

DESIGN OF A *SACCHAROMYCES CEREVISIAE* STRAIN CAPABLE OF
SIMULTANEOUSLY UTILIZING CELLOBIOSE AND XYLOSE

BY

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THESIS

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Abstract

Saccharomyces cerevisiae has been widely utilized as a platform microorganism for bioethanol production from lignocelluloses. However, glucose repression limits efficient ethanol production because glucose in lignocellulosic hydrolysates inhibits xylose and other sugars' utilization. As a result, it is attractive to construct a glucose derepressed *S. cerevisiae* strain for efficient utilization of lignocellulosic sugars.

In this thesis, we proposed and constructed an artificial cellobiose assimilating pathway consisting of a cellobiose transporter and a β -glucosidase in *S. cerevisiae*. A total of six different cellobiose assimilating pathways were constructed and compared in a laboratory *S. cerevisiae* strain capable of xylose utilization and the one with best fermentation performance was selected. The resultant yeast strain showed significantly improved cellobiose and xylose consumption ability and ethanol productivity in both shake-flask and bioreactor fermentation. The xylose consumption rate was enhanced by 42% to $0.68 \text{ g L}^{-1} \text{ h}^{-1}$ in the engineered laboratory strain, and a maximum ethanol productivity of $0.49 \text{ g L}^{-1} \text{ h}^{-1}$ was obtained, with no obvious glucose repression phenomenon observed. The maximum ethanol yield achieved was 0.39 g per g sugar. In addition, the best cellobiose assimilating pathway was also transferred to an industrial yeast strain and the resultant industrial strain showed greatly improved fermentation performance. The ethanol productivity was $0.64 \text{ g L}^{-1} \text{ h}^{-1}$, the ethanol yield was 0.42 g per g sugar, and the cellobiose consumption rate was more than $1.77 \text{ g L}^{-1} \text{ h}^{-1}$, which enables fast and efficient ethanol production from lignocelluloses. Thus this approach has been demonstrated to be a promising method to overcome glucose repression and at the same time enhance ethanol productivity.

It was found that a small amount of glucose was accumulated during either cellobiose fermentation or cellobiose and xylose co-fermentation, which inevitably decreased the ethanol yield and productivity. To address this limitation, the role of mutarotase, also called aldose 1-epimerase, which is capable of converting glucose between two anomers was investigated. Three endogenous mutarotase genes *YHR210c*, *YNR071c* and *GAL10* were identified in *S. cerevisiae* s288c wild type strain. The natural cellobiose assimilating strain *Neurospora crassa* also has a mutarotase gene named *NCU09705*. Overexpression of both *S. cerevisiae* and *N. crassa* aldose 1-epimerases showed improved sugar consumption and ethanol production in cellobiose assimilating *S. cerevisiae* strains and aldose 1-epimerase disrupted *S. cerevisiae* strains derived from the s288c strain showed significant drawbacks in cellobiose utilization.

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Chapter 1. Overview of Sugar Utilization in *Saccharomyces cerevisiae*

1.1 Biofuels from Lignocelluloses

1.1.1 Biofuels

Biofuels are considered as a promising alternative energy source compared to fossil fuels. Biofuels include bioethanol, biodiesel and biogas, which are capable of meeting the huge market demand for traditional fuels such as petroleum and natural gas[1]. Increasing crude oil prices render biofuel prices acceptable day by day. Considering the fact that traditional fuels are a nonrenewable resource, which stands for a continuously reducing stock and correspondingly an increasing price, the renewable biofuels produced from biomass represent a competitive energy source, especially in the case of an energy supply crisis. Also, with crop plants or agricultural residues as a sole feedstock in production, together with a reduction of greenhouse gas released, biofuels are considered as environment-friendly[2]. The wide supply of plant materials for biofuel production also makes biofuels attractive for energy security consideration[2]. Because of these advantages, biofuels have attracted extensive attention as a competitive future energy source and many biofuel compatible machines and engines have been designed and sold to meet the significantly increasing demand[1].

Among various sorts of biofuels, bioethanol is the most widely used and accepted biofuel. In 2005 global ethanol production achieved 45 billion liters, making about 2% of total global gasoline consumption [3]. During 2010 there were 13.2 billion gallons of ethanol

manufactured in the United States, and it is estimated that by the end of 2011 the United States will surpass Brazil to be the world's largest ethanol export nation [4]. However, the disadvantages of bioethanol such as low energy intensity, high vapor pressure, and corrosiveness hinder bioethanol's large-scale application. Extensive efforts are required to make ethanol production more efficient and affordable.

Compared to bioethanol, isopropanol and n-butanol exhibit high energy intensity and low corrosiveness. Isopropanol and n-butanol are longer chain alcohols with more carbons in molecules, which make them better alternative energy source than bioethanol. Natural *Clostridium* species are capable of producing isopropanol and n-butanol, but their slow growth rate and anaerobic growth condition limit further development in industrial fermentation. As a result, platform species such as *Escherichia coli* and *Saccharomyces cerevisiae* were used as host strains for isopropanol and n-butanol production. The *Clostridium* genes involved in isopropanol or n-butanol biosynthesis were overexpressed in *E. coli* or *S. cerevisiae* and only a low level of long chain alcohols production was achieved, which may be due to redox imbalance and excess accumulation of intermediate metabolites *in vivo* [5].

Alkanes and alkenes are also attractive biofuel candidates as they are also components in fossil fuels. Engineered *E. coli* strains were constructed with heterologous genes from natural alkene or alkane producing species. However, the titers of alkanes or alkenes in heterologous hosts are very low and the genetic mechanism for alkane or alkene synthesis has not been totally identified yet [6, 7].

Biodiesel composed of fatty acid esters and fatty alcohols is another potential fossil fuel alternative. Valued by energy intensity, bioethanol's energy intensity is only $2.95\text{E}+05$ seJ/J while biodiesel's average value achieves $4.51\text{E}+05$ seJ/J [8]. Recombinant *E. coli* strains were engineered to produce fatty acid ethyl esters (FAEEs) which are ethyl ester biodiesels [9]. A native thioesterase was overexpressed and several genes involved in fatty acid degradation were deleted to increase the production of fatty acids in the recombinant *E. coli* strain, and heterologous ethanol production enzymes, endogenous wax-ester synthases and hemicellulases were also overexpressed to establish a biodiesel producing strain [9].

1.1.2 Lignocellulose as a Feedstock for Bioethanol Production

Bioethanol can be produced either from sugars and starch derived from food crops, or from lignocelluloses derived from biomass. Generally, in large scale industrial fermentation, sugars and starch are preferred. In the United States, starch from corns is the dominating feedstock for bioethanol production while in Brazil sucrose from sugarcane dominates [10]. However, such resources are also a major source of human and animal food worldwide, which results in relatively high cost of the raw material, and the insufficient supply of food crops leads to a competition between food supply and fuel production, which is a critical ethical issue difficult to solve. Considering these factors, lignocellulose as agricultural residues becomes an attractive alternative feedstock. Lignocellulose requires lower energy and labor input, which may reduce the feedstock cost to a lower level than food crops [11]. The non-food feature also makes it acceptable to the public, with no ethical dilemma.

Before the production of ethanol, lignocelluloses are firstly hydrolyzed to individual small sugar molecules. Lignocellulosic biomass is composed of cellulose, hemicellulose, and lignin, while cellulose and hemicelluloses can be converted to fermentable sugars [12]. Cellulose is a long chain polymer composed of β -linked D-glucose molecules, whereas hemicelluloses is a highly branched chain composed of cross-linked D-glucose, D-xylose, L-arabinose and other sugars [13]. Both cellulose and hemicelluloses have to be converted to small sugar molecules. In the pretreatment process, lignin is removed from the biomass mixture and the remaining components are treated by different means including steam explosion, high temperature treatment, or acid/base treatment to release individual sugar molecules [10]. Enzymatic hydrolysis is an indispensable treatment, aiming to digest large polymer molecules to small soluble sugar molecules sufficiently. During this step, an enzyme cocktail is needed to digest the mixture. Briefly, cellulases including endo-glucanases and cellobiohydrolases are added to cleave off a disaccharide named cellobiose from the polymer chain, then a third enzyme β -glucosidase converts one cellobiose molecule to two glucose molecules for further fermentation [14]. Pentose such as xylose and arabinose is also cleaved from hemicelluloses [11]. The fermentable sugar mixture containing glucose, xylose, arabinose and other sugars is achieved after the enzymatic hydrolysis process.

1.1.3 *Saccharomyces cerevisiae* as a Model Microorganism for Bioethanol Production

Saccharomyces cerevisiae, also known as Baker's yeast, has been used for bread, wine and beer production for thousands of years. As a popular eukaryotic microorganism in

research, its complete genome sequence was obtained in 1996 and many genetic tools have been developed [15]. Multiple genome databases and strain databases such as SGD (Saccharomyces Genome Database) and EUROSCARF (EUROpean Saccharomyces Cerevisiae ARchive for Functional Analysis) were established, providing not only biological materials but also sequence databases to all researchers in the world. Besides well-studied genetic background and well-developed tools, *S. cerevisiae* is suitable for ethanol production also due to its high productivity and tolerance of ethanol. A high ethanol concentration is toxic to many microorganisms, which may induce cell death or slow growth rate. In contrast, *S. cerevisiae* has a relatively high tolerance of ethanol, thus resulting in relatively high ethanol productivity. Additionally, *S. cerevisiae* can tolerate low pH and inhibitors from the upstream pretreatment process, ensuring stable fermentation conditions during fermentation. A low pH environment also eliminates the chance to get contamination from other microorganisms that cannot survive under low pH conditions. Compared to other eukaryotic microorganisms, the fast doubling time of *S. cerevisiae* guarantees high production efficiency and short time. Unfortunately, *S. cerevisiae* cannot utilize pentose to produce ethanol due to lack of key enzymes to introduce pentose into the cellular metabolism [16].

1.2 Glucose Repression in *S. cerevisiae*

1.2.1 Glucose Repression

Glucose repression, also called carbon catabolite repression, is one of the major limitations in mixed sugar fermentation for ethanol production [17]. It exists in almost all microorganisms and presents a significant negative effect on bioethanol production in *S.*

cerevisiae [18]. In the presence of glucose, utilization of other sugars is inhibited, which lowers sugar utilization efficiency and also ethanol production rate. The preference for glucose results in a sequential utilization of xylose after glucose depletion, which greatly limits fermentation efficiency using sugar hydrolysates from lignocelluloses [13, 17, 19, 20].

Glucose repression functions on the transcriptional level, and involves large numbers of genes [17]. Glucose repression effects either by interfering transcription activators, or by activating expression of proteins that have a negative effect on transcription [21]. Elements in the glucose repression pathway include (1) activators such as the Hap2/3/4/5 complex, Gal4, Mal63 and Adr1, which are capable of activating the transcription of key genes involved in the catabolism of pentose and other sugars; (2) repressors such as Mig1/2/3, which play a key role in glucose repression and are capable of binding to a variety of promoters that are repressed by glucose; (3) intermediary elements such as Snf1 and Snf4, which encode protein kinases associating with other proteins; (4) glucose sensors such as Snf3 and Rtg2, which are located on yeast membranes. Under the condition of high concentration of glucose, Snf3 expression is repressed while under the condition of low concentration of glucose, Rtg2 expression is repressed. Thus, Snf3 is considered as a sensor activated on low levels of glucose while Rtg2 is activated on high levels of glucose [19, 20]. However, the complicated glucose repression pathways have not been fully understood and only two pathways were studied, which covers only a small part of the regulation system [17-20].

1.2.2 Glucose Derepression Studies

In order to overcome glucose repression, great efforts have been made in the past decades. Generally, there are two research directions about glucose derepression studies, either to establish a genetic model to study the mechanisms using systems biology tools[22-25], or to construct glucose derepressed strains by gene modulation[21].

In the mechanistic studies, two glucose sensors, Snf3 and Rgt2, were investigated[26]. They are involved in the regulation of sugar transporters. Signal about glucose concentration is transported via Grr1 to the nucleus. At high concentrations of glucose, Grr1 deactivates the repression of Rgt1 on *HXT* genes, thus glucose transportation is inhibited because Hxt proteins are dominant transporters for glucose transportation[26]. Another pathway involving Hxk2 was also studied. Hxk2 is a glycolytic enzyme carrying the intracellular glucose concentration signal to Snf1. It can inactivate Snf1 by a protein phosphatase Glc7-Reg1. Additionally, Snf1 is capable of phosphorylating the Mig1 protein. Only phosphorylated Mig1 can translocate from nucleus to cytosol and then regulates sugar assimilation by binding to promoters or by inducing the repression of relative genes in the assimilation of pentose sugars. Therefore, with high concentrations of glucose, Snf1 is repressed and then dephosphorylated Mig1 represses other sugars' utilization. On the other hand, at low concentrations of glucose, Snf is activated, which phosphorylates Mig1 to translocate into the cytosol, thus avoiding glucose repression [19, 20].

With ^{13}C -labeled glucose, phenotypic characterization of *S. cerevisiae* strains was obtained by metabolic flux analysis [27-29]. The Mig1 family including Mig1, Mig2 and Mig3 was characterized [30], while the relationship between Mig1 and Mig1-dependent

Hxk2 was also analyzed [23]. All these studies aim at clarifying Mig1-related glucose repression mechanisms [23, 30-32]. Based on these studies, Mig1 disrupted *S. cerevisiae* strains were constructed. However, engineered strains ($\Delta mig1$, $\Delta mig2$ or $\Delta mig1\Delta mig2$) did not show significant improvement in glucose derepression as expected [21], which means glucose repression is still a complicated system with lots of unknown parts to study with.

1.3 Engineered *S. cerevisiae* for Sugar Assimilation

1.3.1 Xylose Assimilation in *S. cerevisiae*

Efficient xylose utilization is one of the prerequisites for cellulosic biofuels production. Unfortunately, wild type *S. cerevisiae* cannot ferment pentose to ethanol as it does not possess a complete xylose assimilating pathway. In spite of this, it has the ability to metabolize xylulose via the pentose phosphate pathway to produce ethanol, while xylulose can be produced either from xylose catalyzed by xylose isomerase (XI), or converted from xylitol by xylitol dehydrogenase (XDH), while xylitol is converted from xylose by xylose reductase (XR) [33, 34]. At the same time, there are large numbers of natural fungal and bacterial species that can utilize pentose sugars, though these strains are not suitable for industrial ethanol fermentation [35-38].

