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Introduction of *FLO1* to and characterization of a xylose fermenting *Saccharomyces cerevisiae.*

Master of Science Thesis

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Thesis for the degree of Master of Science

” Introduction of *FLO1* to and characterization of a xylose fermenting *Saccharomyces cerevisiae.*”

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Abstract

The interest for bioethanol as a fuel is increasing rapidly all over the world. This is due to a number of factors including declining oil reserves and a will to use carbon neutral fuels.

Ethanol production today is associated with many problems. The dominating feedstocks are sugar and starch-based agriculture crops. These can be used as food and feedstuff for domesticated animals. Agriculture today is very oil dependent and it is therefore both an ethical as well as an economical issue whether it is reasonable to continue bioethanol production in this way. The ideal feedstock appears to be hydrolyzates from lignocellulosic biomass. Lignocellulosic materials are composed of the three polymers cellulose, hemicellulose and lignin which combined are the structural elements in the plant cell wall. Lignocellulose is available in abundance from a variety of sources all over the world and is currently very cheap.

The common baker's yeast *Saccharomyces cerevisiae* is a good organism for ethanol production and is widely used today. However, it does not perform satisfactory when fermenting lignocellulosic hydrolyzatesbecause 1) The hydrolyzates have a high content of xylose, which *S. cerevisiae* can not utilize as substrate for fermentation; 2) During the hydrolysis process toxic compounds are produced which inhibit growth and decrease ethanol production.

Xylose fermenting *S. cerevisiae* can be obtained through genetic engineering and higher tolerance to inhibitors can be achieved by high cell densities. In this study, we focused on generating high local cell densities through the expression of the *FLO1* gene*,* a gene coding for a protein called flocculin which enables the yeast to flocculate, meaning that the cells clump together in flocs of several thousand cells. We tried to activate *FLO1* in a xylose fermenting strain of *S. cerevisiae* named CEN.PK XXX, based on the CEN.PK113-7D strain. A number of different approaches were conducted in order to achieve this. Among these, the endogenous promoter of *FLO1* was intended to be replaced by a strong constitutive promoter. However it was found that the strain lacks *FLO1* making the attempt impossible. A gene disruption cassette was intended to be constructed including the *FLO1* gene under the control of the *TDH3* promoter. However, the attempt was not successful. When *FLO1* was cloned into a shuttle vector and transformed to competent, recombinase negative *Escherichia coli* cells, a fragment of approximately 2000 bp was lost. This behavior is likely due to the large regions of highly repetitive sequences in the *FLO1* gene which causes some recombination mechanism to occur in the host bacteria.

The CEN.PK XXX strain was characterized under anaerobic conditions in Erlenmayer flasks in media supplemented with 50 g/l glucose, 50 g/l xylose or a mixture of 25 g/l, each of xylose and glucose, respectively. It could be concluded that CEN.PK XXX utilizes xyloseat rates that are among the highest reported in the litterature, however, the strain accumulates xylitol, which is an intermediate of xylose fermentation and this accumulation is likely due to an imbalance of cofactors in the metabolic pathway

Keywords: *Saccharomyces cerevisiae*, ethanol, inhibitors, xylose, flocculation, *FLO1,* CEN.PK113-7D, CEN.PK XXX

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Contents

1 Introduction

The interest for biofuels is rapidly increasing around the world and many countries and organizations are showing great interest in biofuel production for many reasons. Some of these include high oil prices and globally declining oil reserves, concern for emissions of air pollutants, a will to use carbon neutral fuels as well as the possibility of alternative income for agriculture producers [1], which is considered a good way for developing communities and remote villages to become self-sufficient in energy production [2]. Especially bioethanol has drawn considerable interest, due to its long traditional use and easy production. Today it is the most widely used liquid biofuel for motor engines [3].

Compared to the price of the predominant liquid fuel, gasoline, ethanol price is competitive. The prices (updated on 2012-04-10) were 0.60 and 0.87 $/l for ethanol and gasoline respectively. To have a fair comparison between these we need to take into account the energy density of 24 and 34.2 MJ/l [4]. This gives a price of 0.358 $/MJ for ethanol and 0.365 $/MJ for gasoline. The margin is small and has varied historically. As long as the ethanol price is ~70 % or less (based on volume) of that of gasoline, the price per energy unit will be lower for ethanol. In addition to the assumed price benefit, one should also take into account that the combustion of ethanol is cleaner and the emissions of toxic substances are therefore lower [5].

One major issue with ethanol production is that currently only a handful of raw materials can be efficiently utilized as substrates. Sugars, molasses (from sugar cane and sugar beet) and starch (from e.g. maize and potato) are completely dominating [6]. All of these feedstocks compete for arable land with food and animal feedstuff. Therefore it is an economical as well as an ethical issue whether it is reasonable to continue production in this way. Today the ethanol and oil prices are strongly correlated, due to the heavy oil dependence of agriculture [7]. As long as these feedstocks are used, the small margins for ethanol versus gasoline previously described cannot be improved and it is even debated whether the production of bioethanol actually requires more nonrenewable energy than the resulting fuel provides [8]. A better feedstock would be lignocellulosic biomass from wood and straw, consisting of the sugar polymers cellulose and hemicellulose as well as lignin, which is a complex macromolecule, not primarily made up from sugars. Only agricultural waste and crop residues have been estimated to potentially produce close to $5 \cdot 10^{11}$ liters of bioethanol [9], compared to the 2006 global production of $4 \cdot 10^{10}$ liters [10]. They are cheap, easily accessible and abundant, which make them ideal for large-scale ethanol production. These materials come from a wide variety of sources and their use enables local sources to be utilized. In Sweden, the choice of source would naturally fall on wood. The total annual growth of productive forest in Sweden is approximately 110 Million m^3 distributed on 39% Scots pine, 42% Norway spruce and 12% birch [11]. With an estimated sugar composition of 43% glucan, 10% xylan, 2.4% galactan, 1.4% arabinan and 8.7% mannan of total weight dry wood [12], the highest theoretical yield of ethanol is $2.58 \cdot 10^{10}$ liters [13], corresponding to $6.2 \cdot 10^{11}$ MJ. This would be more than enough to replace all gasoline used for transport in Sweden which were $1.6 \cdot 10^{11}$ MJ in 2010. One must also consider that far from all of the forest can be used for ethanol

production, but with an increase of about 35 Million m^3 of total forest volume between the years 1995 to 2005, the potential is still to be considered as good [14].

The organism of choice, and completely dominating for bioethanol production is the common baker's and brewer's yeast *Saccharomyces cerevisiae,* one of the first microorganisms to be domesticated by humans. Many of the problems with effective and sustainable ethanol production presented above are due to limitations related to the yeast and all alternative organisms' fermentative performance. Much effort is put into improving *S. cerevisiae* and its performance in ethanol fermentation: one among other such attempts will be presented in this project.

1.1 Aims

The aim of this project is to be able to create and characterize a flocculating and xylose-fermenting yeast strain. This will be done to increase the robustness of the yeast in the presence of fermentation inhibitors typically found in the wood hydrolysate used for bioethanol production. It is important that the properties introduced into the yeast strain are stable and do not change over time and generations. In order to achieve this I took into consideration the following sub goals:

- Activate the expression of the *FLO1* gene in a xylose fermenting *S. cerevisiae* CEN.PK XXX as well as in the wild type strain CEN.PK113-7D for comparison. The constructed strains should be robust and able to be used for further studies.
- Evaluate and compare fermentative performance of the different strains, co-consumption of xylose and glucose, and sensitivity to fermentation inhibitors.
- Propose how a process for production of ethanol from lignocellulosic hydrolyzate may be designed using flocculating and xylose fermenting yeast.

2 Background

2.1 Lignocellulosic biomass

Lignocellulosic biomass generally consists of the three polymers cellulose, hemicellulose and lignin. These are the main constituents of the plant cell wall and give structure and rigidity. The internal distribution of these components differs between different plants and tissues, but the main structure is preserved. Cellulose molecules form microfibrils which are coated with hemicellulose and make up the main structural matrix. This ordered structure resides in a durable polymeric matrix of lignin as illustrated in Figure 1.

Cellulose is a linear homopolysaccharide polymer composed of units of anhydroglucose linked with β(1-4)-glycosidic bonds. Formation of microfibrils is a spontaneous arrangement of cellulose molecules which occurs due to extensive hydrogen bonding. Microfibrils are the structural components of cellulose fibers. This forms a highly crystalline structure.

Hemicellulose is a heteropolysaccharide with a more irregular structure than cellulose. It consists of both hexose sugars such as glucose, mannose and galactose as well as pentoses such as xylose and arabinose. It also includes 4-O methylglucuronic acid and galacturonic acid. The irregular structure makes hemicellulose mainly amorph, compared to the crystalline structure of cellulose.

The third constituent of lignocellulose is lignin which is mainly made up from variously bonded hydroxy- and methoxy-substituted phenylpropane units. None of these can be utilized as substrate for fermentation which is the reason why lignin is usually unwanted in ethanol production [12].

2.2 Hydrolyzates from lignocellulose

Using lignocellulosic biomass as feedstock for ethanol production requires initial processing and preparation which the currently dominating sugar and starch based feedstocks do not. The process can be divided into two major operations; pretreatment and hydrolysis. The aim of these processes is to make all the sugar monomers available for fermentation. The product is called hydrolyzate. The pretreatment aims to delignify the lignocellulosic biomass, to solubilize and break the cellulose matrix and to release all available sugar monomers from cellulose and hemicellulose. There are a number of alternative methods to achieve this result, among these a few are listed here: 1. physical pretreatment such as milling, pyrolysis and microwave oven heating 2. physicochemical treatment such as steam explosion or autohydrolysis, liquid hot water method, ammonia fiber explosion and CO_2 explosion 3. chemical pretreatment such as acid or alkaline pretreatment, wet oxidation or organosolv pretreatment 4. biological pretreatment where microorgansims are utilized to break the lignocellulose matrix. The hydrolysis is done using enzymes to convert the complex polymers to simple sugar monomers. However a combination of the techniques mentioned above is usually utilized as reviewed in [10].

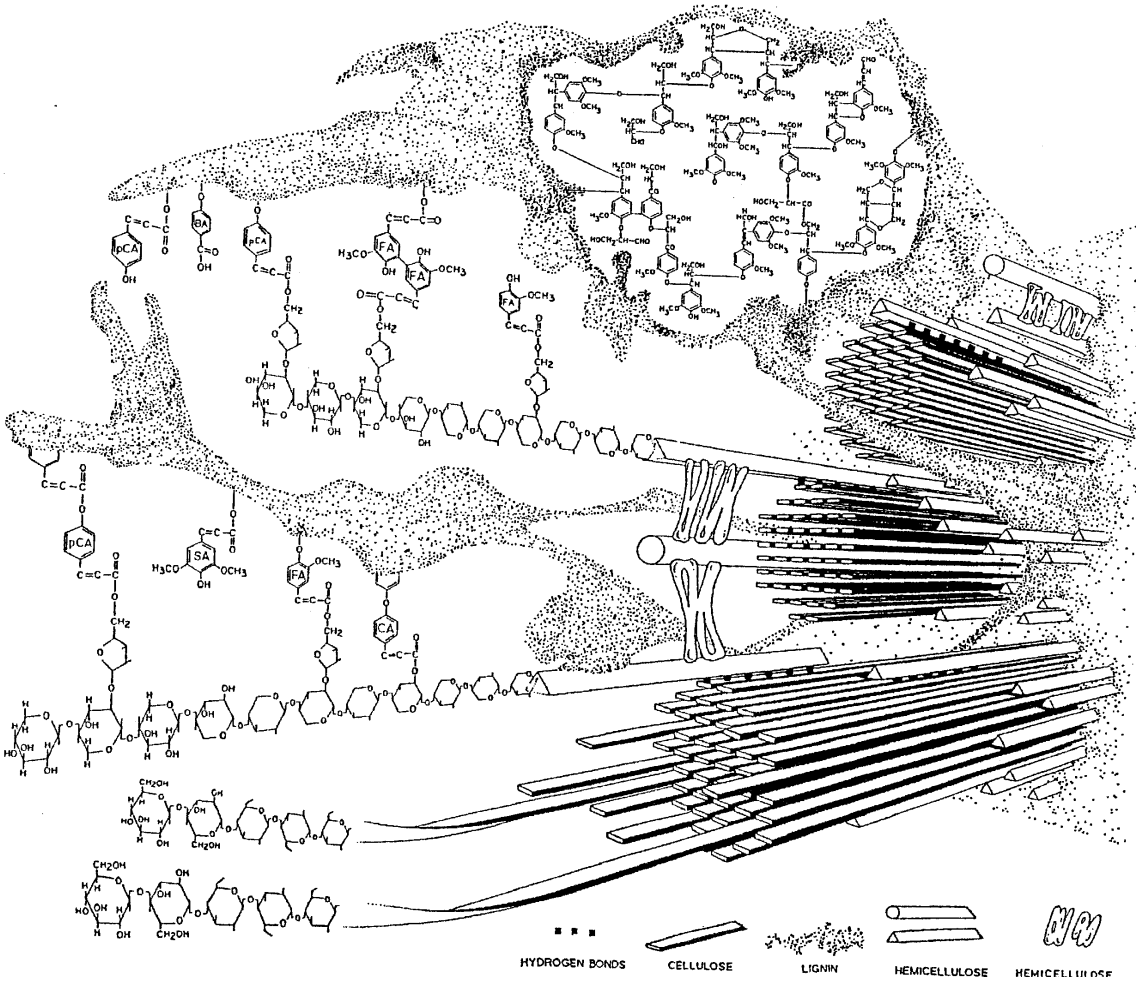

Figure 1. Visualization of the structural arrangement of lignocellulose. Components have been arranged to make the cellulose/hemicellulose structure clearly imbedded in a lignin matrix. Abbreviations BA, *p*CA, SA, FA and CA stands for *p*-hydroxybenzoeic-, *p*-coumaric-, sinapic-, ferulic-, and cinnamic acid respectively. Adapted from [15]

