2. In Cassette 1.1 transform FR 33, FR 27 and FR 34. Cassette 1.2 transform FR 33, FR 28 and FR 35

Name	Template	Primers	Product (size in bp)
FR 33	FR 25 FR 26	XI-3 F	X1-3 UP- RTG-tXKS (3848)
		XKSmid R	
FR 34	FR 29 FR 31 FR 32	XI-CY-F1(TEFp)	CY-URA-XI-3 dw (3761)
		XI-3 dw R	
FR 35	FR 30 FR 31 FR 32	XI-PI-F1(TEFp)	PI-URA-XI-3 dw (3761)
		XI-3 dw R	

Table 11 Primes used for diagnostic PCR for conformation of transformation.

Name	Sequence
TPIp F (XI-3 up)	GTTTTCCGTTTTAGGATATTGACGCCGGATATTTAACTTACTTAGAATAATGCC
RTG control	CTTGAGACCAACTACCGGAAC
MCR short F	TTGGAAGTTATGGATGGTTCTGAC
MCR short R	ATTACTCTTGGACCTCTTGCTCTA
PTA F (TEF1p)	TAGCAATCTAATCTAAGTTTTAATTACAAAAAAACAATGTCTAGAATAATAATG
PTA R (tADH1)	TAATAAAAATCATAAATCATAAGAAATTCGTTATTGTTGTTGTTGAGAGGC

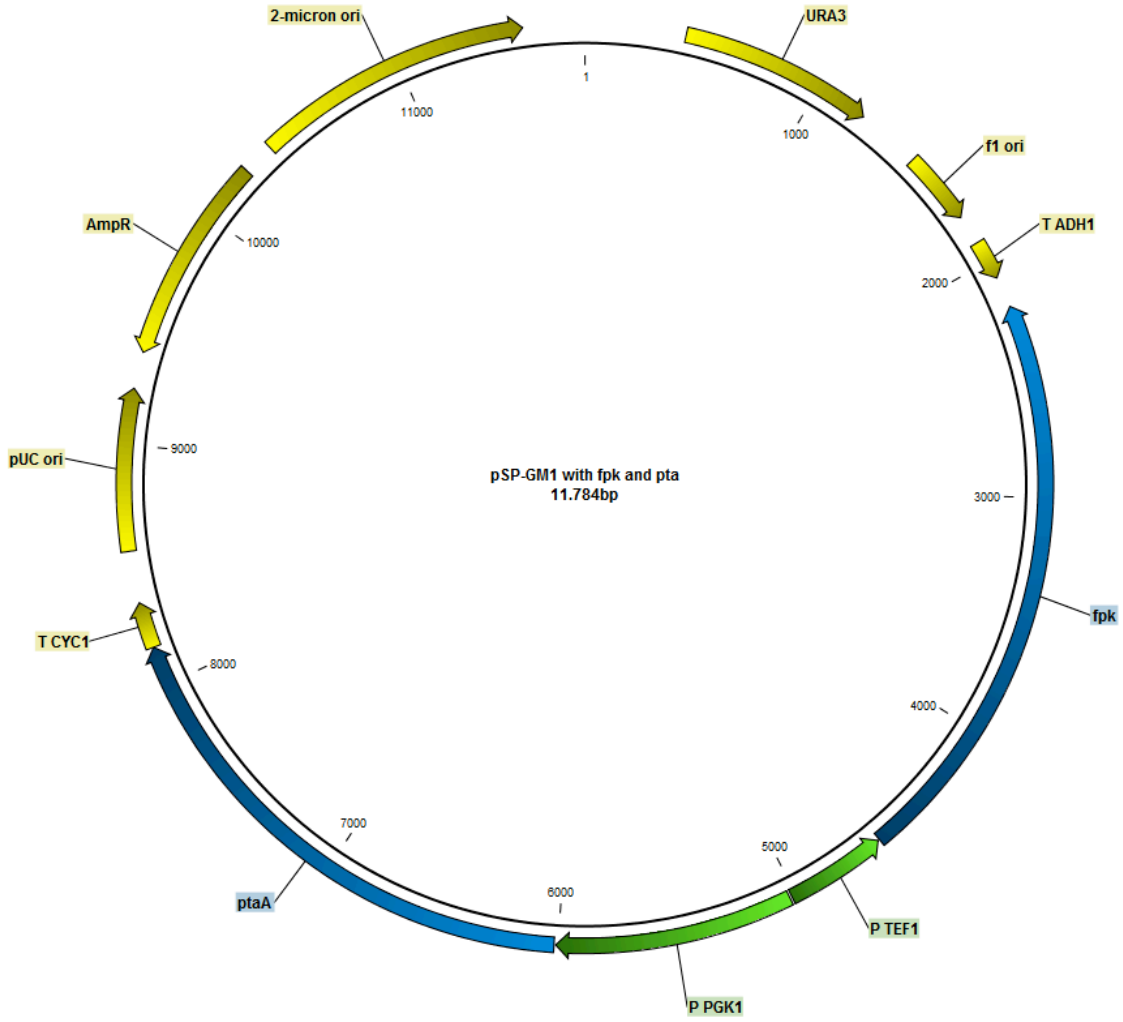

Figure 9 Map of the plasmid composition of cassette 2.2 the composition of cassette 2.1 is identical with the exception of the FPK gene which is replaced with the XPK gene.