Engineered xylose pathways involving XI is able to avoid redox imbalance that is one of the problems in the XR-XDH pathways [39]. However, the low activity and expression level of XI also limits xylose assimilation in engineered *S. cerevisiae* strains [40]. As a result, it is desirable to introduce heterologous enzymes with high activity into wild type *S.*

cerevisiae in order to construct an efficient xylose-assimilating strain. Additional evolutionary engineering approaches are also needed to redirect metabolic flux through the XI based pathway [41]

Among various kinds of xylose-assimilating microorganisms with the XR-XDH pathways, *Pichia stipitis* (taxonomic classification has been changed to *Scheffersomyces stipitis*) is able to ferment xylose to ethanol, with satisfactory ethanol yield and productivity due to its natural XR-XDH xylose utilizing pathway[37]. However, inhibitors derived from pretreatment and enzymatic hydrolysis process inhibit *P. stipitis* fermentation, which is an obstacle in ethanol production. Therefore, the engineered *S. cerevisiae* strain with heterologous genes encoding key xylose-assimilating enzymes from *P. stipitis* has attracted great attentions and promising results have been obtained. *P. stipitis* *XYL1* gene encoding for xylose reductase and *XYL2* gene encoding for xylitol dehydrogenase were introduced into *S. cerevisiae* to obtain an efficient xylose assimilating strain. Additionally, although xylulokinase (XKS) converts xylulose to xylulose-5-phosphate exists in *S. cerevisiae*, overexpression of endogenous *XKS1* gene was also attempted to enhance the xylose consumption rate [42]. *P. stipitis* xylulokinase gene *XYL3* was also expressed in *S. cerevisiae* to obtain a high xylose consumption rate. The resultant engineered *S. cerevisiae* strains with a heterologous xylose assimilating pathway showed improved xylose utilization ability [43].

However, in order to obtain an efficient *S. cerevisiae* strain to convert xylose to ethanol, many more factors should be taken into consideration; the xylose utilization efficiency is not only limited by enzyme activities, but also related to other issues as redox imbalance, low flux of the pentose phosphate pathway, and low sugar uptake rates [44].

For many wild type xylose reductases, there is a strict dependence on NADPH rather than NADH; while for xylitol reductases, NAD^+ is preferred over NADP^+ . This redox imbalance results in low xylose consumption rates due to NADPH depletion and NADH excess, which strongly impairs xylose fermentation. To solve this problem, either the wild type *S. cerevisiae* xylose reductase was replaced by a mutant xylose reductase exhibiting higher preference to NADH, or a mutant xylose reductase was co-expressed with the wild type enzyme to maintain redox balance [43, 44]. In both methods, improved ethanol production and xylose utilization were reported [43, 44].

To eliminate the negative impact of excess xylitol production on ethanol yield and XDH activity, balanced enzyme activity is another key factor in xylose assimilation. The XR/XDH/XKS activity ratio was modified in engineered *S. cerevisiae* strains to minimize xylitol production and to redirect metabolic flux to ethanol production [45]. Other enzymes responsible for xylitol accumulation were also investigated. Endogenous aldo-keto reductase GRE3 was found to possess xylose reductase activity, and deletion of GRE3 gene was proven to reduce xylitol accumulation in xylose assimilating *S. cerevisiae* strains [46].

Another approach to improve xylose assimilation efficiency is to modify the pentose phosphate pathway in xylose assimilating strains. Bera and coworkers observed decreased xylitol accumulation but little improved xylose assimilation by overexpressing four PPP genes TKL1, TAL1, RPE1 and RKI1 in xylose assimilating *S. cerevisiae* strains [47]. Karhumaa and coworkers also reported an engineered xylose assimilating *S. cerevisiae* strain: the overexpression of XR and XDH together with four PPP enzymes and XKS proved to have a higher xylose assimilation rate than the control strain [48].

The gluconeogenesis process was also proved to affect xylose assimilation in *S. cerevisiae* strains. Hector and coworkers constructed gluconeogenesis gene disrupted *S. cerevisiae* strains expressing the *S. stipitis* XR-XDH pathway in plasmid, and found two enzymes including fructose phosphate aldolase (FBA1) and phosphoglucose isomerase (PGI1) that are responsible for recycling fructose-6-phosphate to glucose-6-phosphate were required for efficient aerobic xylose assimilation [49].

1.3.2 Cellobiose Assimilation in *S. cerevisiae*

Cellobiose is a disaccharide composed of two glucose molecules. It usually occurs in sugar hydrolysis, cleaved from cellulose and hemicellulose chains. Catalyzed by β -glucosidase, the β -(1, 4) glucolytic bond that links two glucose molecules is cut off and two β -glucose molecules are released. As wide type *S. cerevisiae* cannot utilize cellobiose directly, the conversion from cellobiose to glucose is required in enzymatic hydrolysis[10].

On the other hand, certain cellulolytic fungi can grow up with cellobiose as a sole carbon source. Previous study of a model cellobiose assimilating strain, *Neurospora crassa*, showed that a high affinity cellobiose transport system is necessary for efficient cellobiose utilization [50]. The cellobiose transport system includes at least a cellobiose transporter which executes sugar uptake function and an intracellular β -glucosidase that converts cellobiose to glucose. As a result, to introduce a cellobiose assimilating pathway into *S. cerevisiae*, the cellobiose transport system should be constructed in the wild type strain. It was reported that with a heterologous *N. crassa* cellobiose transport pathway, engineered *S.*

cerevisiae showed improved cellobiose utilization efficiency and ethanol productivity [14]. Besides *N. crassa*, β -glucosidase also exists in fungi or bacteria such as *Saccharomycopsis fibuligera* and *Candida wickerhamii*. Engineered *S. cerevisiae* with the β -glucosidase gene from *S. fibuligera* also yielded improved ethanol production [51]. However, the low efficiency of cellobiose transportation limits further improvement in cellobiose utilization.

Besides the cellobiose transport system mentioned above, co-expression of a cellobiose phosphorylase and a lactose permease also enabled intracellular cellobiose assimilation in *S. cerevisiae* [51]. Expression of a heterologous lactose permease gene from *Kluyberomyces lactis* facilitates cellobiose transportation; and a cellobiose phosphorylase from *Clostridium stercorarium* converts intracellular cellobiose to glucose-phosphate, which enters glycolysis pathway after hydrolysis, an engineered *S. cerevisiae* strain was also able to utilize cellobiose [51].

Generally, transporters with high affinity of cellobiose and efficient hydrolytic enzymes are two main factors required for construction of a robust cellobiose assimilating *S. cerevisiae* strain. A cellobiose utilizing strain instead of a glucose utilizing strain will be an alternative choice for glucose derepression strain construction.

1.4 Project Overview

This project aimed to construct an engineered *S. cerevisiae* capable of simultaneously utilizing cellobiose and xylose, thereby removing glucose repression and enhancing ethanol

production in mixed sugar fermentation by introducing both cellobiose and xylose utilization pathways into yeast.

It was hypothesized that with a heterologous cellobiose assimilating pathway in *S. cerevisiae*, there should be no glucose repression in xylose uptake because no extracellular glucose exists. After transportation, intracellular cellobiose will be converted to glucose immediately, and glucose will be metabolized in glycolysis swiftly, with a relatively low level of intracellular glucose accumulated that is unable to activate glucose repression. Thus, with the co-expression of a cellobiose transporter and a β -glucosidase, an engineered *S. cerevisiae* strain with xylose-assimilating pathway should be capable of fermenting cellobiose and glucose simultaneously, without glucose repression. This strategy will be useful in practical ethanol production, which reduces the cost of cellulolytic enzyme cocktail and results in a high sugar consumption rate.

In order to obtain a glucose derepressed yeast strain, we first sought to discover a cellobiose assimilating pathway composed of an efficient cellobiose transporter and a high-activity β -glucosidase that can yield high ethanol production in *S. cerevisiae*. Second, we sought to co-express the cellobiose-assimilating pathway with a xylose-assimilating pathway in *S. cerevisiae*. In our group, we have obtained an engineered *S. cerevisiae* strain with relatively high xylose utilization capability, which can be used as the background strain. Third, we evaluated the fermentation performance of the engineered strains using different combinations of sugars, different concentrations of sugars, and different fermentation conditions. In parallel, the glucose derepression performance was also evaluated. Finally,

with a high efficiency strain, we also attempted to obtain an improved strain with reduced glucose accumulation and higher ethanol production.

In total, we constructed six different cellobiose utilization pathways and identified the most efficient one. In shake-flask fermentation and bioreactor fermentation, a mixture of cellobiose and xylose, a mixture of cellobiose and glucose, or a mixture of cellobiose, xylose and glucose were used as substrates for the best engineered laboratory strain, and significantly improved sugar utilization and ethanol production together with eliminated glucose repression were observed. An industrial *S. cerevisiae* strain was also engineered for cellobiose fermentation, which resulted in an even higher ethanol productivity compared to the laboratory strain.

During the cellobiose fermentation process, accumulated glucose was observed which might result from imbalanced conversion between α -glucose and β -glucose. Mutarotases in *S. cerevisiae* named aldose 1-epimerases was concluded as the key enzymes responsible for glucose accumulation and investigation result was reported.

1.4 Conclusions

Biofuels are under extensive investigation due to the increasing concerns on energy security, sustainability and global climate change. Bioconversion from lignocelluloses to ethanol has drawn more and more attention because of its potential to eventually replace fossil fuels [1]. *S. cerevisiae* is the most widely used microorganism for large scale industrial fermentation of ethanol. Unfortunately, *S. cerevisiae* cannot utilize cellobiose or xylose from lignocelluloses hydrolysates, and glucose repression limits the efficient utilization of sugar

mixture from lignocelluloses [15]. Previously reported glucose repression studies mainly focused on mechanisms or single gene disruptions, neither of which led to a glucose derepressed strain with a high ethanol production efficiency [17-20].

Here we designed a novel strategy by introducing a cellobiose-assimilating pathway into an engineered xylose utilizing *S. cerevisiae* strain. The resultant strain exhibited greatly improved ethanol productivity, and was capable of simultaneously utilizing cellobiose and xylose, or even with additional glucose too. Mutarotases playing an important role in glucose conversion were also investigated. Marginal improvement in cellobiose utilization was found in mutarotase overexpression strains.

Chapter 2. Construction of a *S. cerevisiae* Strain Capable of Simultaneously Utilizing Cellobiose and Xylose

2.1 Introduction

Cellobiose is one of the intermediate products from cellulose hydrolysis. Catalyzed by a cellulose cocktail, cellulose can be degraded to cellobiose by exocellulases and endocellulases, and cellobiose can be further converted to glucose by β -glucosidases in the cellulose cocktail [52-56].

In the conventional methods for mixed sugar fermentation in *S. cerevisiae*, a mixture of glucose and pentose sugars derived from lignocellulose are used. As a result, cellobiose is only considered as an intermediate which may inhibit the effect of endoglucanases and cellobiohydrolases in cellulose hydrolysis. To relieve the inhibition effect of cellobiose, β -glucosidases were expressed with other necessary enzymes in simultaneous saccharification and fermentation (SSF) process. Enhanced cellulose hydrolysis efficiency was reported in several publications [53-55].

Without cellobiose transporters, a heterologous β -glucosidase was introduced into *S. cerevisiae* to construct a cellobiose assimilating strain. With the expression of a heterologous *Saccharomycopsis fibuligera* β -glucosidase gene (BGL1) under the control of a constitutive promoter, Gurgu and coworkers observed ethanol production from a recombinant strain [57]. However, without a high affinity transporter of cellobiose, relatively low concentrations of glucose were produced extracellularly due to the low level of secreted β -glucosidase, which

impaired the glucose consumption efficiency. Only marginal ethanol production was achieved and extracellular glucose may inhibit further expression of β -glucosidase.

Other transporters and enzymes with relatively low activities were also expressed in *S. cerevisiae* in order to construct cellobiose assimilating strains. For example, recombinant *S. cerevisiae* strains co-expressing a cellobiose phosphorylase and a lactose permease were constructed, which enabled intracellular cellobiose assimilation in *S. cerevisiae* [51]. Expression of a heterologous lactose permease gene from *Kluyberomyces lactis* facilitated cellobiose transportation; and a cellobiose phosphorylase from *Clostridium stercorarium* converted intracellular cellobiose to glucose-phosphate, which enters the glycolysis pathway after hydrolysis. The resulted engineered *S. cerevisiae* strain was able to utilize cellobiose [51].

However, nearly all these strategies suffered from their low efficiency due to inefficient pathway construction or low activity enzymes. There was not much research about intracellular cellobiose assimilation as researchers usually focused on lignocellulose' SSF but not cellobiose assimilation. The very few publications about ethanol production from cellobiose either lacked an efficient sugar uptake pathway, or used a low efficiency pathway that was hard to improve for ethanol production [51].

Here we design a new strategy in which a cellobiose transporter gene and a β -glucosidase gene are co-expressed in *S. cerevisiae* (Figure 2.1). The high efficiency pathway promises fast cellobiose assimilation and ethanol production, which makes it an attractive platform for mixed sugar fermentation.

Besides, this strategy represents a novel approach to address the glucose repression problem. Cellobiose rather than glucose is used as a main carbon source in our new strategy. A mixture of cellobiose and pentose sugars is used for ethanol production. Cellobiose is transported inside yeast cells via the heterologous cellobiose transporters while xylose is transported by endogenous hexose transporters, thus preventing direct competition between glucose and pentose sugars in the transport process. Once inside yeast cells, cellobiose is converted to glucose by β -glucosidase and immediately consumed by yeast cells, which results in a low intracellular glucose concentration, thereby further alleviating glucose repression. Distinguished from existing glucose derepression methods, there is no gene depletion in yeast strain, and glucose assimilation is not impaired, while xylose assimilation is improved because of synergic effect. This strategy avoids the almost inevitable glucose repression in lignocelluloses fermentation for the first time, and improves both sugars' assimilations at the same time. Based on this engineered cellobiose-xylose co-assimilating strain, evolutionary engineering and metabolic flux modification can be carried out to obtain more efficient ethanol producing strains.