2.3 Xylose fermentation

In order to achieve high yields of ethanol from lignocellulosic materials, all the available sugar monomers ought to be utilized as substrates in the fermentation process. The most abundant monomer is the hexose sugar glucose (Figure 2a), the building block of cellulose. The pentose sugar xylose (Figure 2b) is the second most common monosaccharide building block and has its origin in the hemicellulose fraction of lignocellulose [12, 16, 17]. There are many species of yeast that can convert xylose to ethanol, of which *Candida shehatae* and *Pichia stipitis* (*Scheffersomyces stipitis*) are considered especially good [18]. However *S. cerevisiae* is not one of them [19]. In xylose utilizing strains, such as *P. stipitis* xylose is metabolized via the oxidoreductive pathway (Figure 3a), wherein the first step is xylose reduction to xylitol via the enzyme xylose reductase (XR) which is dependent on reducing cofactors such as NADPH or NADH, showing a higher affinity to NADPH. In the second step, xylitol is oxidized to xylulose via the NAD^+-dependent enzyme xylitol dehydrogenase (XDH). In the third step,

xylulose is phosphorylated to xylose-5-phosphate by xylulokinase (XK) and then enters the pentose-phosphate pathway (PPP) (Figure 3a) [20]. The first two steps generate an imbalance in redox potential, as they prefer different cofactors, which can create an accumulation of xylitol and inhibit the metabolism under anaerobic conditions [21]. The imbalance can however be maintained by making XR more specific to NADH, so that the overall red-ox balance is maintained, which will result in higher ethanol yield and productivity [22-24]. *S. cerevisiae* lacks the enzymes responsible for the first two steps (i.e. XR and XDH), but can slowly metabolize xylulose through XK [25].

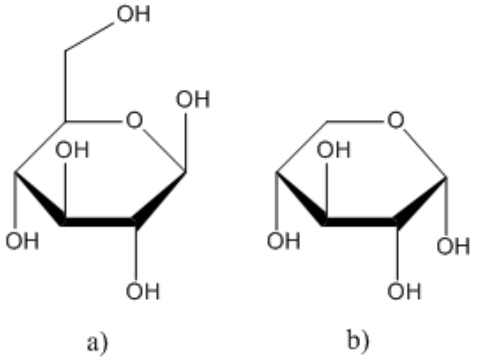

Figure 2. a) Glucose in cyclic configuration b) Xylose in cyclic configuration

An alternative pathway for xylose fermentation is common to many prokaryote microorganisms as well as some plants. It also occurs in the anaerobic fungi belonging to *Piromyces* sp. E2. The pathway utilizes the enzyme xylose isomerase (XI) which directly converts D-xylose to D-xylulose and therefore circumvents the redox imbalance which occur in the XR/XDH pathway, as shown in Figure 3b [26, 27]. The pathway has been introduced to *S. cerevisiae* with promising results [28]. However, a comparative study with both pathways in two strains of identical genetic background showed that the former oxidoreductive XR/XDH pathway is preferable for ethanol production under anaerobic conditions [29]. This conclusion is however debated and others have drawn the opposite conclusion [27].

S. cerevisiae is still an interesting organism to use in large scale production, due to its long traditional use, high ethanol yield, productivity and tolerance. It is also well studied and regarded as safe. This is why many strategies have been formulated to create xylose fermenting variants. Most of them include the introduction of *XYL1* and *XYL2* which encode XR and XDH respectively. In addition to this, attempts have been made to over express the XK enzyme, which is encoded by the *XKS1* gene [20, 30-33].

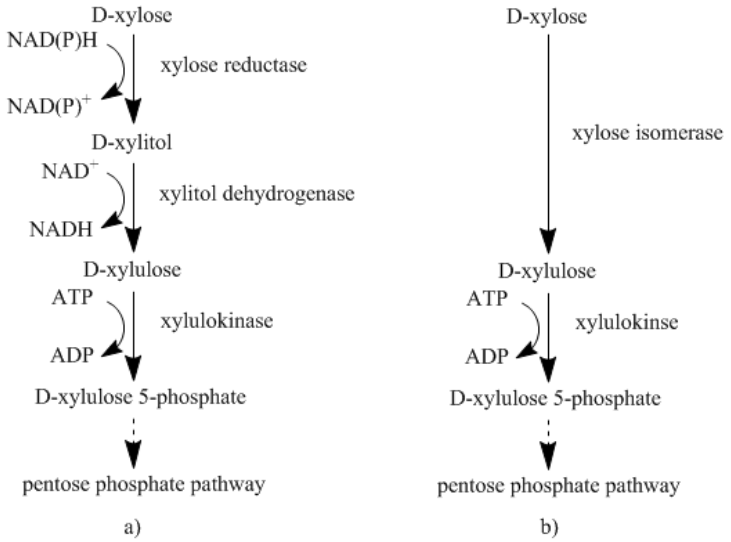

Figure 3 Metabolic degradation of D-xylose. a) oxidoreductive XR/XDH pathway and b) cofactor independent XI pathway.

2.4 Inhibitors from hydrolysis

Methods using concentrated or dilute acids to produce hydrolyzates can be designed to give high yields and relatively quick and cheap production of hydrolyzates [34]. However, these treatments will also result in the production of degradation products such as furfural from pentoses (e.g. xylose), and hydroxymethylfurfural (HMF) from hexoses (e.g. glucose), as shown in Figure 4, as well as other products such as phenolic- and aliphatic compounds and organic acids [35, 36]. These compounds have a growth inhibitory effect on yeast: they reduce enzyme activity, break down DNA, inhibit synthesis of protein and RNA [37-39] and therefore influence fermentation performance and ethanol production. Furfural and HMF are metabolized in *S. cerevisiae* through the same pathways involved in ethanol degradation or production. Figure 5 shows the metabolism for furfural [40, 41]. Processes to remove or reduce these substances are complicated and expensive. This is also the motivation for trying to improve the yeast, rather than the substrates.

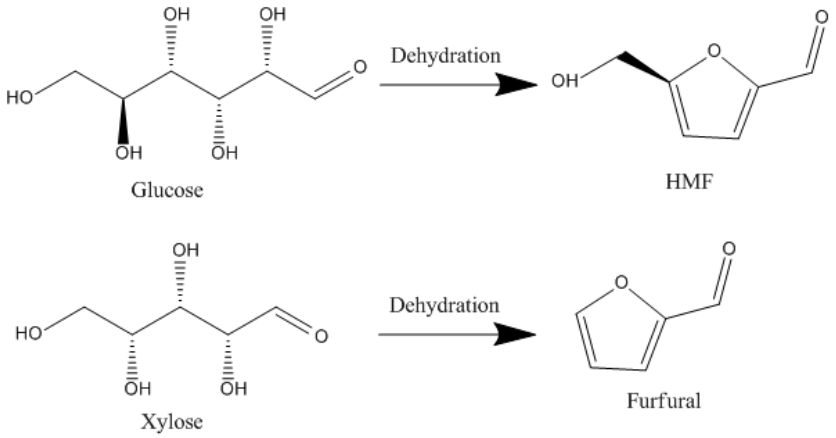

Figure 4 Degradation of sugars to hydroxymethylfurfural and furfural.

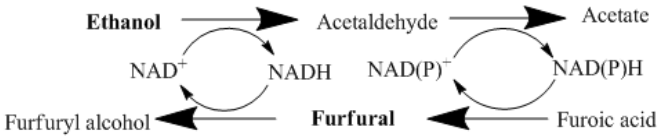

Figure 5 Metabolic degradation of furfural

2.5 Flocculation

The ability of some yeast to reversibly adhere to each other to form flocs, consisting of thousands of cells is called flocculation. Yeast flocculation is natural and has been exploited for centuries in fermentation processes in the brewing industry. The flocs separate from the bulk medium due to sedimentation which makes flocculating yeast an effective, environmental-friendly, simple and cost free way to separate yeast cells from the final product [42].

The flocculation process involves special proteins on the cell wall of the yeast. These proteins are called flocculins and form thin protrusions which bind selectively to mainly mannose residues on the cell wall of neighboring cells. The interaction requires calcium ions in the medium. Removal of calcium ions or addition of complex binders such as EDTA will prevent flocculation and flocs will dissolve [43, 44]. There are two distinct phenotypes of flocculating yeast called Flo 1 phenotype and NewFlo phenotype respectively. Flo 1 is characterized by flocculation being inhibited by mannose, while in NewFlo flocculation is inhibited by mannose as well as glucose, sucrose and maltose [45].

Flocculation is governed by a family of genes called the *FLO*-family. Expression of *FLO1* is mainly responsible for the Flo 1 phenotype. *FLO2* and *FLO4* are alleles (copies) of *FLO1. FLO5* and *FLO9* are highly homologous to *FLO1* (94% similarity) [46]. Expression of these genes is therefore believed to equivalently give rise to the Flo 1 phenotype [47]. In addition to the above mentioned ones, a number of genes are involved, to a variable extent, in the flocculation mechanism. Among these genes there are *FLO11,* which encodes a cell surface flocculin, sometimes responsible for flocculation, but also has a part in the invasive pseudohyphae formation [48-50] and *FLO8* which works as a transcriptional regulator for many of the *FLO*-genes [51]. The characteristics of the NewFlo phenotype may be a result of alterations in the length of tandem repeats in some of the *FLO*-genes [52].

Flocculation in wild type strains is often induced by external factors and stresses. In brewing yeast, flocculation occurs in the later parts of the fermentation, induced by nutrient starvation and/or stress conditions [53], such as carbon (sugar) or nitrogen depletion, oxygen limitation or high ethanol concentration [42].

The interactions of the proteins expressed by the different *FLO*-genes under different conditions and stresses makes the flocculation mechanism complicated and hard to predict, and the fermentative properties can vary widely between a flocculating strain and its non-flocculating counterpart [54, 55]. Flocculation does not usually occur in industrial scale production of bulk ethanol, as commonly used strains lack the ability to flocculate. The common lab strain S288c, as well as many other lab strains, has a nonsense mutation in *FLO8* which inhibits flocculation as *FLO1* and *FLO11* are not expressed [56].

2.6 Flocculation as a tool for better performing yeast in the presence of inhibitors

Good performance of yeast in toxic hydrolyzates has been achieved either by low concentration of inhibitors or by a high cell density [57]. The former is most easily achieved through continuous fermentation with low feed rate [58]. There are several techniques to achieve the high cell density. In continuous cultivations, cell recycling can be used [59, 60]. Cell immobilization can be used with different approaches [61, 62], as well as encapsulation [63, 64]. These techniques show good potential and each have their own advantages. In this context, flocculation appears to be a very good candidate for achieving high cell density and to be used in a very similar way as it has been done in breweries for centuries. When flocculation occurs, the creation of locally high cell densities would be spontaneous and preferably constitutive which would be ideal for large scale production.

The different characteristics displayed by flocculating yeast make it interesting to implement in large scale ethanol production. Flocculating yeast has shown to withstand and detoxify inhibitors in lignocellulosic hydrolyzates to a much higher degree than non-flocculating yeast [65], and to outperform the non-flocculating counterparts also in terms of ethanol productivity [20].

Since inhibitor tolerance and detoxification is one of the major motivations for using flocculating yeast, and the concentration of these substances are highest at the beginning of the fermentation, it is desirable to use a strain which is constitutively flocculating, and not influenced by external factors or induced during the fermentation process [54]. In non-flocculating strains, flocculation can be achieved via constitutive expression of *FLO1*, either by overexpression [66, 67] or by replacing the endogenous and inducible promoters of *FLO1* with strong constitutive ones [55, 68].

2.7 Gene cloning

Cloning is the procedure in which copies of DNA are produced. This can be achieved in a plethora of ways. Here one commonly used method is presented.