2.2 Results

2.2.1 Comparison of Various Cellobiose Assimilating Systems in a Laboratory *S. cerevisiae* Strain

As proof of concept, the mixed sugar fermentation consisting of D-xylose and cellobiose was used as a model system. Specifically, an engineered xylose-utilizing yeast strain HZ3001 was used as a host to co-express a cellobiose transporter gene and a β -glucosidase gene. In this strain, the xylose utilization pathway consisting of xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis* was integrated into the chromosome. Three recently discovered cellobiose transporter genes from *Neurospora crassa*, including *cdt-1*, *NCU00809*, and *cdt-2*[14] and two β -glucosidase genes, one from *N. crassa* (*ghl-1*) and the other from *Aspergillus aculeatus* (*BGL1*), were evaluated. A total of six different strains, referred to as SL01 through SL06, were constructed by introducing a pRS425 plasmid harboring one of the cellobiose transporter genes and one of the β -glucosidase genes into the HZ3001 strain (Figure 2.2). In each plasmid, the cellobiose transporter gene and the β -glucosidase gene were assembled into the multi-copy plasmid pRS425 by the DNA assembler method [58]. The empty pRS425 plasmid was introduced to the HZ3001 strain to yield the SL00 strain, which was used as a negative control. All strains were cultivated in the YPA medium supplemented with 40 g L⁻¹ cellobiose and 50 g L⁻¹ D-xylose in shake-flasks, and their sugar consumption rates, cell growth rates, and ethanol titers were determined (Figure 2.3).

Among all strains, the SL01 strain containing the β -glucosidase gene *ghl-1* from *N. crassa* and the cellobiose transporter gene *cdt-1* showed the highest sugar consumption rate and ethanol productivity. Thus, this strain was selected for further characterization.

2.2.2 Cofermentation of Cellobiose and Xylose in an Engineered

Laboratory *S. cerevisiae* Strain

Both SL01 and SL00 were cultivated using the YPA medium supplemented with 40 g L⁻¹ cellobiose and 50 g L⁻¹ D-xylose in both shake-flasks and bioreactors (Figure 2.4). In the shake-flask cultivation (Figure 2.4 a and b), 83% of the cellobiose was consumed in 96 hours by SL01, with a 41.2% higher overall D-xylose consumption rate (from 0.33 g L⁻¹ h⁻¹ to 0.46 g L⁻¹ h⁻¹) compared to SL00. Consistent with the enhanced sugar consumption rate, 2.3-fold higher overall dry cell weight growth rate was observed (from 0.031 g dry cell weight L⁻¹ h⁻¹ to 0.072 g dry cell weight L⁻¹ h⁻¹). The ethanol productivity was increased by more than 3.1-fold, from 0.07 g L⁻¹ h⁻¹ to 0.23 g L⁻¹ h⁻¹. The highest ethanol yield of 0.31 g per g sugar was reached in 48 hours, and the overall ethanol yield was 0.28 g per g sugar, representing a 23% increase compared to the SL00 strain. In the SL01 cultivation, a faster D-xylose consumption rate was observed, without the lag phase that is the hallmark of glucose repression in cofermentation of glucose and D-xylose. Moreover, improved cell growth and ethanol production were also observed. In the bioreactor cultivation (Figure 2.4c and d), almost all of the cellobiose and 66% of the D-xylose were consumed in 48 hours by SL01, representing 42% increased D-xylose consumption rate (from 0.48 g L⁻¹ h⁻¹ to 0.68 g L⁻¹ h⁻¹) and 1.02-fold increased dry cell weight growth rate (from 0.08 g dry cell weight L⁻¹ h⁻¹ to

0.17 g dry cell weight L⁻¹ h⁻¹) compared to SL00. The ethanol productivity was increased by more than 4.4-fold (from 0.09 g L⁻¹ h⁻¹ to 0.49 g L⁻¹ h⁻¹) and the ethanol yield was 0.39 g per g sugar. Compared to shake-flask cultivations, sugar consumption rates in the first 24 hours were lower due to the low cell density used in the beginning of batch cultivation.

2.2.3 Cofermentation of Cellobiose and Glucose in an Engineered Laboratory *S. cerevisiae* Strain

To analyze whether a small concentration of glucose will repress cellobiose utilization significantly, a mixture of 10 g L⁻¹ glucose and 40 g L⁻¹ cellobiose was tested using the SL01 strain. For SL01, with solely 10 g L⁻¹ glucose, 91.3% glucose was consumed and the maximum ethanol productivity and yield reached 0.40 g L⁻¹ h⁻¹ and 0.32 g per g sugar, respectively, at 9 hours. After that, ethanol was gradually consumed (Figure 2.5 b). In comparison, with 10 g L⁻¹ glucose and 40 g L⁻¹ cellobiose, the ethanol productivity and yield were 0.38 g L⁻¹ h⁻¹ and 0.28 g per g sugar, respectively, at 9 hours, and reached the maximum level (0.44 g L⁻¹ h⁻¹ and 0.30 g per g sugar, respectively) at 24 hours (Figure 2.5 a). Thus, the effect of cellobiose on the maximum ethanol yield and productivity was insignificant. For SL00, with 10 g L⁻¹ glucose and 40 g L⁻¹ cellobiose, no cellobiose consumption was observed (Figure 2.5 c), while with solely 10 g L⁻¹ glucose, the profile of glucose consumption and ethanol production was almost identical to that of SL01. It was found that the presence of cellobiose increased the overall ethanol productivity, but its effect on the maximal ethanol yield and productivity seems to be insignificant.

2.2.4 Cofermentation of Cellobiose, Xylose, and Glucose in an Engineered Laboratory *S. cerevisiae* Strain

A small amount of glucose (less than 10% of total sugars) is typically present in lignocellulosic hydrolysates when cellulose cocktails deficient in β -glucosidase were used to catalyze the hydrolysis of lignocellulosic materials. Thus, the fermentation performance of the engineered SL01 strain was also investigated using a mixture of cellobiose, D-xylose and glucose. Two concentrations of glucose, 5 g L⁻¹ or 10 g L⁻¹, were combined with 40 g L⁻¹ cellobiose and 50 g L⁻¹ D-xylose as a mixed carbon source in bioreactors.

In the batch cultivation with 5 g L⁻¹ glucose (Figure 2.6a and b), 81.5% cellobiose and 69.3% D-xylose were consumed, respectively, by SL01 at 48 hours. Compared to SL00, the D-xylose consumption rate was increased by 89%, from 0.38 g L⁻¹ h⁻¹ to 0.73 g L⁻¹ h⁻¹. The ethanol productivity was increased by 2.2-fold (from 0.13 g L⁻¹ h⁻¹ to 0.43 g L⁻¹ h⁻¹) while the ethanol yield was increased from 0.24 g per g sugar to 0.30 g per g sugar. In the batch cultivation with 10 g L⁻¹ glucose (Figure 2.6 c and d), 74.3% cellobiose and 74.4% D-xylose were consumed, respectively, by SL01 at 48 hours. Compared to SL00, the D-xylose consumption rate was increased by 52%, from 0.51 g L⁻¹ h⁻¹ to 0.77 g L⁻¹ h⁻¹. The ethanol productivity was increased by 1.1-fold (from 0.21 g L⁻¹ h⁻¹ to 0.45 g L⁻¹ h⁻¹) and the ethanol yield was increased from 0.27 g per g sugar to 0.31 g per g sugar at 72 hours.

2.2.5 Construction and Fermentation of Cellobiose Assimilating System in an Industrial *S. cerevisiae* Strain

Compared to laboratory *S. cerevisiae* strains, industrial *S. cerevisiae* strains have much higher ethanol production capability and robustness. However, despite of the advantages of

industrial strains in fermentation, it is difficult to make gene modification based on very limited information of the multi-ploid industrial strains.

To test the performance of cellobiose assimilating system on the platform close to large scale fermentation, construction of an industrial strain capable of utilizing cellobiose is necessary. Based on the cellobiose assimilating system utilized in a laboratory strain, we introduced a multi-copy plasmid harboring the cellobiose pathway (*cdt1-ghl-1*) into an industrial strain, which resulted in the SLI01 strain capable of utilizing cellobiose efficiently. We compared its fermentation performance with that of the wild type industrial strain SLI00.

In shake-flask cultivation with 90 g L⁻¹ cellobiose supplemented (Figure 2.7 a and b), 94.2% cellobiose was consumed by SLI01 in 48 hours, while the wild type strain hardly showed any consumption. The ethanol productivity was 0.64 g L⁻¹ h⁻¹ while the ethanol yield was 0.42 g per g sugar, close to theoretical yield. Biomass production was quite high due to the characteristics of the parent strain.

Based on the cellobiose cultivation result, we asserted that an industrial strain with an engineered cellobiose pathway was capable of efficiently producing ethanol using cellobiose and xylose as carbon sources.

2.3 Discussion

To create an efficient cellobiose utilizing pathway in *S. cerevisiae*, the performance of different combinations of a cellobiose transporter and a β -glucosidase was evaluated because a balance between the cellobiose uptake rate and the cellobiose conversion rate plays an important role in efficient sugar consumption. Three cellobiose transporters from *N. crassa*

and two β -glucosidases were used to create six different cellobiose assimilating pathways in a multicopy plasmid for further overexpression in a target yeast strain. In order to obtain a *S. cerevisiae* strain capable of co-utilizing xylose and cellobiose, a mixture of cellobiose and xylose was used to select the most efficient cellobiose assimilating pathway for further analysis. By comparing sugar consumption rate, ethanol productivity and yield, and biomass production in shake-flask fermentation, the combination with *N. crassa* cellobiose transporter *cdt1* and *N. crassa* β -glucosidase *gh1-1* was selected.

There are two types of *S. cerevisiae* strains, modified laboratory strains and real “wild type” industrial strains. The former was derived from naturally existing *S. cerevisiae* strains, but then modified to make a simple model for genetic studies. As a result, laboratory strains are often used as benchmark strains because of their advantages such as well-studied gene background, available auxotrophic or antibiotic resistant markers, and haploid feature, which enables simple gene modification. The wild type industrial strains were discovered in long term fermentation adaptation and chosen from industrial fermentation process. Usually industrial strains have fast sugar utilization, ethanol production and biomass production. Industrial strains are usually diploid or multi-ploid, non-auxotrophic, and antibiotic resistant markers are not available. Although it is difficult to modify or engineer, industrial strains are robust and efficient ethanol production hosts. Here we tested the cellobiose assimilating pathway in both a laboratory yeast strain and an industrial yeast strain.

In the laboratory yeast strain, I tested different combinations of sugar mixtures, including cellobiose+ xylose, cellobiose+ glucose, and cellobiose + xylose + glucose. The combination of cellobiose with xylose aimed at co-fermentation ability of these two sugars from

lignocelluloses, while added glucose was designed to test whether glucose repression exists in cellobiose based co-fermentation. Different fermentation approaches were also tested. Shake-flask cultivation was used as a simple and easy method and batch cultivation in a bioreactor exhibited better productivity due to its control in limited oxygen supply and pH condition. We found out that in the cellobiose and xylose co-fermentation system, our engineered laboratory strain showed significantly improved sugar utilization, and the synergistic effect made it even better than the separate cellobiose or xylose fermentation system. The ethanol productivity and yield shown here were much higher than what was obtained from single xylose fermentation. The cellobiose-xylose co-fermentation system represents a high-efficiency system with no glucose repression. Besides, from the results with added glucose to single cellobiose, or to the cellobiose-xylose mixture, we still found greatly improved sugar consumption and limited glucose repression, which suggests even with a small amount of glucose derived from sugar hydrolysates, the utilization of cellobiose and xylose is still efficient enough.

To construct the cellobiose-xylose co-utilizing pathway in an industrial strain, we introduced a cellobiose pathway into an industrial strain containing an integrated xylose utilization pathway. The resultant strain showed high ethanol production and sugar utilization, which were much higher than the laboratory strain: ethanol productivity was enhanced from $0.23 \text{ g L}^{-1} \text{ h}^{-1}$ to $0.64 \text{ g L}^{-1} \text{ h}^{-1}$ and cellobiose utilization rate was enhanced from $0.35 \text{ g L}^{-1} \text{ h}^{-1}$ to $1.77 \text{ g L}^{-1} \text{ h}^{-1}$. The robust and efficient industrial yeast strain enables further establishment of the cellobiose-xylose utilizing system. The industrial strain also could serve as a model for glucose derepression study.

2.4 Conclusions and Outlook

Glucose repression is a well-studied regulatory mechanism in *S. cerevisiae*. Various approaches have been attempted to overcome glucose repression, such as evolutionary engineering and deletion of key genes involved in glucose repression. However, these approaches met with only limited success. In our new strategy, the cellobiose will be transported into yeast cells via a heterologous cellobiose transporter, while pentose sugars will be transported into yeast cells by endogenous hexose transporters, thus mitigating the direct competition between glucose and pentose sugars for the same transporters that partly causes glucose repression. Once inside yeast cells, cellobiose will be converted to glucose by β -glucosidase and consumed, which should result in a low intracellular glucose concentration, thereby further alleviating glucose repression.

By co-expressing a cellobiose transporter gene and a β -glucosidase gene either in an engineered D-xylose-utilizing *S. cerevisiae* laboratory strain or in a high-productivity industrial strain, and using sugars including D-xylose and cellobiose or D-xylose, cellobiose, and a small amount of glucose, or single cellobiose as carbon sources, we demonstrated that these sugars can be consumed simultaneously to produce ethanol with high yields.

Overcoming glucose repression in mixed sugar fermentation in *S. cerevisiae* improved the overall sugar utilization efficiency and ethanol productivity, which is highly desirable in Biofuels production. Further studies on the discovery and engineering of more efficient cellobiose transporters and β -glucosidases coupled with engineering of a more efficient xylose-utilizing pathway are in progress.

2.5 Materials and Methods

2.5.1 Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae L2612 (*MAT α leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal+*) was a gift from Professor Yong-su Jin [59]. *Escherichia coli* DH5 α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in un-baffled shake-flasks at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific unless noted otherwise.

2.5.2 Strain and Plasmid Construction

To integrate the D-xylose utilization pathway consisting of D-xylose reductase, xylitol dehydrogenase, and xylulokinase from *Pichia stipitis*, the genes and corresponding promoters and terminators (ADH1 promoter-xylose reductase-ADH1 terminator, pGK1 promoter-xylitoldehydrogenase-CYC1 terminator, pYK1 promoter-xylulokinase-ADH2terminator) were PCR-amplified and cloned into the pRS416 plasmid using the DNA assembler method [60]. BamHI and HindIII were used to remove the DNA fragment encoding the D-xylose utilization pathway and then ligated to the pRS406

plasmid digested by the same two restriction enzymes. The resulting plasmid was then linearized by *ApaI* and integrated into the *URA3* locus on the chromosome of L2612, resulting in a recombinant xylose-utilizing yeast strain, HZ3001. The pRS425 plasmid (New England Biolabs, Ipswich, MA) was used to co-express a cellobiose transporter gene and a β -glucosidase gene. As shown in Figure 2.2, the pRS425 plasmid was digested by *BamHI* and *ApaI*. The *PYK1* promoter and the *ADH1* terminator were added to the N-terminus and C-terminus of the cellobiose transporter, respectively, while the *TEF1* promoter and the *PGK1* terminator were added to the N-terminus and C-terminus of the β -glucosidase, respectively (Table 2.2). These DNA fragments were assembled into the linearized pRS425 shuttle vector using the DNA assembler method. Three cellobiose transporter genes *cdt-1* (GenBank Accession number XM_958708), NCU00809 (GenBank Accession number XM_959259) and *cdt-2* (GenBank Accession number XM_958780) from *N.crassa* and two β -glucosidase genes *ghl-1* (GenBank Accession number XM_951090) from *N. crassa* and *BGL1* (GenBank Accession number D64088) from *Aspergillus aculeatus* were used. There are six combinations in total, each with one cellobiose transporter gene and one β -glucosidase gene (Table 2.1).

Yeast plasmids were then transferred into *E. coli* DH5 α , which were plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Miniprep Kit (QIAGEN). These plasmids were transformed into the L2612 strain individually to yield the following strains: SL01 (containing the plasmid harboring the *cdt-1* cellobiose transporter gene and the *ghl-1* β -glucosidase gene from *N. crassa*), SL02

(containing the plasmid harboring the NCU00809 cellobiose transporter gene and the *ghl-1* β -glucosidase gene from *N. crassa*), SL03 (containing the plasmid harboring the NCU08114 cellobiose transporter gene and the *ghl-1* β -glucosidase gene from *N. crassa*), SL04 (containing the plasmid harboring the *cdt-1* cellobiose transporter gene from *N. crassa* and the BGL1 gene from *A. aculeatus*), SL05 (containing the plasmid harboring the NCU00809 cellobiose transporter gene and the BGL1 gene from *A. aculeatus*), and SL06 (containing the plasmid harboring the *cdt-2* cellobiose transporter gene from *N. crassa* and the *BGL1* gene from *A. aculeatus*). The empty pRS425 plasmid was transformed into the HZ3001 strain to yield the SL00 strain as a negative control. Yeast transformation was carried out using the standard lithium acetate method [61]. The resulting transformation mixtures were plated on SC-Ura-Leu medium supplemented with 2% glucose. To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research, Orange, CA) and then transformed into *E. coli* DH5 α cells. The resulting cells were spread on LB plates containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by diagnostic PCR or restriction digestion using ClaI and HindIII. All restriction enzymes were obtained from New England Biolabs (Ipswich, MA).

2.5.3 Mixed Sugar Fermentation in Shake-Flasks

For each yeast strain, a single colony was first grown up in 2 mL SC-Ura-Leu medium plus 20 g L⁻¹ glucose, and then inoculated into 50 mL of the same medium in a 250 mL

shake-flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 40 g L⁻¹ cellobiose and 50 g L⁻¹ D-xylose; 40 g L⁻¹ cellobiose, 50 g L⁻¹ D-xylose, and 5 g L⁻¹ glucose; or 40 g L⁻¹ cellobiose, 50 g L⁻¹ D-xylose, and 10 g L⁻¹ glucose in a 250 mL un-baffled shake-flask. YPA media supplemented with 10 g L⁻¹ glucose and 40 g L⁻¹ cellobiose or solely 10 g L⁻¹ glucose were also used to determine the ethanol productivity in the presence of cellobiose. Starting from an initial OD₆₀₀ \approx 1, cell cultures were grown at 30 °C at 100 rpm for fermentation under oxygen limited conditions. OD₆₀₀ readings and cell culture samples were taken at various time points. Dry cell weight was measured gravimetrically using an aluminum foil weighing dish after evaporating under 65 °C for approximate 72 hours. Sugars and ethanol concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad Laboratories, Hercules, CA) and Shimadzu RID-10A refractive index detector following the manufacturer's protocol. The HPX-87H column was kept at 65 °C using a Shimadzu CTO-20AC column oven. 0.5mM sulfuric acid solution was used as a mobile phase at a constant flow rate of 0.6 mL/min. 10 μ L of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 25 minutes after the injection. The concentration of the sugars and ethanol were determined using a standard curve generated using a series of external standards. Each data point represented the mean of triplicate samples. The mixed sugar fermentation data for the strains ranging from SL00 to SL06 are shown in Figure 2.3. The best strain SL01 was selected for further characterization. In addition, both SL00 and SL01 were cultivated using the YPA media supplemented with a mixture of 10 g L⁻¹ glucose

and 40 g L⁻¹ cellobiose or solely 1% glucose (Figure 2.5). For SL01, with solely 10 g L⁻¹ glucose, 91.3% glucose was consumed and the maximum ethanol productivity and yield reached 0.40 g L⁻¹ h⁻¹ and 0.32 g per g sugar, respectively, at 9 hours.

2.5.4 Mixed Sugar Fermentation in Bioreactors

The Multifors system (Infors-HT, Bottmingen, Switzerland) was used for mixed sugar fermentation. Each vessel has a total capacity volume of 750 mL. For each vessel, there was one set of a pO₂ sensor, air sparger, exit gas cooler, temperature sensor, inoculation port, spare port, dip tube, antifoam sensor, pH sensor, drive shaft, heater block, rotameter, and peristaltic pump system. The whole bioreactor system was equipped with a Thermo Flex900 cooling system (Thermo Scientific, Waltham, MA). Single colonies of NC801 and L34 were first grown up in 2 mL SC-Ura-Leu medium plus 20g L⁻¹ glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, 10 mL saturated culture were inoculated in 500 mL YPA medium supplemented with 40 g L⁻¹ cellobiose and 50 g L⁻¹ D-xylose; 40 g L⁻¹ cellobiose, 50 g L⁻¹ D-xylose, and 5 g L⁻¹ glucose; or 40 g L⁻¹ cellobiose, 50 g L⁻¹ D-xylose, and 10 g L⁻¹ glucose. The temperature was maintained at 30 °C and the pH was maintained at 5.5, adjusted by addition of either 2NH₂SO₄ or 4N NaOH. In the first 48 hours, the air flow rate was maintained at 0.5 L min⁻¹, with the impeller speed at 250 rpm. Afterwards, the air flow rate was adjusted to 0.2 L min⁻¹ to achieve high ethanol production under oxygen limited conditions. Triplicate samples were taken at various time points and the OD₆₀₀, sugar concentration, and ethanol concentration were determined as described above.

2.6 Tables

Table 2.1 Constructed cellobiose assimilating strains

Strain Name	Cellobiose Transporter	β -Glucosidase
SL01	<i>cdt1</i>	<i>gh1-1</i>
SL02	<i>NCU00809</i>	<i>gh1-1</i>
SL03	<i>cdt2</i>	<i>gh1-1</i>
SL04	<i>cdt1</i>	<i>BGL1</i>
SL05	<i>NCU00809</i>	<i>BGL1</i>
SL06	<i>cdt2</i>	<i>BGL1</i>
SL00	-	-

Table 2.2 List of primers used in pathway construction

	SL01	SL02	SL03
PYK1 promoter-for	5'-TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCTAATACGCAATGCTACTATTTTGG-3'		
PYK1 promoter-rev	TCCTTAGGAA GAGACATTGT GATGATGTTT TATTTGTTTT GATTGGTGTC TTGTAAATAG		
glucosidase-for	5'-TTACAAGACACCAATCAAAACAAATAAAACATCATCACAATGTCTCTTCCTAAGGATTTC-3'		
glucosidase-rev	5'-TGGAGACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTTTAGTCCTTCTTGATCAAAG-3'		
adh1 terminator-for	5'-TCTTTGATCAAGAAGGACTAAAGCTTTGGACTTCTTCGCCAGAGGTTTGGTCAAGTCTCC-3'		
adh1 terminator-rev	5'-TGGAAGAGTAAAAAAGGAGTAGAAACATTTGAAGCTATCATGCCGGTAGAGGTGTGGTC-3'		
TEF1 promoter-for	5'-TAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGATAGCTTCAAAATGTTTC-3'		
TEF1 promoter-rev	5'-CTTCTCGGTGCTGGCCCCGTCATGGGA GCCGTGAGACGACATTTTGTAAATAAAA CTTAG-3'	5'-ATGGGCACCCATGGCCTCCTTTTCGTTTATGCT GTGAGCCATTTTGTAAATAAACTTAG-3'	5'-GTCGACGGCCTGAGCCACGGGCTTCTTGTTGA AGATGCCCATTTTGTAAATAAACTTAG-3'
transporter-for	5'-AGAAAGCATAGCAATCTAATCTAAGTT TTAATTACAAAATGTCGTCTCACGGCTCC CATG-3'	5'-AAAGCATAGCAATCTAATCTAAGTTTAAATTA CAAAATGGCTCACAGCATAAACGAAAAG-3'	5'-AGAAAGCATAGCAATCTAATCTAAGTTTAAAT TACAAAATGGGCATCTTCAACAAGAAGC-3'
transporter-rev	5'-AAAGAAAAAAATTGATCTATCGATTTTC AATTCAATTCAATCTAAGCAACGATAGC TTCGG-3'	5'-AAAAGAAAAAAATTGATCTATCGATTTCAATT CAATTCAATCTAAATTGTAACCTTCTCG-3'	5'-AAAAAATTGATCTATCGATTTCAATTCAATTC AATTCAAGCAACAGACTTGCCCTCATGC-3'
PGK1 terminator-for	5'-CCAGGCCGACGGCCATGTGTCCGAAG CTATCGTTGCTTAGATTGAATTGAATTGA AATCG-3'	5'-CACCATGGGAGCGCCGATGACGAGAAAGTT ACAATTTAGATTGAATTGAATTGAAATCG-3'	5'-GAGATTCACGAGCATGAGGGCAAGTCTGTTGC TTGAATTGAATTGAATTGAAATCGATAG-3'
PGK1 terminator-rev	5'-TCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGCAGGAAGAATACACTATACTGG-3'		

Table 2.2 (cont.) List of primers used in pathway construction

	SL04	SL05	SL06
PYK1 promoter-for	5'-TCACGACGTTGTAAAACGACGGCCAGTGAGCGCGCTAATACGCAATGCTACTATTTTGG-3'		
PYK1 promoter-rev	5'-GAGGGATAGAATGGAGGAGAGAACGCCAGTTCATCCATTGTGATGATGTTTTATTG-3'		
glucosidase-for	5'-CAAGACACCAATCAAAACAAATAAAACATCATCACAATGGATGAACTGGCGTTC-3'		
glucosidase-rev	5'-ATTGGAGACTTGACCAAACCTCTGGCGAAGAAGTCCAAAGCTCTATTGCACCTTCGGGAG-3'		
adh1 terminator-for	5'-AGCTGCCCCCTTCACGCAGCGCTCCCGAAGGTGCAATAGAGCTTTGGACTTCTTCGCCAG-3'		
adh1 terminator-rev	5'-TGGAAGAGTAAAAAAGGAGTAGAAACATTTTGAAGCTATCATGCCGGTAGAGGTGTGGTC-3'		
TEF1 promoter-for	5'-TAGCATGAGGTCGCTCTTATTGACCACACCTCTACCGGCATGATAGCTTCAAAATGTTTC-3'		
TEF1 promoter-rev	5'-CTTCTCGGTGCTGGCCCCGTCATGGGA GCCGTGAGACGACATTTTGTAAATAAAA CTTAG-3'	5'-ATGGGCACCCATGGCCTCCTTTTCGTTTATGCT GTGAGCCATTTTGTAAATAAAACTTAG-3'	5'-GTCGACGGCCTGAGCCACGGGCTTCTTGTTGA AGATGCCCATTTTGTAAATAAAACTTAG-3'
transporter-for	5'-AGAAAGCATAGCAATCTAATCTAAGTT TTAATTACAAAATGTCGTCTCACGGCTCC CATG-3'	5'-AAAGCATAGCAATCTAATCTAAGTTTAAATTA CAAAATGGCTCACAGCATAAACGAAAAG-3'	5'-AGAAAGCATAGCAATCTAATCTAAGTTTAAAT TACAAAATGGGCATCTTCAACAAGAAGC-3'
transporter-rev	5'-AAAGAAAAAAATTGATCTATCGATTTC AATTCAATTCAATCTAAGCAACGATAGC TTCGG-3'	5'-AAAAGAAAAAAATTGATCTATCGATTTC CAATTCAATCTAAATTGTAACCTTCTCG-3'	5'-AAAAAATTGATCTATCGATTTC AATTCAAGCAACAGACTTGCCCTCATGC-3'
PGK1 terminator-for	5'-CCAGGCCGACGGCCATGTGTCCGAAG CTATCGTTGCTTAGATTGAATTGAATTGA AATCG-3'	5'-CACCATGGGAGCGCCGATGACGAGAAAGTT ACAATTTAGATTGAATTGAATTGAAATCG-3'	5'-GAGATTACGAGCATGAGGGCAAGTCTGTTGC TTGAATTGAATTGAATTGAAATCGATAG-3'
PGK1 terminator-rev	5'-TCACTAAAGGGAACAAAAGCTGGAGCTCCACCGCGGTGCAGGAAGAATACACTATACTGG-3'		