2.7.1 Vectors

Vectors are the name given to plasmids which are used to carry DNA during cloning procedures. Plasmids are circular DNA-fragments, which are distinctly separated from and have the ability to replicate independently from the chromosomal DNA. A vector is usually partly engineered to be a convenient tool for cloning genetic material. Commercially available vectors are in many cases designed to be functional in different organisms, i.e. *Escherichia coli* and yeast. A vector with the ability to function in different host organisms is called shuttle vector. Vectors include different functional regions which enable them to operate as desired. Some of these regions are:

1. Origin of replication (ORI): is necessary for replication of the vector in the specific host. ORIs specific for both host organisms are present in shuttle vectors.
2. Selection marker: can be either of dominant or complementation type. Dominant markers give resistance to some antibiotic, e.g. ampicillin for *E. coli* or kanamycin for yeast. Complementation markers are genes which complement an auxotrophic mutation, usually mutations that impair the biosynthesis of some amino acid or nucleic bases.
3. Multiple cloning site (MCS): is a region containing a very high density of restriction enzyme sites. This region is typically used to insert DNA-sequences of interest.

There are some distinct types of shuttle vectors for the *E. coli*/*S. cerevisiae* system.

1. Integrative vectors: are designed to be integrated in yeast genomic DNA. They lack the ability to autonomously replicate in yeast and therefore have to be integrated through homologous recombination. These vectors result in stable gene integration and expression, usually a gene is inserted as single copy in laboratory strains exploiting the phenomenon of homologous recombination.
2. Episomal vectors: have the ability to autonomously replicate in yeast. This makes the plasmid easy to transform and present at high copy number, however selective pressure must be applied to guarantee plasmid replication and stable gene expression.
3. Centromeric vectors: are designed to mimic chromosomes and mimic chromosome behavior in meiosis and mitosis. They contain an autonomous replicating sequence (ARS) and a centromere, assuring segregation of the plasmid as it occurs for chromosomes. They are usually quite unstable and present at low copy number and mostly used for yeast genomic libraries [69].

2.7.2 Vector construction

The process has been described by Sambrook et al 2001 [70]. The general process to engineer a construct on a vector is:

1. Amplification of the desired DNA fragment.

   This is achieved through the polymerase chain reaction (PCR) technique. The sequence of the primers is typically made of two regions. One region is specific for the nucleotide sequence that has to be amplified (red in Figure 6) and is flanked by a nucleotide sequence containing specific restriction sites which have been chosen to match sites on the vector where the fragment is to be inserted (Figure 6). These regions do not anneal to the template (the desired sequence to be multiplied) but the final product will be flanked by the restriction sites.

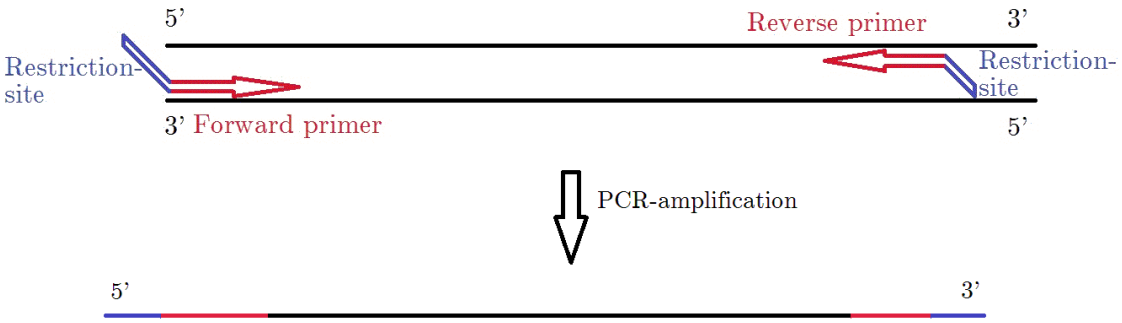

Figure 6 Primers with restriction sites. The blue regions do not anneal to the template, but as the content between the primers is amplified, it will be flanked by the restriction sites.

2. Digestion.

   Restriction enzymes cut double stranded DNA at specific sites of recognition. Using enzymes which leave *sticky ends*, leaving a few bases overhang on either strand (i.e. generating either 5' protruding or 3' protruding extremities), is advantageous. If both vector and insert are cut with the same enzymes, the two fragments could specifically anneal to each other. The cut plasmid will not be able to reclose and the insert will only anneal in one direction when two different enzymes are used (Figure 7a and b).

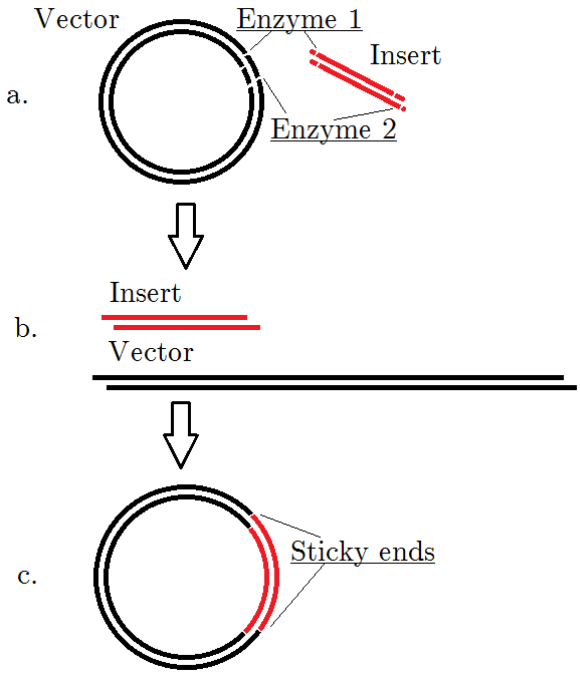

Figure 7 Restriction and annealing of double stranded DNA a. Restriction enzymes cut both DNA molecules at specific sites. b. Vector becomes linearized, small fragments from digestion are removed. c. Sticky ends anneal to each other.

3. Ligation.

   The two digested fragments are mixed and a ligase enzyme is added. The sticky ends will anneal to one another and the ligase will close the gaps, creating a new plasmid with the desired fragment integrated in the accurate position (Figure 7c).

2.7.3 Vector amplification

The newly constructed plasmid vector must be amplified and purified. This is achieved by transforming it into *E. coli*, which is used because it is well studied, easy to transform, fast growing, requires low maintenance and has GRAS status.

In transformation, exogenous DNA is taken up or incorporated to be expressed by a cell. *E. coli* has to undergo a procedure to become what is called competent, meaning susceptible for transformation. This is done by treating *E .coli* cells with calcium ions before transformation. For methodology on heat and cold treatment see Appendix 8.2.1. Far from every cell will have been transformed, and in order to select the ones that carry the plasmid the cells are streaked on to an agar plate in which there is an antibiotic that disable growth for untransformed cells. Transformed cells are able to grow and form colonies on the plate [71]. As long as the cells are kept growing in media with the required antibiotic they will all include copies of the plasmid vector, which can then be harvested and purified.

2.7.4 Transformation of *S. cerevisiae*

To create a stable transformation of *S. cerevisiae* the procedure is substantially different from the one in *E. coli*. Integration to the chromosomal DNA is done not only for stability, but also to avoid having constant selective pressure. One method to do this was developed by Güldener et al 1996 [72] and has been developed especially for gene knock out. The process can be explained in the following steps:

1. The plasmid including the constructed cloning (or disruption) cassette is purified from an over-night culture of *E. coli* according to the methodology described above. First the cells are lysed and the cell debris is removed through centrifugation. The DNA is then captured on a silica based membrane. After several washing steps, the DNA is eluted in pure water.
2. Amplification of cassette

   Through a PCR reaction, the entire cloning cassette is amplified. The primers used for this are flanked with regions which are homologous for a specific region in the *S. cerevisiae* genome where the cassette has to be integrated. This PCR product is called *gene disruption cassette* due to its ability to knock out a targeted gene. The cassette holds a loxP-KAN-loxP system in which the KAN-gene (including promoter and terminator) is used as a selection marker, to produce resistance to the antibiotic kanamycin. The loxP-flanking can be used together with Cre-recombinase to delete the KAN gene through Cre-Lox recombination [73].

3. Transformation

   The transformation procedure for *S. cerevisiae* is similar to the one for *E. coli* but includes treatment of the cells with LiAc to make them competent. The transformation takes place in 30°C for 30 min followed by 15 min in 42°C [74].

4. Homologous recombination and integration

   Once the disruption cassette is transferred into yeast cells, the flanking regions will anneal to the homologous regions in the specific chromosomal locus. The process of homologous recombination will lead to stable integration into chromosomal DNA of Yeast (Figure 8).

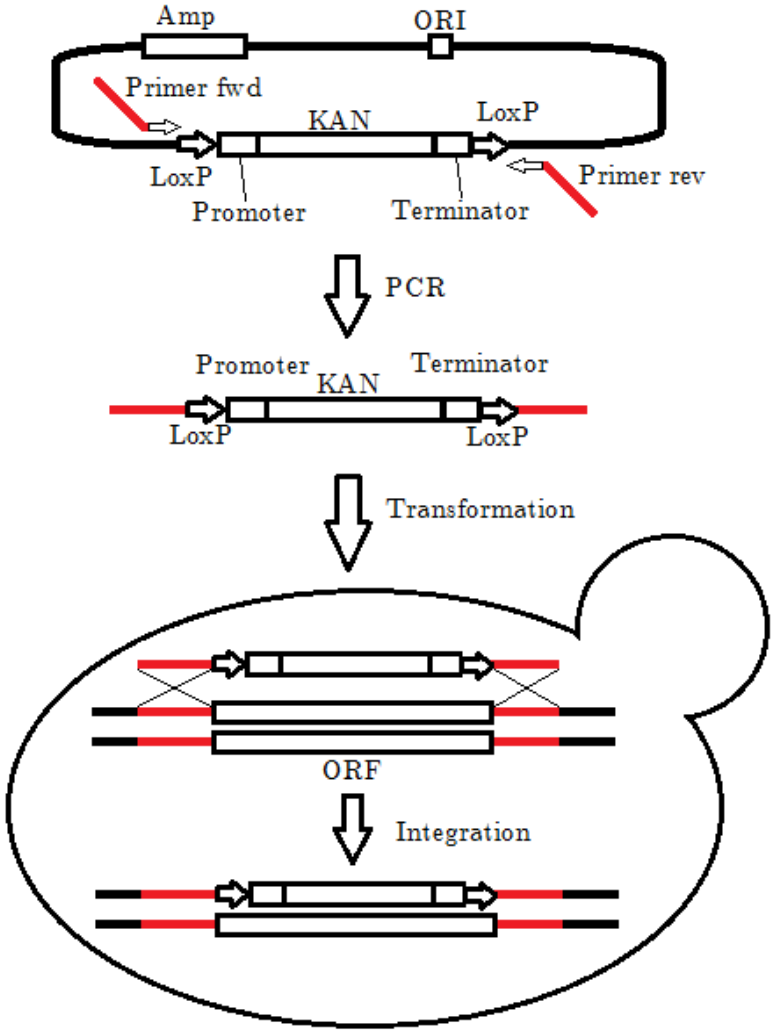

Figure 8 Principle of gene disruption in a target locus of the *Saccharomyces cerevisiae* genome. Red regions are homologous for left and right flanking of target open reading frame (ORF). After the LoxP site on the 3' end the gene of interest can be cloned, together with its functional cassette. In the first step PCR primers (primer fwd, primer rev) anneal to the gene disruption cassette and amplify this in a PCR reaction. The cassette is then transformed into *S. cerevisiae*. The ends of the primers anneal to the flanking regions of the target loci which enables recombination. Successful integration will make the host cell resistant to kanamycin which can be used as selection marker. Redrawn and modified from [72].

3 Materials and Method

Table 1 Plasmids used, created or planned during the project.

Name	Appendix figure	Ref.
pUG6	A	[72]
YEp181_TDH3p-CYC1t	B	**
YEp181_KAN- TDH3p-CYC1t	C	*
pUG6_TDH3p	D	**
YIp211	E	[75]
YIp211_KAN-TDH3p	F	*
YIp211_TDH3p-CYC1t	G	**
YIp211_TDH3p-FLO1-CYC1t	H	*
YIp211_KAN-TDH3p-FLO1-CYC1t	I	*

A map of the plasmids can be seen in Appendix 8.7.
* *This project*
** *Mapelli, Unpublished*

3.1 Strains and media

3.1.1 *E. coli*

For plasmid construction in *E. coli* the strain DH5α was used as a host. All *E.coli* were cultivated in LB-media consisting of 1 g glucose, 10 g peptone, 5g yeast extract and 10 g NaCl in 1 l milliQ (mQ) H_2O (Millipore). All components were mixed and immediately autoclaved. All chemicals were purchased from MERCK (Darmstadt, Germany). For solid media on plates, 20 g/l agar was added before autoclavation and approximately 30 ml was poured into each plate. For selective media LB medium was supplemented with 100 µg/ml ampicillin (LB-Amp) (Appendix 8.1.1).

3.1.2 *S. cerevisiae*

The strain used as reference was CEN.PK 113-7D (*MAT*a, *MAL2-8C, SUC2*) kindly provided by Dr. Peter Kötter (Max, von Laue Str. 9, Biozentrum N250, 60438 Frankfurg Germany) [76]. The xylose fermenting strain was constructed by Dr. Nicklas Bonander and named CEN.PK XXX (Unpublished). CEN.PK XXX harbours the *XYL1* gene under the control of the *S. cerevisiae PGK1* promoter, the *XYL2* gene under the control of the *S. cerevisiae TDH3* promoter, both genes are from *Pichia stipitis,* and the *XKS1* gene from *S. cerevisiae* under the control of the *S. cerevisiae TPI1* promoter. The genes have been codon optimized for expression in S. cerevisiae, cloned in sequence and integrated through homologous recombination in the *GRE3* locus. CEN.PK XXX was constructed from the prototrophic CEN.PK 113-7D.