2.7 Figures

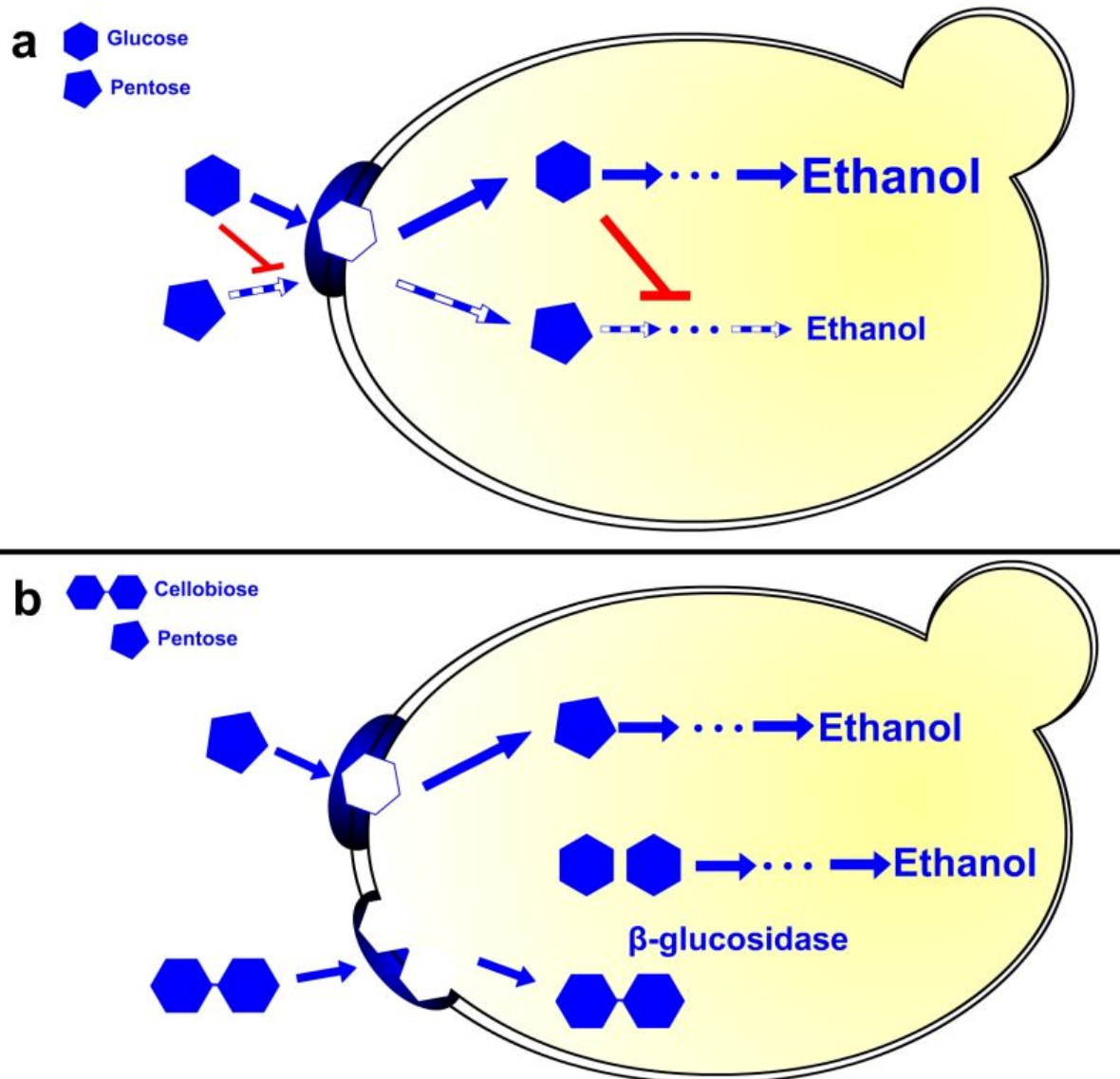


Figure 2.1 Schematic of glucose repression mechanism in co-fermentation of glucose and pentose sugars (a); proposed glucose de-repression mechanism of the strain co-expressing a cellobiose transporter and a β -glucosidase (b).

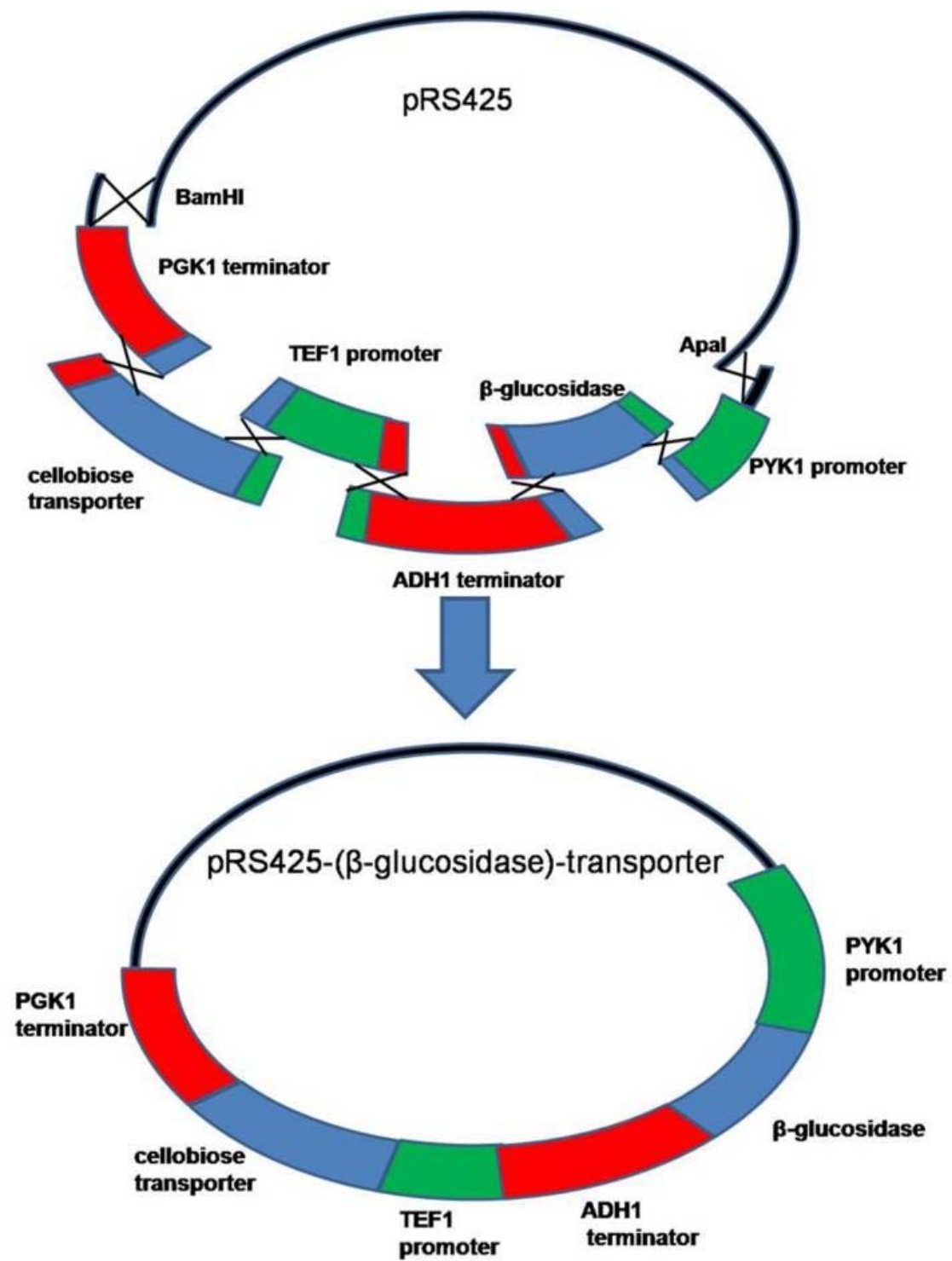


Figure 2.2 Scheme of plasmid construction

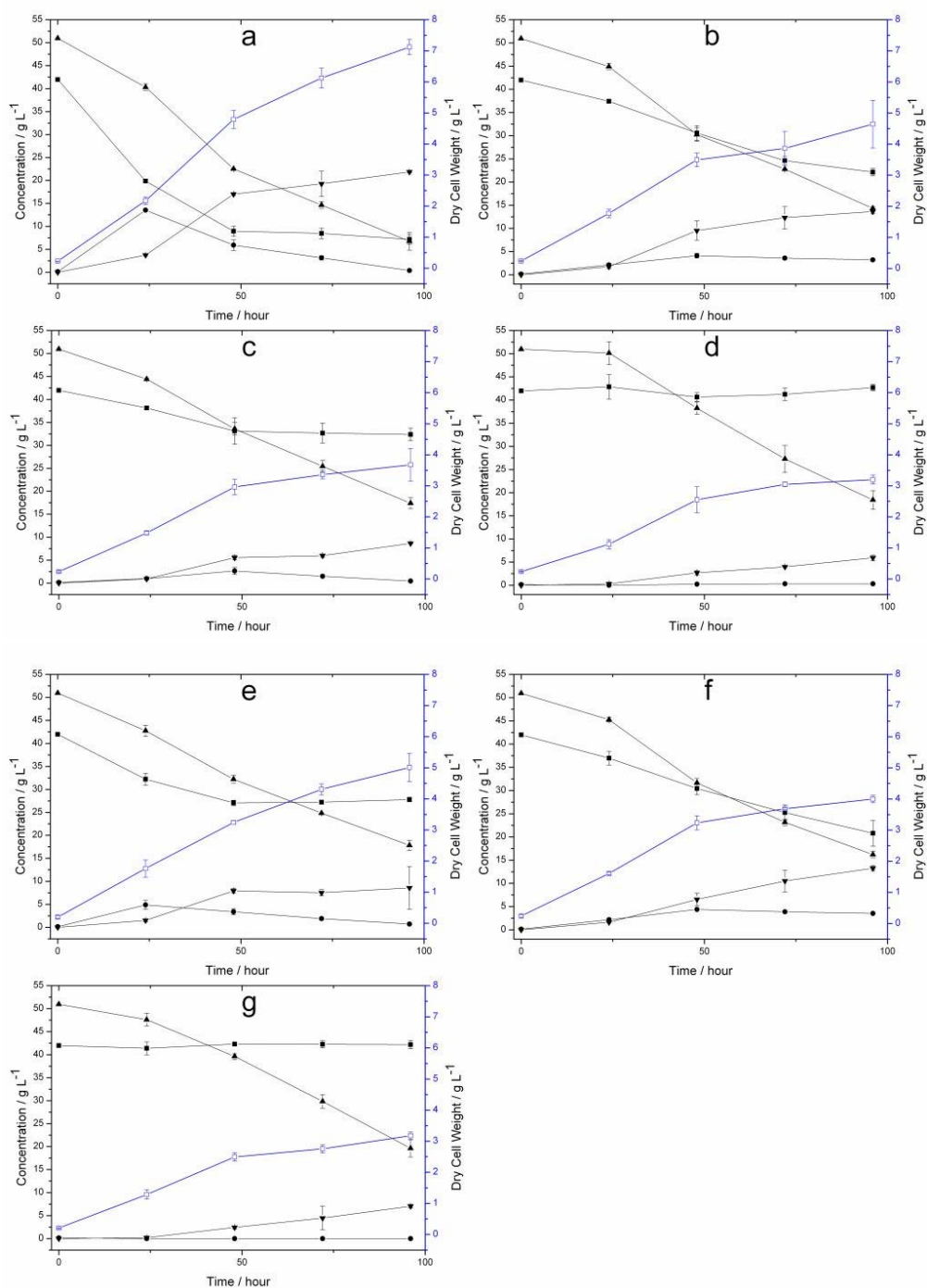


Figure 2.3 Concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and dry cell weight (□) in the cofermentation of 40 g L⁻¹ cellobiose and 50 g L⁻¹ xylose of SL01 (a), SL02 (c), SL03 (e), SL04 (b), SL05 (d), SL06 (f), and SL00 (g), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

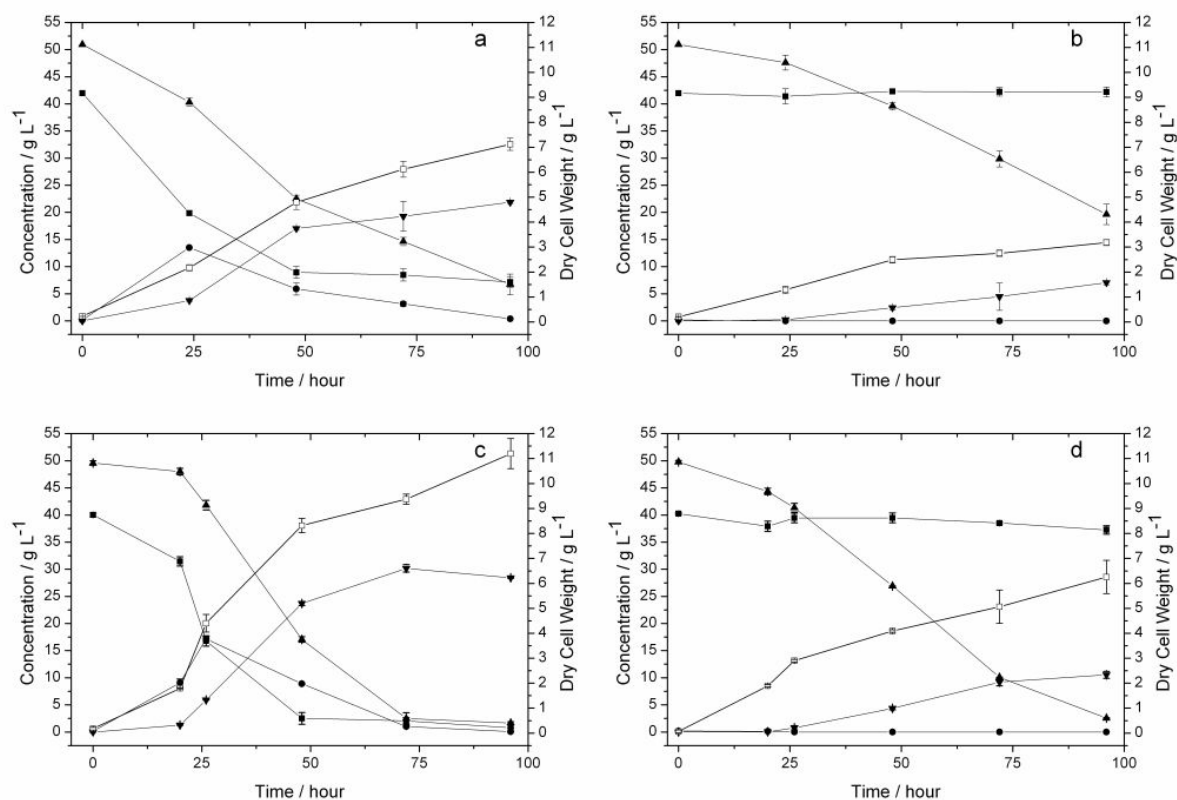


Figure 2.4 Concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and dry cell weight (□) of strains SL01 (a, c) and SL00 (b, d) in YPA medium supplemented with 40 g L⁻¹ cellobiose and 50 g L⁻¹ xylose in shake-flasks (a, b) and bioreactors (c, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