CEN.PK 113-7D was maintained on YEPD-agar plates consisting of 15 g of agar, 10 g of yeast extract, 20 g of soy peptone and 20 g of D-glucose as an additional carbon source in 1 liter mQ H_2O (Appendix 8.1.2). CEN.PK XXX was maintained on minimal media plates with 20 g/l xylose (Appendix 8.1.3)

For yeast cultivation in liquid medium, a defined medium was used containing per liter solution: 7.5 g $(NH_4)_2SO_4$, 3.5 g KH_2PO_4, 0.75 g

$MgSO_4 \cdot 7H_2O$, 30 mg $EDTA \cdot 2H_2O$, as well as trace metal and vitamin solutions (Appendix 8.1.4.) The medium was supplemented with either 50 g/l D-glucose, 50 g/l D-xylose or a mixture of 25 g/l D-glucose and 25 g/l D-xylose as carbon source. Salts, trace metals and carbon source were autoclaved separately. The vitamin solution was filter sterilized. For anaerobic cultivations the medium was supplemented with 10 mg ergosterol and 0.42 mg Tween 80, which was prepared by dissolving the chemicals in boiling absolute ethanol and kept boiling for 10 minutes [77].

3.2 Cultivations

All cultivations were performed in triplicates.

3.2.1 Pre-culture

50 ml defined medium with 50 g/l glucose was poured into 100 ml sterile Erlenmayer shakeflasks. One yeast colony from a YEPD or minimal media plate was inoculated. The flask was sealed with a cotton stopper and left to incubate on a shaker at 150 rpm in 30°C overnight. Cells were harvested by centrifugation at 3000 rcf for 5 min and the supernatant was decanted. Cells were resuspended in 50 ml fresh defined medium with 50 g/l glucose and poured into sterile 100 ml Erlenmayer flasks and incubated as before for 2 h. This step is done to make sure that all cells are in a vegetative state. Cells were once again harvested, washed and resuspended in sterile milliQ H_2O twice.

3.2.2 Main culture

Pre-cultured cells were inoculated to OD_{600} 0.0015 in sterile 250 ml Erlenmeyer flasks with 100 ml defined medium, supplemented with 10 mg/l ergosterol solution, together with the appropriate carbon source. CEN.PK 113-7D was grown in 50 g/l glucose, CEN.PK XXX was grown in 50 g/l glucose, 50 g/l xylose and a mixture of the two carbon sources (25 g/l each). Flasks were sealed with water-lock-loops for anaerobic cultivation. Cells were cultivated at 30°C under orbital continuous shaking at 150 rpm.

Sampling was performed using a syringe connected to a thin metal tube in contact with the cultivation broth, 0.5 ml sample was first discarded, and thereafter the supernatant sample was obtained by filtering 1 ml of culture broth through a 0.2 µm nylon filter and stored at -20°C until analysis. The concentrations of glucose, ethanol, glycerol, acetate and pyruvate were determined by HPLC (Ultimate 3000, Dionex Corp., Sunnyvale, USA) fitted with an Aminexff HPX-87H column (Bio-Rad Laboratories, Inc.) kept at 45°C and using 5mM H_2SO_4 as mobile phase at a flow rate of 0.6 ml/min. All compounds were detected by a refractive index detector RI-101 (Dionex Corp., Sunnyvale, USA) and variable wave length detector VWD 3100 (Dionex Corp., Sunnyvale, USA) at a fixed wave length of 210 nm.

3.2.3 Determination of dry cell mass

After cultivation the cell suspension was filtered using dried and individually weighed filter papers and washed with milliQ H_2O twice. Filter papers were

then dried in a microwave oven for 15 min at 150 W. The filters were then weighed (Appendix 8.4) [78, 79].

3.2.4 Calculations

Since no measurements of cell mass were performed during-, but only at the end of the cultivations, no accurate measurement of specific growth rate can be obtained. However, this can be estimated by the sugar consumption under the assumption that sugar consumption and biomass are linearly dependent.

$$\mu = \frac{\ln(\frac{X_2}{X_1})}{t_2 - t_1} \approx \frac{\ln(\frac{S_2}{S_1})}{t_2 - t_1} \quad (1)$$

Where X is the biomass (g/l), S is the amount of consumed sugar (g/l) and t is the time (h). Specific growth rate was calculated over the entire interval of logarithmic growth. A linear regression of the logarithmic values of sugar concentration was done using MS Excel, the slope of the line is equal to the specific growth rate.

Standard deviations were calculated using MS Excel command STDEV which uses the formula:

$$\sigma = \sqrt{\frac{\sum(x - \bar{x})^2}{(n-1)}} \quad (2)$$

Where $\bar{x}$ is the sample mean and n is the sample size.

An estimation of the consumption rates are calculated by the formula:

$$q_S \approx \frac{S_1 - S_2}{X_{est(1-2)}(t_2 - t_1)} \quad (3)$$

Where S is the concentration consumed compound (g/l) (for production rates, the formula is the same but S_1 is subtracted from S_2 instead), t is the time (h) and $X_{est(1-2)}$ is an estimate of the biomass at a time point halfway between t_2 and t_1(g/l). $X_{est(1-2)}$ was calculated based on ethanol production, with the assumption that ethanol concentration and biomass concentration are linearly dependent. A constant which related ethanol concentration with biomass concentration was calculated by dividing the final biomass concentration with the final ethanol concentration. $X_{est(1-2)}$ could then be calculated by doing an exponential regression for the time point between t_2 andt_1.

Yields were calculated using the formula:

$$Y_C = \frac{C_{end} - C_0}{S_0 - S_{end}} \quad (4)$$

Where C is the concentration of some product (g/l) and S is the concentration of the total sugar (g/l).

3.3 Plasmid construction

In order to construct the entire gene disruption cassette for *S. cerevisiae*, including *FLO1* two different approaches were conducted. All plasmids used in this study are listed in Table 1.

3.3.1 First approach

The pUG6 plasmid was used as template to amplify KAN (when referring to KAN, the entire KAN-cassette containing LoxP-flanking, TEF2p, TEF2t and KAN ORFis included) using oligonucleotides DrdI_KAN_fwd and SapI_KAN_rev (Table 2) as primers for a PCR-reaction (Appendix 8.2.6). The obtained fragment was purified on a 0.8% (w/v) agarose gel (Appendix 8.3) the fragment was in turn used as template for a new PCR reaction using the same primers. This product was later used for the ligation.

The plasmid YEp181_TDH3p-CYC1t carrying the *S. cerevisiae TDH3* promoter and the *CYC1* terminator was used as vector for the ligation. 2 µg of plasmid and 0.5 µg of the amplified KAN-cassette were digested with DrdI and SapI (all restriction enzymes were purchased from Fermentas) (Appendix 8.2.7). The linearized vector was loaded on a 0.8 % (w/v) agarose gel and purified using Illustra GFX PCR DNA and gel band purification kit (GE) (Appendix 8.2.3). The vector was then dephosphorylated using Shrimp Alkaline Phosphatase (Fermentas) (Appendix 8.2.5). In order to remove residues from dephosphorylation and digestion, an additional purification step using GeneJET PCR Purification Kit (Fermentas) was performed (Appendix 8.2.4). After quantification of the vector and plasmid using NanoDrop 2000 (Thermo Scientific), they were ligated (Appendix 8.2.8) and transformed into competent *E. coli* (Appendix 8.2.1), to form YEp_KAN. Cells were cultivated on LB-Amp plates. Growing colonies were tested through verification of colony PCR (Appendix 8.2.9) using the same primers as for the KAN-cassette amplification.

3.3.2 Second approach

The TDH3 promoter was amplified from genomic *S. cereviviae* S288c DNA using oligonucleotides EcoRV_TDH3p_fwd and SpeI_TDH3p_rev (Table 2) as primers in a PCR reaction. The primers introduced EcoRV and SpeI at the respective ends of the PCR product. The *TDH3* promoter was purified using the GeneJET PCR Purification Kit. The pUG6 plasmid was used as vector for the ligation. pUG6 and the *TDH3* promoter were digested with EcoRV and SpeI. The linearized vector was purified on a gel, dephosphorylated and purified, thereafter ligated with the *TDH3* promoter and transformed to competent *E. coli* in the same way as in 3.3.1. Positive colonies were inoculated into 7 ml LB-Amp media and incubated overnight in 37°C. New plasmids were extracted using the GeneJET Plasmid Miniprep Kit (Fermentas) (Appendix 8.2.2). The positive plasmid was designated pUG6_TDH3p.

A DNA fragment containing the KAN cassette together with the TDH3p was amplified from previously created pUG6_TDH3p plasmid using oligonucleotides SphI_KAN_fwd-(a) and SalI_TDH3p_rev (Table 2) as primers in a PCR reaction. As the wrong sized fragment was amplified, the experiment was repeated using oligonucleotide SphI_KAN_fwd-(b) as primer instead of SphI_KAN_fwd-(a). The fragment was cloned into the YIp211 plasmid between the SphI and SalI sites, in the same way as described above. The created plasmid was called YIp211_KAN-TDH3p.

FLO1 (4600 bp) gene was amplified from genomic S288c DNA using oligonucleotides SalI_FLO1_fwd and BamHI_FLO1_rev (Table 2) as primers through a PCR reaction. The fragment was cloned into the plasmid YIp211_CYC1t between SalI and BamHI sites in the same way as described above. The correctness of the cloned sequences was tested via DNA sequencing (Eurofins, Germany).

3.4 Oligonucleotides

Table 2 Oligonucleotides used in this project.

Name	Sequence (5' → 3')	Template
	Plasmid construction	
DrdI_KAN_fwd	CAGACTTGAGCGTCATAACTTCG TATAATG	pUG6
SapI_KAN_rev	CAGCTCTTCCGCTCCTAATAACT TCGTATAG	pUG6
EcoRV_TDH3p_fwd	ATGATATCCAGTTCGAGTTTATC ATTATC	CEN.PK113-7D
SpeI_TDH3p_rev	TACTAGTGTGTGTTTATTCGAAA CTAAG	CEN.PK113-7D
SphI_KAN_fwd-(a)	ATGGTGCATGCCCCTTAATATAA CTTCGTATAATG	pUG6_TDH3p
SalI_TDH3p_rev	TACGTCGACGTGTGTTTATTCGA AACTAAG	pUG6_TDH3p
SphI_KAN_fwd-(b)	ATAATGCATGCTTCGTACGCTGC AGGTAGACAAC	pUG6_TDH3p
SalI_FLO1_fwd	CATGTCGACATGACAATGCCTCA TCGCTATAT	S288c
BamHI_FLO1_rev	CTGGATCCTTAAATAATTGCCAG CAATAAG	S288c

Restriction sites are indicated in the name and underlined in the sequence. Names for restriction site introducing oligonucleotides are created as; restriction enzyme name_Name of region annealing on template_forward or reverse

4 Results

The project was initially intended to be one sequential process based on two distinct parts: i) creating flocculation in the xylose fermenting yeast strain CEN.PK XXX, which is based on CEN.PK 113-7D and ii) characterization of the same, in comparison with the background strains. However the first part of the project was not successful, which meant that the project came to be separated into the two distinct parts; attempts to construct a flocculating and xylose fermenting yeast strain, and characterization of CEN.PK XXX.

4.1 Construction of a flocculating and xylose fermenting yeast strain

The two methods described below were designed to create plasmids with the same basic ability to create flocculation in non-flocculating yeast. It was important to include resistance to kanamycin through the KAN-cassette, as CEN.PK XXX lacks auxotrophies that can be exploited for transformation with vectors carrying auxotrophic markers. Both approaches aimed at eventually obtaining a plasmid including KAN-TDH3p-FLO1-CYC1t which could be used as a gene disruption cassette in the genomic HO locus.

4.1.1 First approach

The construction of the plasmid YEp181_KAN_TDH3p-CYC1t as described in section 3.3.1 was not successful. The *TDH3* promoter fragment amplified through PCR to be cloned into the YEp181_TDH3p-CYC1t plasmid was of the right size but was not successfully integrated into the plasmid, which became obvious when conducting verification by colony PCR. Primers used for the control were the same that were used to amplify the KAN cassette. The experiment remained unsuccessful despite repeated attempts. Colonies were formed to a certain extent on LB-amp plates after transformation. These were probably the result of undigested plasmids.

After the first approach was abandoned it was realized that the failure to clone the desired fragment into the plasmid was most likely due to an error in the SapI_KAN_rev primer. The error lies in the sequence of the restriction site which was wrong, therefore the enzyme did not cut the fragment preventing the annealing to the cut vector.