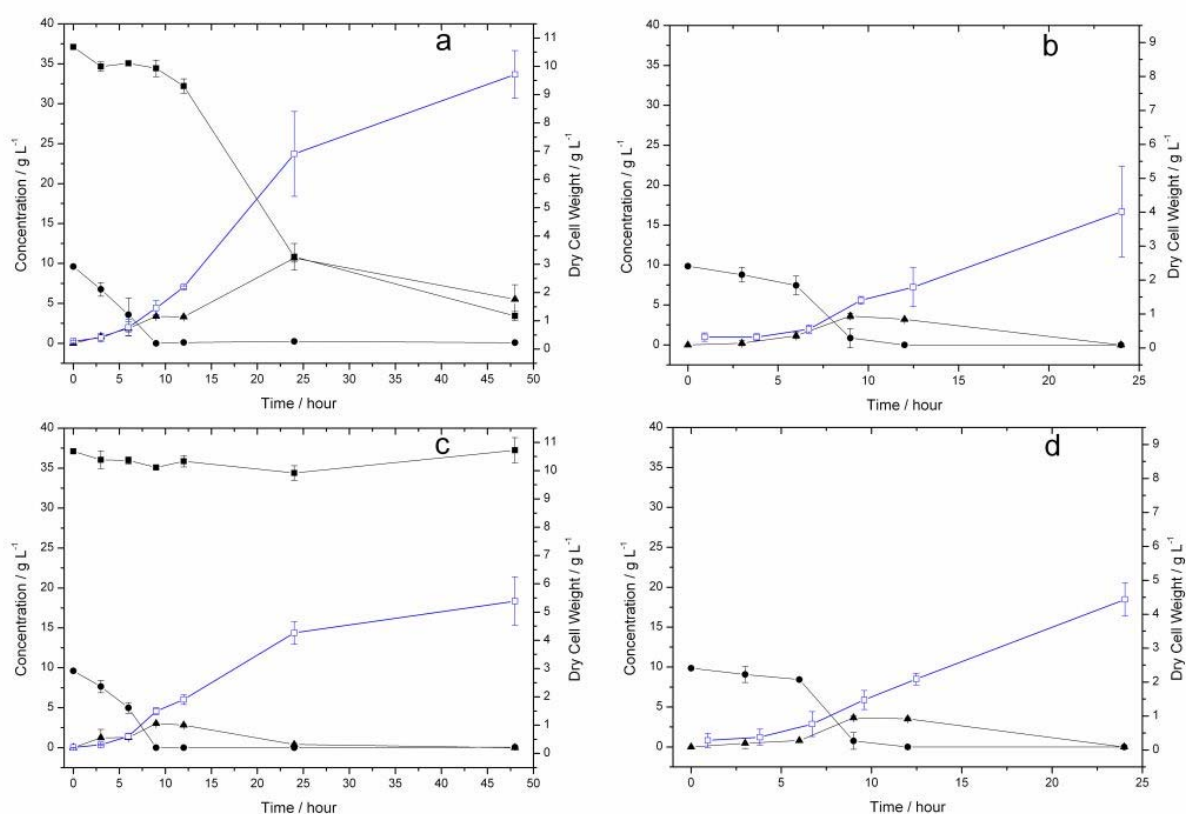


Figure 2.5 Concentrations of cellobiose (■), glucose (●), ethanol (▲), and dry cell weight (□) of SL01 (a, b) and SL00 (c,d) in the co-fermentation of 40 g L⁻¹ cellobiose and 10 g L⁻¹ glucose (a, c), or 10 g L⁻¹ glucose (b, d), plotted as a function of time. Error-bars indicate standard deviations of triplicate samples.

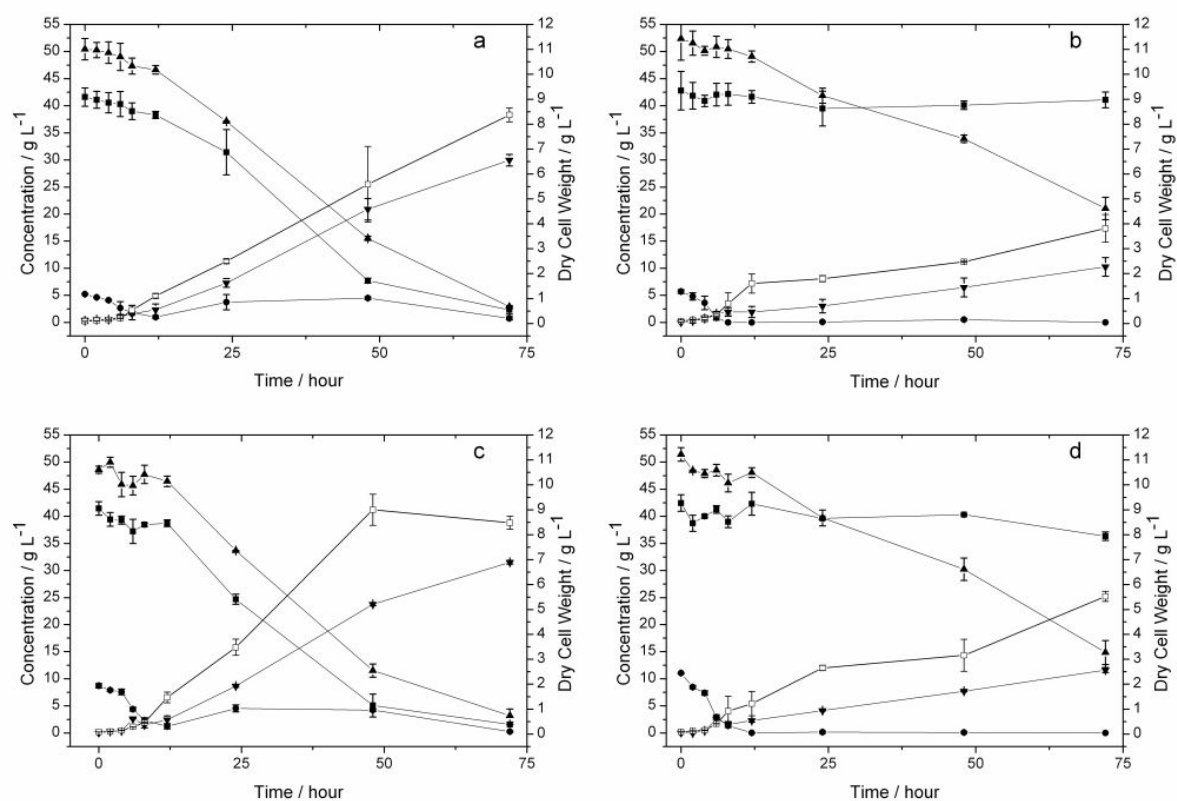


Figure 2.6 Concentrations of cellobiose (■), glucose (●), D-xylose (▲), ethanol (▼), and dry cell weight (□) of strains SL01 (a, c) and SL00 (b, d) in YPA medium supplemented with 5 g L^{-1} glucose–40 g L^{-1} cellobiose–50 g L^{-1} xylose (a, b) or 10 g L^{-1} glucose–40 g L^{-1} cellobiose–50 g L^{-1} xylose (c, d) in bioreactors, plotted as a function of time. Error-bars indicate standard deviations of duplicate samples.

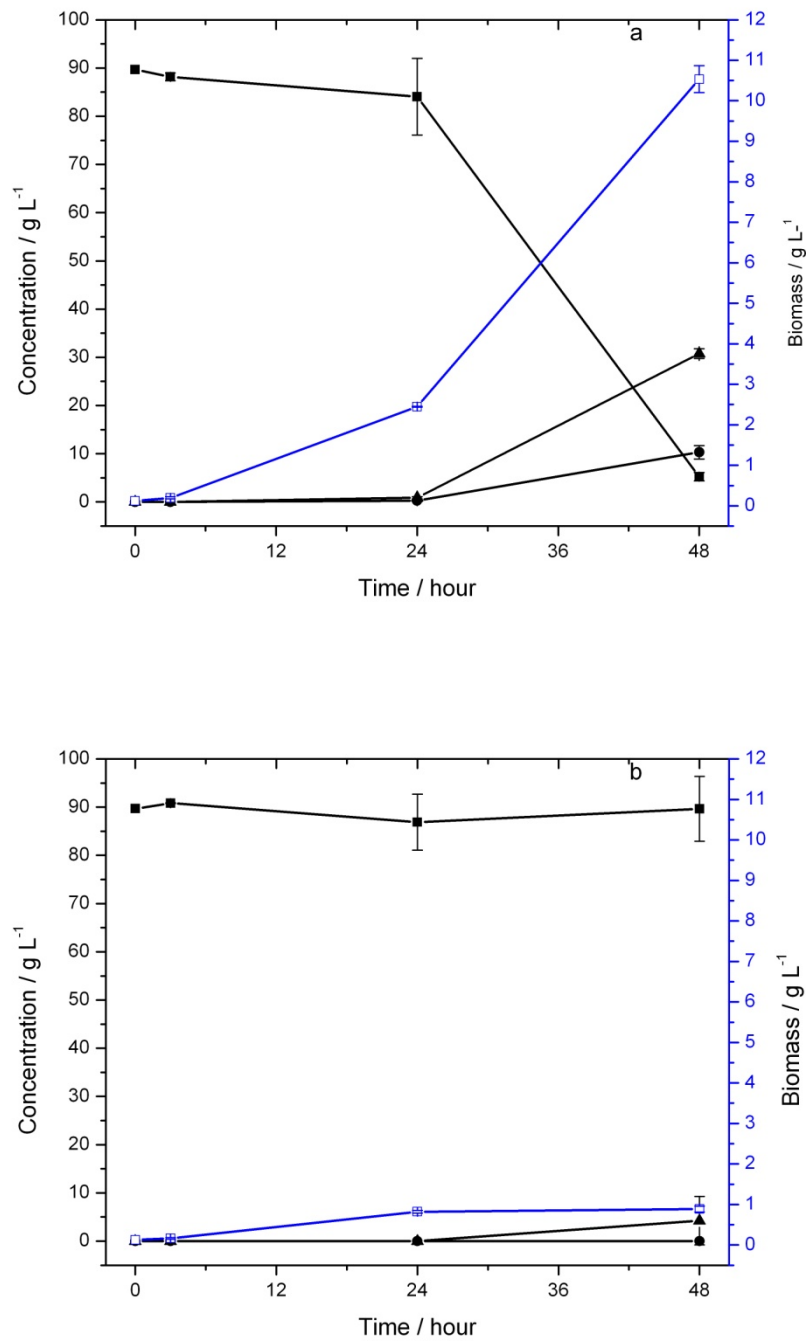


Figure 2.7 Concentrations of cellobiose (■), glucose (●), ethanol (▲), and dry cell weight (□) of strains SLI01 (a) and SLI00 (b) in YPA medium supplemented with 80 g L⁻¹ cellobiose in shake-flasks, plotted as a function of time. Error-bars indicate standard deviations of duplicate samples.

Chapter 3. Investigating the Role of Aldose 1-Epimerases in Cellobiose Utilization

3.1 Introduction

As mentioned in Chapter 2, in the fermentation of cellobiose, it was found that a small amount of glucose was accumulated in the middle of fermentation. Although no glucose was supplemented at the beginning of fermentation, a glucose peak was detected by HPLC analysis of the culture broth. Specifically, in shake-flask fermentation of the laboratory strain SL01, with 40 g L⁻¹ cellobiose and 50 g L⁻¹ xylose, glucose concentration reached a maximum of 12 g L⁻¹ in the middle of fermentation, and in the bioreactor study, this value achieved 17 g L⁻¹. Despite of such high glucose concentration, no obvious glucose repression was observed in all cultivations, which was different from those glucose repression studies reported in the literature [13, 17, 19, 20].

Cellobiose is composed of two β -glucoses and when it is cleaved by a β -glucosidase, only β -glucose is produced. In aqueous solution, there are two anomers of glucose, α -glucose and β -glucose, which maintain a swift equilibrium between these two compounds [62-65]. α -Glucose and β -glucose are two predominant pyranose structures, which differ from each other in the configuration of the hydroxyl group at carbon-1 of the ring [63]. It was hypothesized that β -glucose is not preferred in glycolysis reaction whereby it cannot activate glucose repression. The hypothesis about the preference between α -sugar and β -sugar is supported by a galactose utilization study: although α -glucose is phosphorylated by glucokinase in glycolysis, β -galactose has to be transformed to α -galactose before

phosphorylation by galactokinase [62]. In contrast, glucose and galactose dehydrogenases exhibit specificity for the β -form of their respective sugars [62, 66, 67].

In general, in traditional bioethanol production processes, cellobiose is converted to β -glucose in the sugar hydrolysis step, where part of β -glucose is converted to α -glucose swiftly to maintain the equilibrium between two forms of glucose; then glucose is transported into *S. cerevisiae* to enter the glycolysis pathway and finally produce ethanol. In contrast, in our new strategy, cellobiose is transported into *S. cerevisiae* directly by a cellobiose transporter and *in vivo* only β -glucose is produced by β -glucosidase; and it takes more time to convert β -glucose to α -glucose inside yeast cells. In fact, it has been proven that though the anomers will interconvert in water, the rate of interconversion in the cytoplasm does not seem to be sufficient enough to provide the needs of metabolic pathways. Indeed, the conditions prevailing in the cytoplasm may be very repressive for interconversion that very little spontaneous interconversion occurs. Thus we can get to the conclusion that the accumulated glucose is from excess β -glucose, which showed limited effect in glucose repression.

Excess β -glucose not only limits sugar consumption rate thus limits ethanol productivity, but at the same time extracellular β -glucose released from yeast cell to culture medium may induce contamination from other glucose-assimilating microorganism and inhibits cellobiose utilization. So the excessive β -glucose accumulation due to inefficient conversion between β - and α - forms of glucose is a limiting factor for efficiently utilizing cellobiose in engineered *S. cerevisiae* strains.

Considering that natural conversion between β -glucose and α -glucose inside yeast cell is too slow to accept, it is reasonable to get the idea of introducing heterologous enzymes which are capable of catalyzing the conversion reaction between β -glucose and α -glucose. Engineered strain should show much high sugar consumption rate and ethanol productivity due to efficient utilization of glucose. Aldose 1-epimerase, or named mutarotase, is able to catalyze interconversion between α -anomers and β -anomers of hexose sugars, such as glucose or galactose. It has been found in a wide range of organisms including bacteria, fungi, plants, and mammals such as human too. From published work since now, aldose 1-epimerase (AEP) functional study mainly focuses on lactose utilization to convert β -galactose to α -galactose. There are also epimerase genes existing in wild type *S. cerevisiae*. One typical aldose 1-epimerase is GAL10, distinct from other aldose 1-epimerases: it is a fusion protein to another enzyme of the Leloir pathway, named UDP-glucose-4-epimerase. Our purpose is to analyze the function of AEP in *S. cerevisiae*, finally trying to facilitate cellobiose utilization efficiently.

3.2 Results

3.2.1 Genome mining of candidate aldose 1-epimerase genes

To search for active aldose 1-epimerase genes, GAL10 was selected as a model protein to identify more epimerases sharing high sequence identity using BLAST search (<http://www.ncbi.nlm.nih.gov/>). Resultant genes include *YHR210c* and *YNR071c*, both

existing in wild type *S. cerevisiae*, as well as *NCU09705* which is a gene in *N. crassa*. All the genes were selected for further analysis.

3.2.2 Overexpression of Aldose 1-Epimerase in Cellobiose Assimilating Strain

Two AEP genes were amplified respectively into multi-copy shuttle vector plasmid pRS424 (New England Biolabs, Beverly, MA) with a HXT7 promoter and a HXT7 terminator using the DNA assembler method[60]. Yeast plasmids isolated from transformants were retransferred into *E. coli* DH5 α . Isolated *E. coli* plasmids were first checked by diagnostic PCR using the primers originally used to amplify the AEP genes.

After cloning, *E. coli* plasmids containing AEP genes were transformed into cellobiose assimilating strain SL01; control strain with blank pRS424 plasmid was also constructed (Table 3.1). Resultant strains were then tested using YPA medium supplemented with 80 g L⁻¹ cellobiose in shake-flasks.

As shown in Figure 3.1, comparing to the control strain, AEP overexpression strains showed slightly improved biomass growth, cellobiose consumption rate, ethanol production, and decreased glucose accumulation. At 36h, the cellobiose consumption rate was enhanced by 27%, from 1.17 g L⁻¹ h⁻¹ to 1.49 g L⁻¹ h⁻¹ in the YHR210c-overexpressing strain AEP1; and the cellobiose consumption rate was enhanced by 21%, from 1.17 g L⁻¹ h⁻¹ to 1.42 g L⁻¹ h⁻¹ in *NCU09705*-overexpressing strain AEP2; for ethanol production rate, it was either enhanced by 11%, from 0.15 g L⁻¹ h⁻¹ to 0.17 g L⁻¹ h⁻¹, or enhanced by 20%, from 0.15 g L⁻¹

h^{-1} to $0.19 \text{ g L}^{-1} \text{ h}^{-1}$ in AEP1 and AEP2 strains respectively; glucose accumulation rate was decreased in both AEP1 and AEP2 strains: at 36 h, AEP1 strain showed a 20 % less glucose accumulation, from 7.2 g L^{-1} in control AEP0 to 5.7 g L^{-1} in AEP1 strain, while AEP2 strain showed a 9.1 % less glucose accumulation, from 7.2 g L^{-1} in control AEP0 to 6.6 g L^{-1} . Besides, at the end of fermentation, AEP1 strain showed a 32% less glucose accumulation while AEP2 strain showed a 9.5% less glucose accumulation than the control strain (5.8, 7.8 and 8.6 g L^{-1}). It is reasonable that most advantages from overexpression strains exhibiting in the middle of fermentation, because β -glucose can still be converted to α -form without AEP catalysis, but with a much slower rate. So in the middle of fermentation AEP showed high activity and catalyzed the interconversion in engineered strain, but at last in control strain interconversion can still be completed by natural conversion. As a result, the main advantage of AEP overexpression is to accelerate cellobiose fermentation rate and reduce the chance to get contamination due to extracellular glucose.

Generally, AEP overexpression has been proven to be a plausible approach to accelerate cellobiose assimilating process. The overexpressed AEP plays an important role in glucose interconversion in the middle of fermentation, engineered AEP enzymes are asserted to facilitate cellobiose assimilation to a much high extent. Besides, the AEP overexpression assay also proves that glucose interconversion is a key step during the ethanol production process which calls for more attentions and research.

3.2.3 Cellobiose Fermentation Analysis of AEP-Disrupted Cellobiose

Assimilating Strains

There have been at least three AEP genes identified in wild type *S. cerevisiae* from BLAST search: *YHR210c*, *YNR071c* and *GAL10*. We hypothesized that with disrupted aldose 1-epimerase activity, a cellobiose assimilating *S. cerevisiae* strain should exhibit repressed sugar consumption and enhanced glucose accumulation, which corresponds with the AEP overexpression assay result. Besides, with the comparison of different AEP knockout strains, it will be straightforward to figure out the dominant aldose 1-epimerase in wild type *S. cerevisiae*. Also it is concluded that with all known AEP genes disruption, it is possible to obtain an engineered cellobiose assimilating strain with little or no cellobiose consumption ability.

Strains lacking AEP genes were constructed with the loxP-kanMX-loxP/Cre recombinase system and the short flanking homology PCR technology [68]. The primers used for the replacement PCR constructs are listed in Table 3.2.

3.2.3.1 HZ3001 Strain Lacks *YNR071c* Gene

The *AEP* gene knockout construction was accomplished based on the HZ3001 strain, which was the parent strain of cellobiose assimilating strain SL01. After multiple knockout assays and sequencing assays, it was found that the HZ3001 strain, different from strain listed in the NCBI database, had no *YNR071c* homologous gene in the chromosome.

3.2.3.2 Cellobiose Fermentation Analysis in *AEP* Disrupted Strain SL01

Based on sequencing result of the *YNR071c* gene, there were only two known AEP genes in the SL01 strain: *YHR210c* and *GAL10*. Finally three AEP disrupted strains were constructed, including two single knockout strains ΔYHR and ΔGAL , and one double knockout strain ΔHG . Cellobiose cultivation was tested based on these three knockout strains and one wild type SL01 strain was used as a control. The resultant strains were then tested using YPA medium supplemented with 80 g L⁻¹ cellobiose in the shake-flasks.

As shown in Figure 3.2, the ΔYHR strain exhibited repressed cellobiose consumption, ethanol production during the fermentation process; biomass production was also repressed in the end of the fermentation. In contrast, the ΔGAL strain exhibited a trend totally different from expected—the cellobiose consumption, biomass production, and ethanol production were all higher than the control strain. This indicates GAL10 regulation is more complicated than expected. Due to the repression of the YHR gene knockout and the improvement of the GAL10 gene knockout, double knockout strain ΔHG showed a fermentation performance on the same level with the control strain.

Based on the data of the *GAL10* strain and the GAL10 overexpression strain, we concluded that the GAL10 activity is related to the expression level: sugar consumption got improved with both low and high concentrations of GAL10 enzyme. Instead, the YHR enzyme expression had a simple linear correlation with the epimerase activity. Thus, GAL10 plays a key role in the aldose 1-epimerase activity in the yeast strain.

It was observed that the AEP double knockout strain Δ HG still had the ability to utilize cellobiose and glucose accumulation was not as high as expected, which indicates that besides YHR210c and GAL10, there might still be other unknown proteins with aldose 1-epimerase activity.

3.2.3.3 Cellobiose Fermentation Analysis in AEP Disrupted YKO Strains

YKO strains, in short for Yeast Knock-Out strains, are commercially available *S. cerevisiae* strains developed by the Saccharomyces Genome Deletion Project (insert citation). YKO strains include more than 6,000 knock-out strains covering 96% of the yeast genome, which renders a unique tool for functional analysis of the yeast genes. Parent strains of YKO include BY4741 and BY4742, which are two mating types of the same *S. cerevisiae* strain. Using PCR-based gene knockout technology, target genes are replaced by an antibiotic kanamycin resistant cassette, which functions as a selection marker too. Due to the lack of genome sequencing information in the HZ3001 strain and the lack of YNR071c gene, YKO strains with disrupted aldose 1-epimerase genes which we purchased from Open Biosystems (Huntsville, AL) were utilized for analysis using YPA medium supplemented with 80 g L⁻¹ cellobiose in shake-flasks.

In the background of BY4741 strain with the mating type of a, three single knockout strains Δ YHR, Δ YNR, Δ GAL and the wild type strain were introduced with a plasmid harboring the cellobiose utilizing pathway and then tested with cellobiose cultivation (Figure 3.3). It was found that the Δ YHR and Δ YNR strains showed better cellobiose consumption

than the wild type strain, while the ΔGAL strain showed hardly any growth on the cellobiose medium. As to biomass production represented by OD₆₀₀ absorbance, biomass production was enhanced from 24.3 in the wild type strain to 37.0 in the ΔYHR strain, while in the ΔYNR strain it was enhanced from 24.3 to 30.1, which showed 52.3% or 24.1% more biomass production than the wild type strain respectively. As to cellobiose consumption, in the ΔYHR strain, the cellobiose consumption rate was enhanced from 0.78 g L⁻¹ h⁻¹ to 1.26 g L⁻¹ h⁻¹, while in the ΔYNR strain it was enhanced from 0.78 g L⁻¹ h⁻¹ to 1.25 g L⁻¹ h⁻¹, which showed either 60.3% or 59.9% improvement than the wild type strain respectively. As to glucose accumulation, during the whole fermentation process, the ΔYHR and ΔYNR strains showed higher glucose accumulation than the wild type strain, but it was in proportional to cellobiose consumption in ΔYHR and ΔYNR strains. Thus, there was no obvious difference in glucose accumulation observed. However, compared to the HZ3001 strain used in the AEP disruption assay, glucose accumulation was much less in the BY strains. Only one-third of glucose accumulation was observed in BY strain, which might be due to different genetic backgrounds between these two strains. Ethanol production was correlated to cellobiose consumption and the ΔYHR strain showed the highest ethanol productivity among all strains.

However, in the ΔGAL strain, only slight cell growth was observed (maximum OD₆₀₀ = 4.4), which resulted from little cellobiose consumption during the entire fermentation process. At the end of fermentation, only 12.0 g L⁻¹ cellobiose reduction was found, which might be still due to cellobiose hydrolysis in the aqueous solution. As a result, no glucose accumulation or ethanol production was found. Thus, it was concluded that the GAL10

epimerase plays a dominating role in cellobiose utilization in the BY strain that without *GAL10* expression, the aldose 1-epimerase function cannot be executed by other homologous enzymes.

A similar case occurred in the BY 4742 (mating type α) Δ *GAL* strain (Figure 3.4). The cell density reached only OD₆₀₀ of 4.4 at the end of fermentation, while less than 5.0 g L⁻¹ cellobiose was consumed. Generally speaking, based on the performance of the Δ *GAL* strain derived from both BY4741 and BY4742, we can conclude that *GAL10* is the key aldose 1-epimerase gene in the BY strain, while *YHR210c* and *YNR071c* only have some marginal effect.

3.2.4 Growth Assay of AEP-Disrupted YKO Strains

To further analyze AEP-disrupted YKO strains, real-time OD was measured on different sugars. 80 g L⁻¹ cellobiose or 20 g L⁻¹ glucose were selected as a sole carbon source for the cell growth assay. A 48-hour growth assay was performed by a temperature-controlled incubator/reader Bioscreen C MBR. Data was collected every 30 minutes.

Using cellobiose as a sole carbon source, the BY4741 Δ *GAL* strain grew to OD = 1.5 during the first 18 hours and then maintained this value as a maximum during all the following cultivation time. In comparison, the Δ *YHR* and Δ *YNR* strains reached OD = 2 as a maximum (Figure 3.5). The BY4742 Δ *GAL* strain also followed the same trend, growing to OD = 1.5 during the first 18 hours but maintained this value as a maximum during all the following cultivation time, while all other strains reached OD = 2 as a maximum (Figure 3.6).

Compared to fermentation with glucose as a sole carbon source, in both the BY4741 (Figure 3.7) and BY4742 genetic backgrounds (Figure 3.8), the ΔGAL strain showed highest growth in 48hours, which was totally different from the fermentation performance based on cellobiose. In addition, the ΔYHR and ΔYNR strains, which showed high biomass production in cellobiose medium, showed low biomass production compared to the ΔGAL strain and the wild type strain. This suggests that the functional role of *GAL10* is dominant. Furthermore, the totally opposite performance based on the cellobiose or glucose medium indicates that aldose 1-epimerase regulates certain key reactions in the cellobiose-glucose utilization pathways.

Besides cellobiose and glucose, an artificial disaccharide, *p*-nitrophenyl-D-glucoside(PNPG), which was used to test the glucosidase activity, was utilized to analyze the aldose 1-epimerase activity. PNPG was composed of one fermentable glucose molecule and one non-fermentable nitrophenyl group, both of which were attached together by either α or β -glucolytic bond. PNPG cannot be utilized by *S. cerevisiae* directly but after digested by glucosidase, soluble glucose can be released and the remaining nitrophenyl group will show a clear yellow color that is easy to detect by a spectrophotometer or eyes directly. Here we hypothesized that with PNPG as sole carbon source, the AEP-disrupted BY strains will be able to convert PNPG to β -glucose but the conversion between β -glucose and α -glucose was limited due to the lack of aldose 1-epimerases. Thus, by analyzing repressed growth performance of different AEP-disrupted strains, it would be possible to analyze and to compare the activity of various aldose 1-epimerases.

As shown in Figure 3.9 and Figure 3.10, we discovered that although with various growth curves, PNPG fermentation assay cannot give a clear illustration of the AEP activity. As the maximal OD in this culture was below OD 1, it was possible that fermentation was inadequate. Thus the deficient performance cannot reflect the growth trend in different strains. Besides, in HPLC analysis of the supernatant collected from the PNPG fermentation broth, little reduction of the PNPG concentration was observed, which means though PNPG is a good substrate for *in vitro* glucosidase activity assay, it may not be a good carbon source for cell growth assay.

3.2.5 AEP Enzymatic Assay

To further compare the activity of various AEPs directly, the specific activity of mutarotase was determined using cellobiose assimilating BY strains growing on cellobiose or glucose as a sole carbon source.