4.1.2 Second approach

In the first step, *TDH3*p was successfully cloned between EcoRV and SpeI in the pUG6 plasmid which created the pUG6_TDH3p plasmid. In the second step, when first using oligonucleotide SphI_KAN_fwd-(a) with SalI_TDH3p_rev as primers to amplify the KAN-TDH3p construct, a shorter fragment than intended was obtained. This is likely due to that the primer was designed to anneal to the 5' end of the LoxP-site. The LoxP site is present at two locations on the plasmid as can be seen in Appendix 8.7 on both sides of the KAN-region. Therefore the primer can anneal to either of these regions, and the amplification of the shorter fragment was favored over the longer one. The new SphI_KAN_fwd-(b) primer was designed to anneal further upstream where the sequence is unique. The primer was also designed to introduce a point mutation

in the sequence to silence a SalI-site. When using the SphI_KAN_fwd-(b) primer the right size (~2200 bp) fragment was amplified. This was successfully cloned into the integrative plasmid YIp211 between SphI and SalI to create YIp211_KAN-TDH3p. This plasmid was intended to be a platform of either two procedures to activate expression of *FLO1*. As *FLO1* is often present in non-flocculating *S. cerevisiae* but for some reason inactivated, as is the case with the S288c strain (See chapter 2.5), the endogenous promoter of *FLO1*could possibly be replaced by the *TDH3*p. However, during the progress of the project, it was found that CEN.PK113-7D, which was recently sequenced, completely lack *FLO1* [80], which made this procedure not feasible. The alternative to the above mentioned procedure was to clone *FLO1* into the KAN-TDH3p cassette and transfer it into the HO-locus of CEN.PK XXX. Either it could be possible to use the endogenous terminator of the HO-locus, alternatively clone in the *CYC1* terminator into the cassette. Both of these alternatives were taken into consideration.

FLO1 (sequence in Appendix 8.6) was amplified from S288c genomic DNA and cloned between SalI and BamHI into both YIp211_KAN-TDH3p and YIp211_TDH3p-CYC1t. Only cloning into YIp211_TDH3p-CYC1t was successful, which created the YIp211_TDH3p-FLO1-CYC1t plasmid. From this plasmid the FLO1-CYC1t sequence was intended to be cut out and cloned into the YIp211_KAN-TDH3p to create the final disruption cassette on a plasmid called YIp211_KAN-TDH3p-FLO1-CYC1t. However it soon became obvious that the fragment which had been cloned into YIp211_TDH3p-CYC1t was significantly shorter than the *FLO1* fragment which had been amplified through PCR. The plasmid was sent for sequencing and it was found that a fragment of approximately 2000 bp had been lost. This was consistently repeated over several attempts. The method was modified to include gel band purification of the PCR product to ensure that the right fragment was amplified and cloned, but with the same result. This behavior has previously been observed in some studies and is likely due to that *FLO1* contains large regions of highly repetitive sequences which enables recombination when transferred to *E. coli* [81, 82]. After this, the project had to be abandoned due to lack of time.

4.2 Strain characterization through shake flask cultivation

CEN.PK XXX was characterized by cultivation in different defined media under anaerobic conditions. CEN.PK113-7D was used as reference as it is the parental strain of CEN.PK XXX. The cultivation method was originally designed to be applicable for flocculating yeast, therefore, as it is troublesome to take samples of optical density and biomass in flocculating yeast due to large local differences within the cultivation, only a final dry cell mass measurement was conducted after the cultivation. The initial method also included a washing step with citrate buffer after the second pre-culture. This was intended to dissolve flocks by citrate complex binding Ca^{2+} ions so that they no longer are available for the flocculation mechanism. However this was modified to two steps of washing with mQ water.

When comparing the pre-culture of CEN.PK113-7D and CEN.PK XXX, it was shown that the former had a faster growth rate, and only needed to be

cultured over night to transform the clear medium to a cloudy broth. CEN.PK113-7D was harvested after about 24 hours. The latter strain required much longer and the growth varied a lot between different flasks. The cells had to be left for 48 hours in order to bring all of the flasks to the opaque, cloudy state. The fact that the different strains were pre-cultured for different amounts of time was not considered significant as a second pre-culture with fresh medium ensured that all the cells were in a vegetative state.

An indirect measurement of the specific growth rate was calculated based on the total sugar consumption using Equation 1. The results are given in Table 3.

Table 3 Specific growth rate estimated from total sugar consumption

Strain	Medium	μ
CEN.PK113-7D	50 g/l glucose	0.35 ± 0.03 *
CEN.PK XXX	50 g/l glucose	0.26 ± 0.03
CEN.PK XXX	50 g/l xylose	0.08 ± 0.03
CEN.PK XXX	25 g/l glucose, 25 g/l xylose	0.26 ± 0.06
CEN.PK XXX (Xylose phase)**	25 g/l glucose, 25 g/l xylose	0.04 ± 0.00

CEN.PK XXX was grown in different media with different carbon source and compared to the parental CEN.PK113-7D strain.

* *Data given with ± shows the standard deviation based on triplicate measurements.*

*** Based only on xylose consumption after depletion of glucose.*

CEN.PK113-7D finished its fermentation after approximately 24 hours, which could be observed as no gas was bubbling through the water lock. CEN.PK XXX grew considerably slower, which can also be observed in the carbohydrate consumption and production of extracellular metabolites (Figure 9-12). Xylose fermentation was considerably slower, both by ocular observation of the cloudiness (cell density), as well as generation of CO_2 gas.

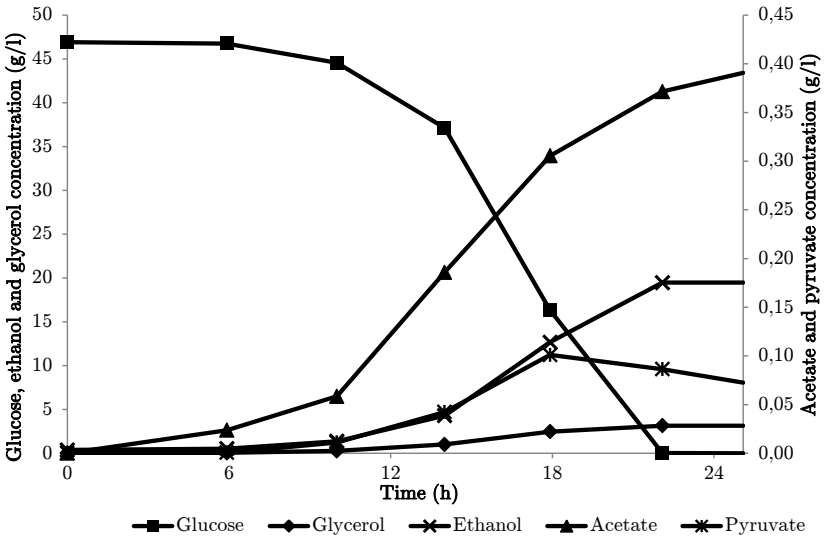

Figure 9 Extracellular metabolites from shake flask cultivations of CEN.PK113-7D in defined medium with 50 g/l glucose as carbon source. The values shown are the average of triplicate cultures.

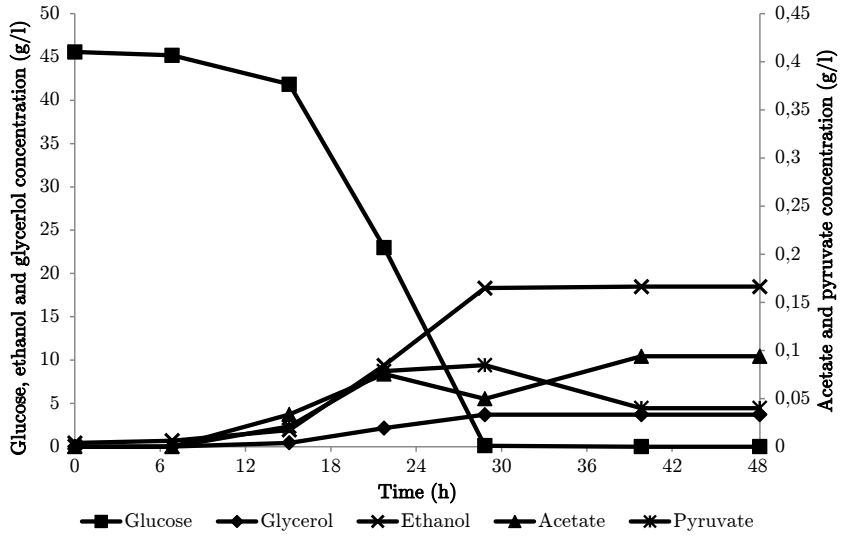

Figure 10 Extracellular metabolites from shake flask cultivations of CEN.PK XXX in defined medium with 50 g/l glucose as carbon source. The values shown are the average of triplicate cultures.

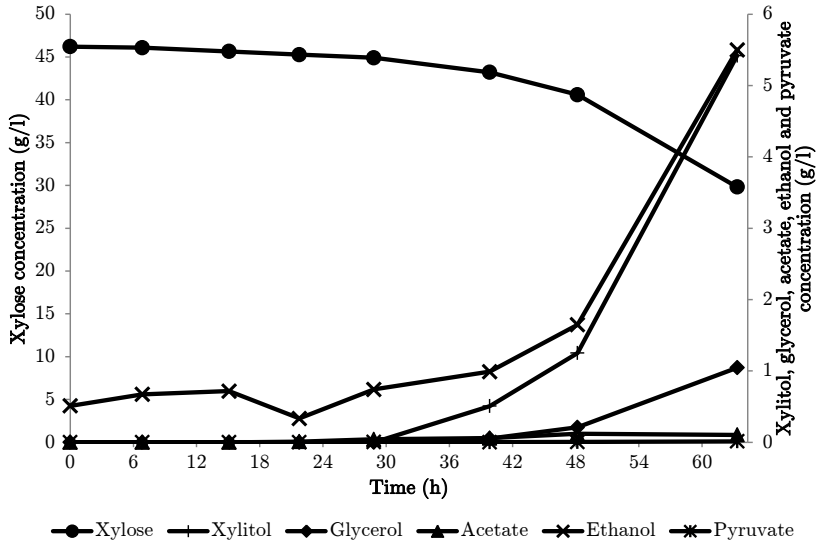

Figure 11 Extracellular metabolites from shake flask cultivations of CEN.PK XXX in defined medium with 50 g/l xylose as carbon source. The values shown are the average of triplicate cultures.

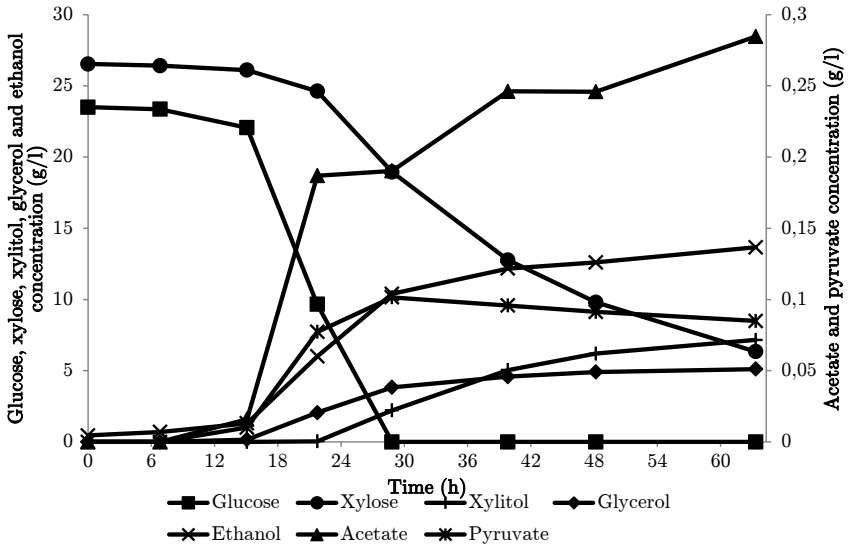

Figure 12 Extracellular metabolites from shake flask cultivations of CEN.PK XXX in defined medium with 25 g/l glucose and 25 g/l xylose as carbon source. The values shown are the average of triplicate cultures.

Glucose was depleted after approximately 19 hours for CEN.PK113-7D in glucose medium and about 25 hours for CEN.PK XXX in the same medium (Figure 9 and 10). At this point the fermentation stopped as the carbon source become limiting. The extracellular ethanol concentration was kept more or less constant after this point and changes were mostly due to evaporation. Xylose fermentation was slow which is why CEN.PK XXX was left to ferment for more than 60 hours compared to 25 hours for CEN.PK113-7D. Despite the long cultivation time, the yeast fermenting pure xylose had hardly started to consume xylose to any higher extent; only about 35% of the initial amount had been consumed after 63 hours and 20 minutes. For the mixed medium, glucose was rapidly consumed and depleted in about the same time as for the pure glucose medium. Xylose was consumed to a higher extent compared to the yeast in pure xylose medium. After 63 hours and 20 minutes only about 24% of the initial xylose present remained. This can be explained by the presence of glucose, which enables the culture to rapidly grow to a high cell density.

The dry cell mass measurements were not completely comparable among the different conditions as not all available carbon had been utilized for both cultivations containing xylose. It can also be seen in Figure 13 where cultures containing xylose show similar, low cell mass.

Estimations of glucose and xylose consumption rates and ethanol production rates, calculated using Equation 3 are given in table 4. The ethanol production rate is estimated for two different time points for CEN.PK XXX in mixed media, first during exponential growth on glucose (glucose phase) and second right after depletion of the glucose, when xylose consumption is as fastest (xylose phase) which occurs after approximately 25 hours. High standard deviation can be seen for many of the values. This can be explained by the fact that all calculations are done during exponential consumption and productions, during which large differences can occur in the different replicates.

Table 4 estimation of sugar consumption rates and ethanol production rate.

Strain	Medium	Phase	Production and consumption rates (g g^{-1} h^{-1})		
			$q_{glucose}$	q_{xylose}	$q_{ethanol}$
CEN.PK 113-7D	50 g/l glucose	-	4.48 ± 0.31*	-	1.77 ± 0.09
CEN.PK XXX	50 g/l glucose	-	3.90 ± 3.26	-	1.54 ± 1.22
CEN.PK XXX	50 g/l xylose	-	-	0.63 ± 0.16	0.23 ± 0.10
CEN.PK XXX	25 g/l glucose and xylose	Glucose	4.30 ± 2.3	-	1.63 ± 1.01
CEN.PK XXX	25 g/l glucose and xylose	Xylose	-	0.35 ± 0.05	0.10 ± 0.03

** Data given with ± shows the standard deviation based on triplicate measurements.*

Yields of different metabolites on total sugars consumed, calculated using Equation 4, are given in Table 5. Most interesting is the ethanol yield which did not differ much between the different cultivations, however the yield from the cultivations containing xylose is somewhat lower. The accumulation of xylitol is high, especially for the pure xylose medium, where its concentration is as high as that of ethanol.

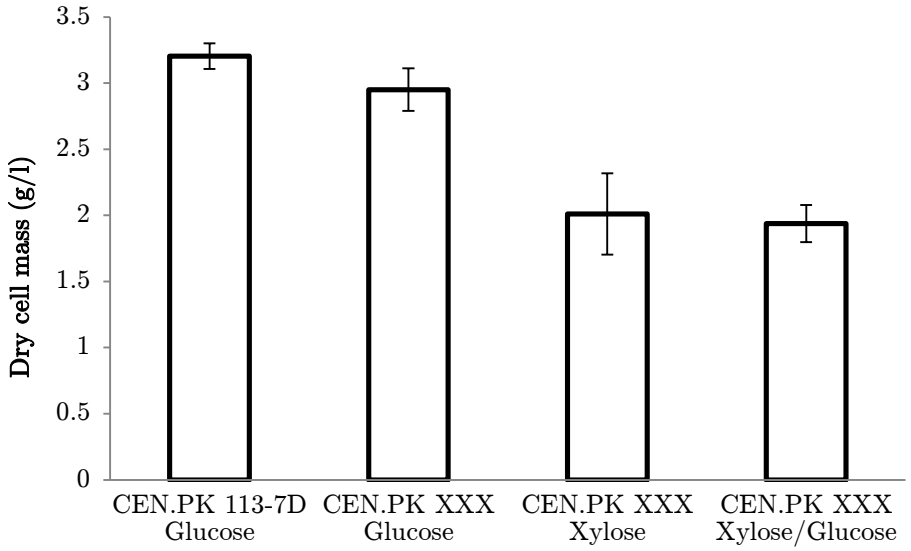

Figure 13 Measurements of dry cell mass after finished cultivation in different defined media. CEN.PK113-7D was harvested after 25 hours and 5 min. CEN.PK XXX was harvested after 63 hours and 20 min. Error bars indicate standard deviation.

Table 5 Yields of different carbohydrates and biomass.

Strain	Media	Yields (g/g total sugars)					
		Xylitol	Glycerol	Acetate	Ethanol	Pyruvic acid	Biomass*
CEN.PK1 33-7D	50 g/l glucose	-	0.067	0.0083	0.41	0.0015	0.069
CEN.PK XXX	50 g/l glucose	-	0.080	0.0030	0.38	$1.7 \cdot 10^{-4}$	0.067
CEN.PK XXX	50 g/l xylose	0.33	0.064	0.0066	0.30	$7.7 \cdot 10^{-4}$	0.13
CEN.PK XXX	25 g/l glucose and xylose	0.36**	0.12	0.0066	0.30	0.0019	0.043

**Yield of biomass was estimated by total dry cell mass at the end of the cultivations, the inoculum was not considered.*

*** Xylose yield in mixed media is based only on xylose consumption (g/g xylose)*

5 Discussion

5.1 Construction of a flocculating and xylose fermenting yeast strain

The error in the oligonucleotide SapI_KAN_rev used as primer for the PCR reaction in the first approach to construct a disruption cassette was due to a human error. The sequence for the restriction site for SapI introduced by the oligonucleotide in the 3' end of the amplified fragment is GCTCTTC, which is also the sequence in the oligonucleotide. However, since SapI_KAN_rev is the reverse primer, in order for the restriction site to be correct in the finished product, it needs to be reversed and complementary to the original sequence. Therefore a functional reverse primer for the intended fragment would have to look like: CAGAAGAGCCGCTCCTAATAACTTCGTATAG. In many cases this reverse complementation is not necessary as restriction sites are commonly *inverted repeat palindromes*, meaning that they read the same backwards and forward, but found on different complementary DNA strands. In this specific case, the mistake made major difference to the outcome and eventual failure of the project.

Another approach was initiated which had the same aim to create a disruption cassette. In the end, the problem was that a large part of the *FLO1* gene was lost when transformed into *E. coli*. This problem would likely have arisen whether or not the first or the second construction approach was used. The problem has been described before, and the incomplete gene has been introduced in *S. cerevisiae* which then displayed some weak flocculation [82]. This was not investigated in this study. One of the requirements for the intended flocculating strain was that it should be robust with possibility to be used in further studies. A strain with an incomplete *FLO1* and weak flocculation would therefore not be desirable.

The decision to change to an integrative from an episomal plasmid in the second approach did not greatly affect the outline of the study, but introduced the possibility to take advantage of the ability of the integrative plasmid to be directly integrated in the genomic DNA of yeast. However this was not the main intention for the construct.

In the first study where the complete *FLO1* gene was isolated and sequenced, the same problem with deletion of nucleotides occurred. They were eventually able to locate a colony from transformation which had a plasmid with the right size *FLO1* [81]. Other studies have been published where this problem was not noticed [67]. At least in one of these studies the right size fragment was confirmed.

The evidence from the literature indicates that it is possible to overcome the obstacles encountered during this study. It is probably mostly a matter of trying until successful.

5.2 Characterization of CEN.PK XXX

The CEN.PK XXX was constructed with the intention of it being a template for further studies and improvements. It would have been a good and comprehensive study to compare the different strains CEN.PK113-7D, CEN.PK XXX and CEN.PK XXX(*FLO1*+), as they all have the same background and their differences could be isolated to their respective different genotypes. In this

context the characterization of CEN.PK XXX compared to CEN.PK113-7D is still a valid study.

In a wide perspective, the ability of CEN.PK XXX to utilize xylose is slow. After the 63 hours and 20 minutes that the cells were cultivated, about 35% of the total xylose was consumed. The corresponding time for glucose consumption by CEN.PK113-7D was approximately 15 hours, and the specific growth rate based on sugar consumption was less than a quarter for CEN.PK XXX grown on xylose compared to the parental strain grown on glucose. The mixed medium is a more applicable model for lignocellulosic hydrolyzates. Co-consumption of xylose and glucose is advantageous compared to pure xylose. Glucose can quickly be consumed and the cells can maintain a high growth rate to reach high cell densities. When glucose is depleted xylose consumption will be faster. In this cultivation 87% of all available sugars and 76% of all xylose is consumed after 63 hours and 20 minutes. The apparent tendency of the yeast to decrease its xylose consumption as seen in Figure 12 was likely due to a kinetic limitation of the xylose transport which is mediated by hexose transporters [83]. The transporters typically have much higher affinity for hexose sugars such as glucose [84], which is why almost no co-consumption of xylose and glucose takes place. When the xylose is decreasing, the activity of the enzyme is also decreasing. It is likely that a similar tendency would have been observed after some additional time in the pure xylose medium as well.

The large accumulation of xylitol in both xylose containing cultivations is a consequence of the redox imbalance in the XR and XDH reactions as there is no external electron acceptor (oxygen) that can regenerate NADH to NAD^+ as described in chapter 2.3.

A comparative study between different xylose fermenting strains of *Saccharomyces cerevisiae* that was conducted by Kuyper et al. (2005) [85] is presented in Table 6. In this context, the CEN.PK XXX performs well, especially for a first approach for a xylose fermenting strain. The top performer in this comparison, RWB 217 is constructed to over express a whole lot more enzymes in the pentose phosphate pathway. A more fair comparison would be the RWB 202-AFX strain which only has the XI over expressed. Compared to the other XR/XDH-strains CEN.PK XXX consumption rate of xylose is high. One must also notice the limitation of the comparison, since the calculations done for CEN.PK XXX are based on a number of assumptions, which may not be completely accurate. One indication of this is the extraordinary high biomass yield received for when the yeast was cultivated in pure xylose (Table 5). This is hard to explain, since the same strain grown in mixed media consumed a lot more sugars, but still resulted in less biomass.

Table 6 Xylose consumption rates of xylose-metabolising *S. cerevisiae* strains in literature.

Strain	Description*	Cultivation	Sugar concentration (g/l)	q_{xylose} (g/g h)	$q_{xylitol}$ (g/g h)	Ref
TMB 3001	XR, XDH, XK	Anaerobic batch	Glucose, 50; xylose 50	0.06	0.01	[86]
TMB 3001	XR, XDH, XK	Anaerobic batch	Xylose, 50	0.13	0.038	[87]
TMB 3001	XR, XDH, XK	Anaerobic chemostat	Glucose, 5; xylose, 15	0.2	0.078	[88]
TMB 3001C1	XR, XDH, XK	Anaerobic batch	Xylose, 10	0.6	0.198	[89]
424A(LNH -ST)	XR, XDH, XK	Oxygen limited batch	Glucose, 73; xylose, 40	0.22**	0.024	[90]
Industrial strain A4	XR, XDH, XK	Anaerobic batch	Glucose, 50; xylose 50	0.21	0.057	[86]
H1691	XR, XDH, XK	Anaerobic batch	Xylose, 50	0.20	0.083	[91]
RWB 202-AFX	XI	Anaerobic batch	Xylose, 20	0.34	0.007	[92]
RWB 217	XI, XKS, RPE, RPI, TAL, TKL	Anaerobic batch	Xylose, 20	1.06	0.002	[85]
CEN.PK XXX	XR, XDH, XK	Anaerobic batch	Xylose, 50	0.63	0.245	***
CEN.PK XXX	XR, XDH, XK	Anaerobic batch	Glucose, 25; xylose, 25	0.35**	0.163	***

* *Introduced and/or overexpressed genes; XR, xylose reductase; XDH, xylitol dehydrogenase; XK, xylulokinase; XI, xylose isomerase; RPE, ribose phosphate epimerase; RPI, ribose phosphate isomerase; TAL, transaldolase; TKL, transketolase.*
** *Calculated from the xylose consumption phase.*
*** *This study*

5.3 Future perspectives

Since the major motivation for the research field is to create new and effective ways to ferment lignocellulosic hydrolyzates, CEN.PK XXX is at present not a feasible organism to be used in any large scale applications. In comparison to glucose, the xylose uptake is still much slower, and in order to receive a good economy on fermentation the process needs to be fast. Therefore xylose consumption rate should be comparable to that of glucose. The large accumulation of xylitol is also a problem as this carbon mass that could otherwise have been used for ethanol. However, the strain is still a good tool for investigating different aspects of genetic engineering in *S. cerevisiae.* And there is still good reason to investigate the effect of flocculation. As a proof of concept, it would perhaps be enough to use the incomplete *FLO1* gene to only create weak flocculation. It is very well possible to find transformed colonies which includes the gene of the right size, and it might just be a matter of time and patience to find it.

The flocculation approach to the problem of coping with inhibitory compounds in lignocellulosic hydrolyzates should still be investigated and it could be worth the effort to continue with the method described in this study. A faster approach would be to purchase plasmids where the cloning of the full length *FLO1* has actually been successful.

One interesting approach is to synthesize the gene and adjust the nucleotide sequence so that the same amino acid sequence is received, but the repetitive regions are eliminated on nucleotide level. This would likely decrease the tendency of recombination in *E. coli*.

6 Conclusions

Activation of *FLO1* in xylose fermenting *S. cerevisiae* was not successful in this study. The method described requires more time to be successful, but there is no reason to believe that it is impossible. Similar methods have been used to successfully activate *FLO1* in previous studies

Evaluation and comparison between different strains was performed but in a largely modified manner as no flocculating strain could be characterized. A xylose fermenting strain was characterized and it could be shown that it has the ability to utilize xylose as sole carbon source, however not very efficiently. It accumulates plenty of xylitol which indicate that a cofactor imbalance is being accumulated during anaerobic fermentation. However, as a potential industrial ethanol producer, the investigated strain shows some promise. It will be hard to receive fast xylose utilization as long as the yeast wild type hexose transporters are being used. Introduction of a good pentose sugar transporter may very well be the missing link to receive a fast xylose fermenter. The use of flocculation may also improve the ability to transport xylose in the yeast as chemical gradients within the flocs could work similarly as in encapsulated yeast.

To propose a process to produce ethanol from lignocellulosic hydrolyzates as described in the aims of this project is hard based on the achieved results. It is important to first of all find a good organism to be used for fermentation that can utilize all available sugars to a high yield of ethanol at a fast rate. The organism studied here, unfortunately does not display these abilities. It is reasonable to believe that its performance could be improved with the introduction of flocculation, but it will hardly make it qualify for use in large scale ethanol production. However it is possible to imagine a system, under the best possible conditions, using a good flocculating yeast strain with strong ability to convert xylose to ethanol. Such a system would preferably be used in large batch reactors under anaerobic conditions. The yeast could be inoculated from the beginning and would grow in the medium and flocculate spontaneously. The major problem I can see with this is that a small inoculum into a large volume of hydrolyzate will create a long lag time. One way to cope with this is to let the cells adapt to the hydrolyzate medium before inoculation, and the flocculation may very well result in substantially decreased lag time. After the fermentation the flocs will efficiently sediment to the bottom of the reactor where they can be collected for reuse and the liquid will be more or less free from cells. This type of process would fit well with the industrial demands for low maintenance and efficient fermentation.

7 References

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xylulokinase (*XKS1*) and oxygen availability," *Metabolic Engineering,* vol. 3, pp. 236-249, 2001.

[92] M. Kuyper, A. A. Winkler, J. P. Dijken, and J. T. Pronk, "Minimal metabolic engineering of *Saccharomyces cerevisiae* for efficient anaerobic xylose fermentation: a proof of principle," *FEMS yeast research,* vol. 4, pp. 655-664, 2004.

8 Appendix

8.1 Media

8.1.1 LB medium

Compound	Amount
Glucose	1 g
Peptone	10 g
Yeast Extract	5 g
NaCl	10 g
dH_2O (fill to V_{final}=1l)	

Mix all ingredients and autoclave.

For solid media in plates; add 20 g agar to the above mixture.

For ampicillin media; final ampicillin concentration of 100 µg/ml from a 1000X stock solution (100 mg/ml dissolved in dH_2O, sterilized by filtration and stored at -20°C.

When adding ampicilline to solid media, the medium must cool down a bit (not enough for it to solidify), otherwise ampicillin will be damaged. Pour medium into the plates immediately after ampicillin has been added.

Store LB+Amp plates at 4°C

8.1.2 YEPD-agar plates

Compound	Amount
Agar	15 g
Yeast extract	10 g
Peptone	20 g
D-Glucose	20 g
dH_2O (fill to V_{final}=1 l)	

Mix all ingredients and autoclave.

When the solution cools to a temperature so that it is possible to hold the flask in your hand, pour the plates into plates and let cool. Keep at 4°C.

8.1.3 Minimal media plates with 20 g/l xylose

Table 7 Prepare in flask 1.

Compound	Amount
Agar	12 g
YNB	1.026 g
Ammoniumsulphate	3 g
dH_2O (fill to V_{final}=0.54 l)	

Include stirring bar in bottle

Table 8 Xylose stock solution

Compound	Amount
Xylose	300 g
dH_2O (fill to V_{final}=1 l)	

Table 9 Base salt stock solution

Compound	Amount
$(NH_4)_2SO_4$	100 g
KH_2SO_4	60 g
$MgSO_4$·7H2O	10 g
dH_2O (fill to V_{final}=1 l)	

Autoclave all three bottles separately.

Add 20 ml base salt stock solution and 40 ml xylose stock solution to 0.54 l agar-YNB-ammoniumsulphate solution.

Stir mixture for a minute and cast into dishes.

8.1.4 Defined media

Table 10 $(NH_4)_2SO_4$ 50X stock solution

Compound	Amount
$(NH_4)_2SO_4$	375 g
dH_2O (fill to V_{final}=1 l)	

Table 11 KH_2PO_4 50X stock solution

Compound	Amount
KH_2PO_4	175 g
dH_2O (fill to V_{final}=1 l)	

Table 12 $MgSO_4$ 200X stock solution

Compound	Amount
$MgSO_4 \cdot 7H_2O$	150 g
dH_2O (fill to V_{final}=1 l)	

Table 13 Trace metal 100X stock solution

Compound	Amount
$CaCl_2 \cdot 2H_2O$	0.9 g
$ZnSO_4 \cdot 2H_2O$	0.9 g
$FeSO_4 \cdot 7H_2O$	0.6 g
$H_3BO_3 \cdot 7H_2O$	0.2 g
$MnCl_2 \cdot 2H_2O$	0.155 g
$Na_2MoO_4 \cdot H_2O$	0.08 g
$CoCl_2 \cdot 6H_2O$	0.06 g
$CuSO_4 \cdot 5H_2O$	0.06 g
KI	0.02 g
$EDTA \cdot 2H_2O$	3 g
dH_2O (fill to V_{final}=1 l)	

All the above solutions are mixed and autoclaved separately.

Vitamin 1000X stock solution:

- Dissolve 25 mg d-biotin in 10 ml 0.1 M NaOH.
- Add the dissolved biotin to app. 300 ml dH_2O and adjust pH to 6.5 using 0.1 M HCl.
- Add the following vitamins:

  p-amino benzoic acid (PABA): 100 mg

  Nicotinic acid: 500 mg

  Ca-Panthothenate: 500 mg

  Pyridoxine, HCl: 500 mg

  Thiamine, HCL: 500 mg
- Adjust pH to 6.5 (with 2M NaOH), adjust to 500 ml with dH_2O and sterile filter into 50 ml Falcon tubes. Keep at 4°C

Carbon source 2X stock solution:

- Dissolve the carbon source (50g glucose, xylose or 25 g each of glucose and xylose for final media concentration of 50 g/l) in about 300 ml dH_2O. Fill up with dH_2O to 500 ml. Autoclave immediately.

Ergosterol/Tween 80 1000X stock solution:

- Dissoleve 1 g ergosterol in a minimum volume of boiling absolute ethanol (approx. 25 ml), add 42 g Tween 80, boil for 10 min. Adjust to 100 ml with pure ethanol. Transfer to eppendorff tubes. Keep at -20 °C

Table 14 Final preparation of defined medium. For one liter media.

Compound	Amount
$(NH_4)_2SO_4$ 50X stock solution	20 ml
KH_2PO_4 50X stock solution	20 ml
$MgSO_4$ 200X stock solution	5 ml
Trace metal 100X stock solution	10 ml
Vitamin 1000X stock solution	1 ml
dH_2O (Fill to V_{final}=500 ml)	
Sterile filter solution into the 2X carbon source solution	

For anaerobic cultures, add 1 ml ergosterol/Tween 80 1000X stocksolution per liter mixture.

8.2 Strain construction

8.2.1 Transformation

A. To transform, mix 0.1 ml aliquots of competent cells with DNA (1-10 ng). Use positive (empty plasmid compared to constructed plasmid) and negative control (no DNA). Leave on ice for 10-30 min. *Use volumes: Positive control 2 µl; sample of 2 and 5 µl.*

B. Heat chock at 42°C for 2 min.

C. Add 1 ml LB (see Appendix 8.1.1) to the shocked cells and allow expression for 30-60 min at 37°C

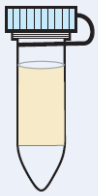
D. Centrifuge at 5000 x rcf for 8 min. Pour off the supernatant, resuspend with the fluid left in the tube and streak (approximately 80 µl) on plates. Incubate at 37°C over night.

8.2.2 Plasmid extraction of DNA from *E. coli*

QuickProtocol™

GeneJET™ Plasmid Miniprep Kit

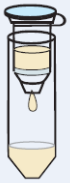
Note. All steps should be carried out at room temperature.
All centrifugations should be carried out in a microcentrifuge at ≥ 12000 x g (~11000rpm).

1 

Resuspend Cells, Lyse and **Neutralize**

Add to the pelleted cells:
250 µl of Resuspension Solution and vortex.
250 µl of Lysis Solution and invert the tube 4-6 times.
350 µl of Neutralization Solution and invert the tube 4-6 times.
Centrifuge 5 min.

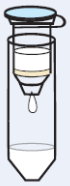
↓

2 

Bind DNA

Load the supernatant to GeneJET™ spin column.
Centrifuge 1 min.

↓

3 

Wash the column

Add 500 µl of Wash Solution and centrifuge for 30-60 s. Discard the flow-through. } x 2 times
Centrifuge empty column for 1 min.

↓

4 

Elute purified DNA

Transfer the column into a new tube.
Add 50µl of Elution Buffer to the column and incubate 2 min.
Centrifuge 2 min.
Collect the flow-through.

8.2.3 Gel band purification

Quick Reference Protocol Card

28-9034-70 (100 purifications)
28-9034-71 (250 purifications)

illustra™ GFX™ PCR DNA and Gel Band Purification Kit

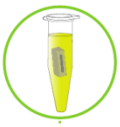
B. Protocol for purification of DNA from TAE and TBE agarose gels

- Check appropriate volume of ethanol added to **Wash buffer type 1**

:Add :Spin :Incubate

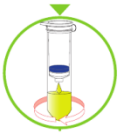
1. Sample capture

- Weigh a DNase-free 1.5 ml microcentrifuge tube
- Excise band of interest and place in microcentrifuge tube
- Weigh microcentrifuge tube plus agarose gel band
- Calculate weight of agarose gel slice
- 10 µl Capture buffer type 3 for each 10 mg agarose gel slice
- Mix by inversion
- 60°C until agarose is completely dissolved
- Check color of Capture buffer type 3-sample mix is yellow or pale orange



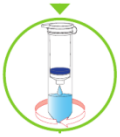
2. Sample binding

- 600 µl Capture buffer type 3-sample mix to assembled GFX MicroSpin™ column and Collection tube
- 60 s room temperature
- 30 s 16 000 × g. Discard flow through
- Place GFX MicroSpin column inside the same Collection tube
- Repeat Sample Binding step until all sample is loaded



3. Wash & dry

- 500 µl Wash buffer type 1
- 30 s 16 000 × g
- Discard Collection tube. Transfer GFX MicroSpin column to a clean 1.5ml DNase-free microcentrifuge tube.



4. Elution

- OR 10–50 µl Elution buffer type 4 OR type 6
- 60 s room temperature
- 60 s 16 000 × g
- Retain flow through
- Store purified sample DNA at -20°C

imagination at work

8.2.4 PCR product purification

PURIFICATION PROTOCOL

Note

- All purification steps should be carried out at **room temperature**.
- All centrifugations should be carried out in a table-top microcentrifuge at **>12000 x g** (10 000-14 000 rpm, depending on the rotor type).

Step	
1	Add a 1:1 volume of **Binding Buffer** to completed PCR mixture (e.g. for every 100 µl of reaction mixture, add 100 µl of Binding Buffer). Mix thoroughly. Check the color of the solution. A yellow color indicates an optimal pH for DNA binding. If the color of the solution is orange or violet, add 10 µl of 3 M sodium acetate, pH 5.2 solution and mix. The color of the mix will become yellow.
2 for DNA ≤500 bp	Optional: if the DNA fragment is ≤500 bp, add a 1:2 volume of 100% isopropanol (e.g., 100 µl of isopropanol should be added to 100 µl of PCR mixture combined with 100 µl of Binding Buffer). Mix thoroughly. **Note**. If PCR mixture contains primer-dimers, purification without isopropanol is recommended. However, the yield of the target DNA fragment will be lower.
3	Transfer up to 800 µl of the solution from step 1 (or optional step 2) to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through. **Note**. If the total volume exceeds 800 µl, the solution can be added to the column in stages. After the addition of 800 µl of solution, centrifuge the column for 30-60 s and discard flowthrough. Repeat until the entire solution has been added to the column membrane.
4	Add 700 µl of **Wash Buffer** to the GeneJET purification column. Centrifuge for 30-60 s. Discard the flow-through and place the purification column back into the collection tube.
5	Centrifuge the empty GeneJET purification column for an additional 1 min to completely remove any residual wash buffer. **Note**. This step is essential as the presence of residual ethanol in the DNA sample may inhibit subsequent reactions.
6	Transfer the GeneJET purification column to a clean 1.5 ml microcentrifuge tube. Add 35-50 µl of Elution Buffer to the center of the GeneJET purification column membrane and centrifuge for 1 min. **Note** • For low DNA amounts the elution volumes can be reduced to increase DNA concentration. An elution volume between 20-50 µl does not significantly reduce the DNA yield. However, elution volumes less than 10 µl are not

	recommended. • If DNA fragment is >10 kb, prewarm Elution Buffer to 65°C before applying to column. • If the elution volume is 10 µl and DNA amount is ≥5 µg, incubate column for 1 min at room temperature before centrifugation.
7	Discard the GeneJET purification column and store the purified DNA at -20°C.

8.2.5 Dephosphorylation treatment

For Fermentas shrimp alkaline phosphatase prepare:

- DNA 50 µl
- Shrimp alkaline phosphatase (SAP) 4 µl
- SAP buffer 10X 6 µl

8.2.6 Preparation of PCR

General protocol for Fermentas High Fidelity PCR Enzyme Mix:

1. Gently vortex and briefly centrifuge all solutions after thawing.
2. Place a thin-walled PCR tube on ice and add the following components for each 50 µl reaction:

10X High Fidelity PCR buffer with 15 mM $MgCl_2$	5 µl
dNTP Mix, 2 mM of each nucleotide	5 µl
Forward primer 10 µM	5 µl
Reverse primer 10 µM	5 µl
Template DNA	10 pg - 1 ng
High Fidelity PCR Enzyme Mix	0.5 µl
Water, nuclease-free	To 50 µl

3. Gently vortex and briefly centrifuge to collect all drops.
4. If the thermal cycler is not equipped with a heated lid, overlay the reaction mixture with a half volume of mineral oil.
5. Place the samples in a cycler and immediately start PCR.

Thermal cycling conditions:

Step	Temperature °C	Time	No. cycles
Initial denaturation	94	3 min	1
Denaturation	95	30 s	5
Annealing	Tm1*-5	1 min	
Extension	72	1 min/kb	
Denaturation	95	30 s	15-25
Annealing	Tm-5	1 min	
Extension	72	1 min/kb	
Final extension	72	10 min	1
Hold temperature	12	∞	Hold

** Tm1 is the melting temperature for the part of the primer which anneals to the template and is calculated by the formula: 69.3+0.41·GC-650/N where GC is the percentage of nucleotides G and C. N is the number of annealing nucleotides.'*

8.2.7 Digestion

For Fermentas FastDigest Restriction enzymes mix in PCR tubes:

	Plasmid (vector)	Insert DNA
FastDigest 10X or FastDigest green 10X buffer	4 µl	3 µl
DNA	2 µg	0.5 µg
FastDigest enzyme	2+2 µl	1+1 µl
Water, nuclease free	up to 40 µl	up to 30 µl

Place in thermo cycler (PCR) machine and run program: 1. 20 min at 37 °C 2. 10 min at 80 °C 3. Keep at 4 °C

8.2.8 Ligation

For Fermentas T4 DNA Ligase, mix in PCR tubes:

Linear vector	50-100 ng
Insert DNA	1:1-10:1 molar ratio over vector
T4 DNA Ligase buffer 10X	2 µl
T4 DNA Ligase	0.7 µl
dATP 5mM	2 µl
Nuclease free water	up to 20 µl

If vector and insert are to dilute, it might be necissary to prepare 40 µl total volume. Buffer, Ligase and dATP are then doubled in volume.

Leave at room temperature overnight.

8.2.9 PCR control of colonies

Pick one colony with the tip of a sterile pipette tip and dissolve it in 50 µl nuclease free water.

Cells are lysed with heat/cold cycling in PCR machine (Table 15). The lysed samples are then used as template for PCR reaction and analyzed on a gel.

Table 15 PCR program for lysis of cells

Step	Temperature °C	Time
1	65	30 s
2	8	30 s
3	65	1.30 min
4	97	3 min
5	8	1 min
6	65	3 min
7	97	1 min
8	80	1 min
9	4	∞

Table 16 PCR mix for Fermentas DreamTaq polymerase.

PCR mix	Vol µl
Lysed cells (Table 15)	5
Forward primer 10 µM	2.5
Reverse primer 10 µM	2.5
DreamTaq buffer 10X	5
dNTP mix 2 µM	5
DreamTaq polymerase	0.25
Nuclease free water	Up to 50 µl

Table 17 PCR program for control of colonies

Step	Temperature °C	Time	No. cycles
Initial denaturation	94	3 min	1
Denaturation	95	30 s	25
Annealing	Tm-5	1 min	
Extension	72	1 min/kb	
Final extension	72	10 min	1
Hold temperature	12	∞	Hold

8.3 0.8% (w/v) agarose gel

- Weigh 3.2 g agarose and put in a 500 ml flask.
- Add water to 400 ml, include magnetic stirrer.
- Heat in microwave until boiling.
- Stir until all agarose is dissolved. Keep at 65 °C

To cast a gel, add 2 µl gel red 10000X (BIOTIUM) to the mold. Then pour 20 ml of agarose solution into the mold and mix thoroughly. Attach the comb and let to polymerize for 1 h.

8.4 Determination of dry cell mass

1. Dry 0.45 µm filter papers on a glass dish in a microwave oven at 150 W for 10 minutes. Put tissue paper between the glass and the filter to avoid the filter adhering to the glass.
2. Put the filter in a desiccator and allow to cool for 10-15 minutes.
3. Weigh the filters.
4. Filter the cell suspension and wash with deionized water twice.
5. Dry the filter in a microwave oven for 15 minutes at 150 W.
6. Put the filter in a desiccator and allow to cool for at least 1 hour.
7. Weigh the filter.

If more than 30 mg dry weight is present the time in the microwave oven should be checked to make sure it is sufficient.

8.5 XXX-construct

```
>XXX-construct
GGTACCCACCTGCATGTCTTCACTGGTTACTCTTAATAACGGTCTGAAAATGCCCCTAGTCGGCTTA
GGGTGGTTTAAACTAAGGGATATAGAAGCAAATAGTTGTCAGTGCAATCCTTCAAGACGATTGGGAA
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CCCATACTTGCAACAACCAAGATTGATCGAATTTGCTCAATCCAGAGGTATTGCTGTCACTGCTTAC
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```

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8.6 ORF of *FLO1*

>YAR050W Chr 1
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8.7 Plasmids

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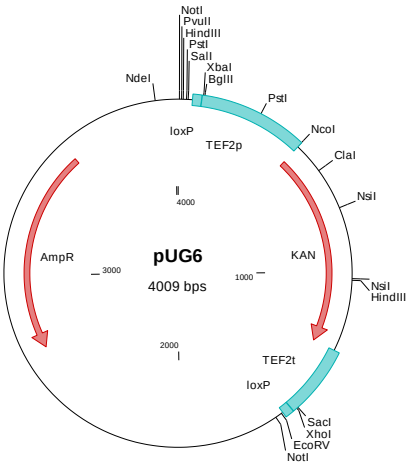

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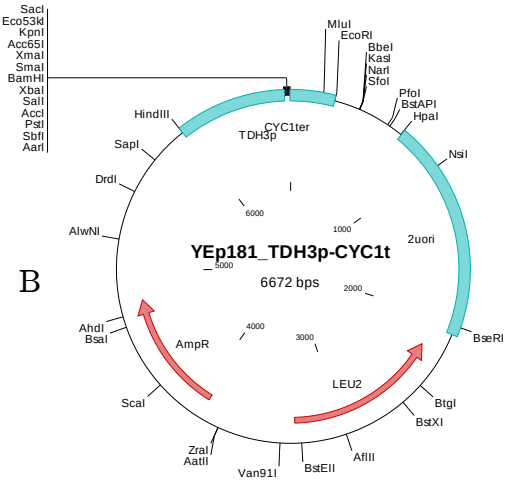

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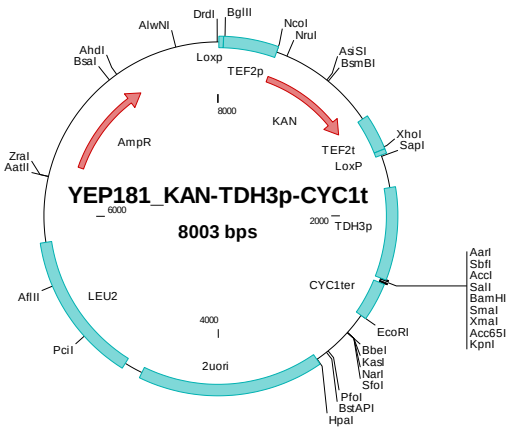

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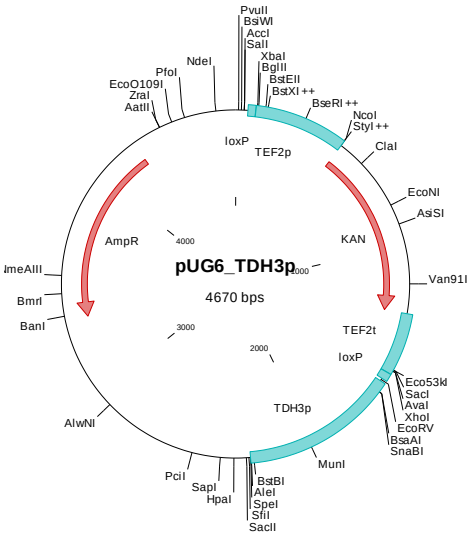

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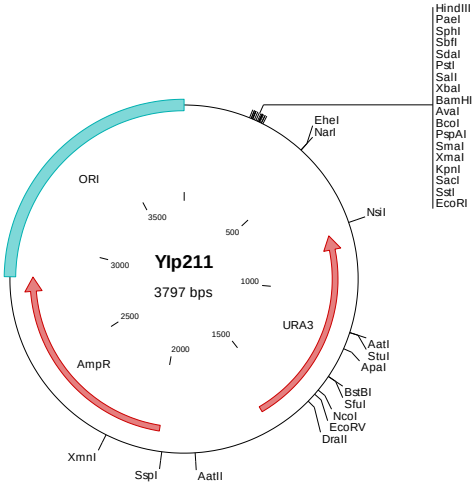
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KpnI
SacI
SstI
EcoRI
EheI
NarI
NsiI
AatI
StuI
ApaI
BstBI
SfuI
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DraII
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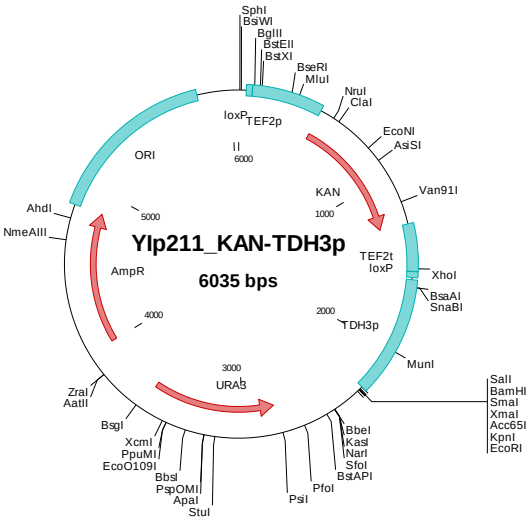
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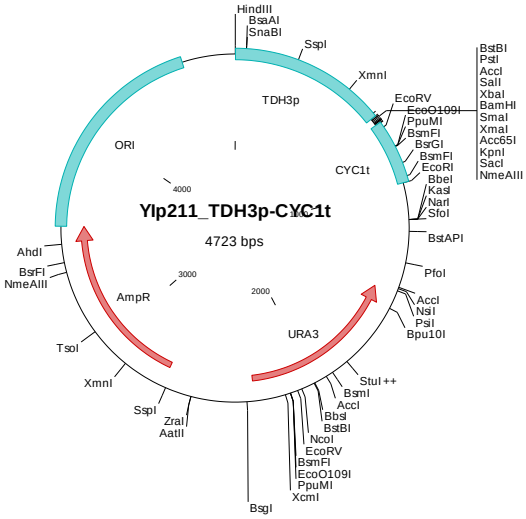
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BsgI
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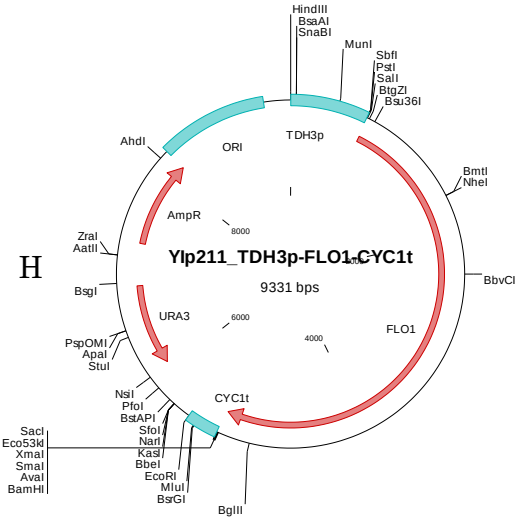
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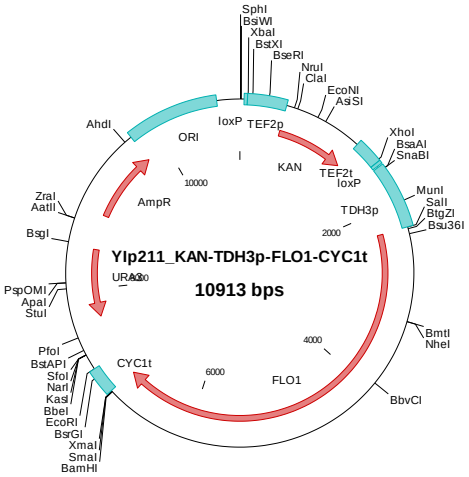
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