Figure 3.11 showed the specific activity of mutarotase in the BY4742 AEP disrupted strains harboring cellobiose assimilating plasmids which were cultured with glucose as a sole carbon source. In the BY4742 ΔYHR strain, the specific mutarotase activity was 5.74×10^{-7} $\mu\text{mol}/\text{min}/\text{mg}$ total protein, which was on the same level with that in the ΔYNR (6.30×10^{-7} $\mu\text{mol}/\text{min}/\text{mg}$ total protein). The ΔGAL strain and the wild type strain showed approximately activities, 2.28×10^{-7} $\mu\text{mol}/\text{min}/\text{mg}$ total protein and 1.95×10^{-7} $\mu\text{mol}/\text{min}/\text{mg}$ total protein, respectively. We hypothesized that under glucose condition, the expression of GAL10 mutarotase was inhibited by the YHR or YNR mutarotases. As a result, in the ΔYHR and

ΔYNR strains, disrupted regulation resulted in overexpression of the GAL10 mutarotase, exhibiting high activity. In the ΔGAL strain, the GAL10 mutarotase possessing the highest activity was disrupted and only the YHR and YNR mutarotases were expressed, which induced a much smaller activity. Based on this result, we concluded that GAL10 is the dominant mutarotase among all three mutarotases in *S. cerevisiae*, but with limited requirement of mutarotase, its expression is regulated by the other two mutarotases.

Figure 3.12 showed the specific mutarotase activities in the BY4742 AEP disrupted strains harboring the cellobiose assimilating plasmids which were cultured with cellobiose as a sole carbon source. The specific mutarotase activity of the ΔYHR strain was $3.9E-07$ $\mu\text{mol}/\text{min}/\text{mg}$ total protein, while the specific mutarotase activity of the ΔYNR strain was $7.64 E-07$ $\mu\text{mol}/\text{min}/\text{mg}$ total protein and the wild type strain exhibited the highest activity of $8.54E-07$ $\mu\text{mol}/\text{min}/\text{mg}$ total protein. The ΔGAL strain showed limited growth in the cellobiose medium, thus no activity was detected. Combined with the activities measured in the glucose medium, it was concluded that the dominant GAL10 mutarotase expression was activated when β -glucose conversion was needed, and the YHR mutarotase possessed a higher activity than the YNR mutarotase because the ΔYNR strain showed a higher activity than the ΔYHR strain.

Figure 3.13 showed the specific mutarotase activities in the BY4742 strain harboring the cellobiose assimilating plasmid and the AEP overexpressed in a multi-copy plasmid which was cultured in glucose. Unexpectedly, the mutarotase activity in all three

AEP-overexpressed strains were on the same level with the wild type strain, which means mutarotase regulation functions in overexpression strains.

3.3 Discussion

There are at least three aldose 1-epimerase genes in *S. cerevisiae*, *YHR210c*, *YNR071c* and *GAL10*. AEP catalyzes interconversion between α -glucose and β -glucose, thus relieving the imbalanced β -glucose accumulation in the cellobiose assimilating strains, which may be the reason of extracellular accumulation of glucose in cellobiose fermentation. Overexpression of the AEP genes in the cellobiose assimilating strains should improve the cellobiose utilization rate and AEP-disrupted strains were concluded to present repressed sugar consumption ability.

From genome mining and DNA sequencing, it was found that the HZ3001 strain does not have the *YNR071c* gene in its chromosome, which has not been reported. The complexity and uncertainty due to genome difference adds more difficulties to the AEP study.

From the overexpression assay in the SL01 strain, it was found that overexpression of all aldose 1-epimerase genes were able to facilitate cellobiose consumption. As a result, the fermentation time could be shortened and ethanol productivity could be improved. The benefits of AEP overexpression mainly occurred in the middle of fermentation when the cellobiose consumption rate was high but natural interconversion in the aqueous environment between α -glucose and β -glucose was deficient.

Two batches of AEP-disrupted strains were constructed and tested, including the SL01 strain and the BY4741/4742 strain. Due to the lack of the *YNR071c* gene, two single

knockout strains and one double knockout strain were constructed based on SL01 while three single knockouts of the BY strains were evaluated. The performance of the AEP knockout strains has been proved to be delicate and complicated. In the cellobiose cultivation of the SL01 AEP knockout strains, the ΔGAL strain showed improved sugar consumption while other strains reported repressed or similar ability to utilize cellobiose compared to the wild type strain. Though the result is different from what was expected, we can still get an important conclusion that the *GAL10* gene is the key factor for glucose interconversion and cellobiose utilization. In the YKO strains derived from BY4741/4742, cellobiose fermentation was tested. In both mating types, the ΔGAL strain showed almost no ability to utilize cellobiose, which proves the significance of the *GAL10* gene.

In the continuous cell growth assay, using cellobiose, glucose or artificial disaccharide PNPG as a sole carbon source, the cell growth of the BY AEP knockout strains was analyzed. The ΔGAL strain showed a high ability to utilize glucose, but low ability to utilize cellobiose. This can be a promising evidence that *GAL10* catalyzes both sugar conversion and utilization in the cellobiose assimilating strains.

PNPG was used to test the aldose 1-epimerase activity as indicated by cell growth. However, PNPG was a good substrate for the *in vitro* enzyme activity assay but not suitable for fermentation as a pure carbon source. It was found that no cellobiose assimilating strains could grow up on the PNPG medium.

To get a direct view of the expression of the AEP enzymes in the cellobiose assimilating strain, specific mutarotase activity assay was performed in the BY4742 AEP

disrupted strains and the AEP overexpression strains. A complex regulation system was observed: when glucose interconversion is needed, the expression of the dominant mutarotase GAL10 is activated; and when glucose is sufficient, low activity mutarotases such as YHR and YNR are expressed but the GAL10 expression is repressed.

3.4 Conclusions and Outlook

Extracellular glucose accumulation was observed in the cellobiose fermentation, which may be due to inefficient conversion between β -glucose and α -glucose. It was also proved that α -sugar is preferred in metabolic reactions to some extent. Although interconversion between two anomers can be executed swiftly in aqueous environment, it is more difficult to complete this reaction inside *S. cerevisiae* cells. To facilitate cellobiose consumption and eliminate possible contamination, glucose interconversion should be accelerated. One reasonable approach is to introduce aldose 1-epimerase which can catalyze the interconversion into cellobiose assimilating *S. cerevisiae*. Overexpression of related aldose 1-epimerase genes has been proved to show some benefits on the cellobiose consumption rate. However, to study the AEP function, analysis of the AEP disrupted strains showed much more complicated performance than expected. From the results we obtained so far, we can conclude that *GAL10* is the dominant aldose 1-epimerase gene which can regulate sugar utilization in a cellobiose assimilating strain. The totally different trend in cellobiose fermentation and glucose fermentation shows that cellobiose-glucose utilization is a system more complicated than we expected, and aldose 1-epimerase working together with other proteins regulates cellobiose and glucose utilization by a delicate mechanism. The study

about aldose 1-epimerase may lead to significant improvement in sugar metabolism, which will facilitate bioethanol production.

3.5 Materials and Methods

3.5.1 Strains, Media and Cultivation Conditions

Saccharomyces cerevisiae L2612 (*MATa leu2-3 leu2-112 ura3-52 trp1-298 can1 cyn1 gal+*) was a gift from Professor Yong-su Jin[59]. BY4741 (*MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0*), BY4742 (*MATa ; his3Δ1; leu2Δ0; lys2Δ0; ura3Δ0*), and corresponding YKO strains (Name listed in Table 3.3) were purchased from Open Biosystems Products, (Huntsville, AL). *Saccharomyces cerevisiae* Classic was purchased from Homebrew full name (city, state). *Escherichia coli* DH5α was used for recombinant DNA manipulation. Yeast strains were cultivated in synthetic dropout media to maintain plasmids (0.17% Difco yeast nitrogen base without amino acids and ammonium sulfate, 0.5% ammonium sulfate, 0.05% amino acid dropout mix). YPA medium (1% yeast extract, 2% peptone, 0.01% adenine hemisulfate) with 2% D-glucose was used to grow yeast strains. *E. coli* strains were grown in Luria broth (Fisher Scientific, Pittsburgh, PA). *S. cerevisiae* strains were grown in un-baffled shake-flasks at 30 °C and 250 rpm for aerobic growth, and 30 °C and 100 rpm for oxygen limited condition. *E. coli* strains were grown at 37 °C and 250 rpm. All chemicals were purchased from Sigma Aldrich or Fisher Scientific.

3.5.2 Strain and Plasmid Construction

To integrate the AEP genes into a multi-copy plasmid, corresponding AEP genes were PCR-amplified and cloned together with the HXT7 promoter and the HXT7 terminator into pRS424 plasmid using the DNA assembler method (insert citation). The resulting plasmid was then transferred into *E. coli* DH5 α , which was plated on LB plates containing 100 mg/L ampicillin. Single colonies of the *E. coli* transformants were then inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN). These plasmids were transformed into the HZ3001 strain with pRS425-*cdt-1-ghl-1* plasmid individually to yield AEP1 (containing the plasmid harboring the YHR210c gene from *S. cerevisiae*) and AEP2 (containing the plasmid harboring the NCU09705 gene from *N. crassa*). The empty pRS424 plasmid was transformed into the HZ3001 strain together with the cellobiose-assimilating pRS425-*cdt-1-ghl-1* plasmid to yield the AEP0 strain as a negative control. Yeast transformation was carried out using the standard lithium acetate method[61]. The resulting transformation mixtures were plated on SC-Trp-Leu medium supplemented with 2% glucose. To confirm the proper construction of plasmids using the DNA assembler method, plasmids were isolated from yeast cells using the Zymoprep Yeast Plasmid Miniprep II kit (ZymoResearch, Orange, CA) and then transformed into *E. coli* DH5 α cells. The resulting cells were spread on LB plates containing 100 mg/L ampicillin. Single *E. coli* colonies were inoculated into LB liquid media. Plasmids were isolated from *E. coli* using the QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) and checked by

diagnostic PCR or restriction digestion using EcoRI and HindIII. All restriction enzymes were obtained from New England Biolabs (Ipswich, MA).

To construct single AEP knockout strains in SL01, corresponding ORFs were removed by *loxP-kanMX-loxP* disruption cassette using DNA assembler method. The kanamycin resistance marker was then rescued with *cre*-bearing pSH47 plasmid and the double AEP knockout strain was constructed following the same protocol. Both SL01 AEP-disrupted strains and the YKO strains were transformed with the pRS425--*cdt-1-ghl-1* plasmid individually to enable the cellobiose assimilating ability.

3.5.3 Cellobiose Fermentation in Shake-Flasks

For each AEP overexpression strain, a single colony was first grown up in 2 mL SC-Ura-Leu medium plus 20 g L⁻¹ glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. For each AEP disrupted strain, single colony was first grown up in 2 mL SC-Leu medium plus 20 g L⁻¹ glucose, and then inoculated into 50 mL of the same medium in a 250 mL shake flask to obtain enough cells for mixed sugar fermentation studies. After one day of growth, cells were spun down and inoculated into 50 mL of YPA medium supplemented with 80 g L⁻¹ cellobiose. Starting from an initial OD600 \approx 1, cell cultures were grown at 30 °C at 100 rpm for fermentation under oxygen limited conditions. OD600 readings and cell culture samples were taken at various time points. Sugars and ethanol concentrations were determined using Shimadzu HPLC equipped with a Bio-Rad HPX-87H column (Bio-Rad

Laboratories, Hercules, CA) and Shimadzu RID-10A refractive index detector following the manufacturer's protocol. The HPX-87H column was kept at 65°C using a Shimadzu CTO-20AC column oven. 0.5mM sulfuric acid solution was used as mobile phase at a constant flow rate of 0.6 mL/min. 10 µL of filtered sample was injected into the HPLC system with a Shimadzu SIL-20AC HT auto sampler, and each run was stopped at 25 minutes after the injection. The concentrations of the sugars and ethanol were determined using a standard curve generated using a series of external standards. Each data point represented the mean of duplicate samples.

3.5.4 Continuous Micro-culture Growth Assay

Microculture growth of yeast was performed in the Bioscreen C incubator/plate reader from Thermo Laboratory Systems (Franklin, MA) using the honeycomb plate. Cells were pre-cultured in YPA medium complemented with 20 g L⁻¹ glucose, 80 g L⁻¹ cellobiose or 20 g L⁻¹ PNPD individually in shake-flasks to provide yeast cells in balanced growth to inoculate micro-cultures in the Bioscreen C. Starting at OD~0.2, 200 µL culture medium was transferred to a well with a volume of 350 µl under 30 °C and continuous shaking. OD600 measurements were routinely collected at 30-min intervals.

3.5.5 Mutarotase Activity Assay

The mutarotase activities in cellobiose assimilating *S. cerevisiae* strains were determined using the BY4742 strains. Both AEP-disrupted strains and AEP-overexpressed strains with a cellobiose assimilating pathway were investigated. In the mutarotase activity

assay for the AEP-disrupted strains, cell cultures were grown in tubes filled with 5 mL YPA medium supplemented with 20 g L⁻¹ glucose or 80 g L⁻¹ cellobiose. The culture tubes were grown at 30°C at 250 rpm for 48 hours. And in AEP-overexpressed strains, cell cultures were grown in tubes filled with 5 mL SC medium supplemented with 20 g L⁻¹ glucose. The culture tubes were grown at 30°C at 250 rpm for 48 hours. Cells were resuspended in Y-PER Extraction Reagent (Thermal Scientific, Rockford, IL) following the manufacturer's instructions. Supernatants were then collected for measurement of protein concentration and mutarotase activity.

To determine the total protein concentration, BCA Protein Assay Reagent (Thermal Scientific, Rockford, IL) was used following the manufacturer's instructions. A Synergy 2 Multi-Mode Microplate Reader was used to measure the change of absorbance. Total protein concentration was calculated following the manufacturer's instructions.

To determine the mutarotase activity, a mixture containing 0.34 mM NAD⁺, 0.05U of glucose dehydrogenase and 50 mM Tris hydrochloride (pH 7.2) was made. 820 µL of the mixture was pipetted into a UV cuvette and then 130 µL mutarotase containing solution was added. Then, 50 µL of a 166 µM freshly prepared α-glucose was added to the cuvette and the increase in absorption at 340 nm was recorded for 3 minutes.

3.6 Tables

Table 3.1 AEP genes overexpressed in the SL01 strain

Strain Name	Gene	Source
AEP1	<i>YHR210c</i>	<i>S. cerevisiae</i>
AEP2	<i>NCU09705</i>	<i>N. crassa</i>
AEP0	-	

Table 3.2 List of primers in AEP disruption

AEP overexpression	
YHR-for	5'-CAAAAAGTTTTTTTAATTTTAATCAAAAATGTCAAATAATAAGGCTGGCGGTGAATAT-3'
YHR-rev	5'-GATCATGAATTAATAAAAGTGTTTCGCAAACCTACACCGCAAACGATATCGAGTCTTAGAA-3'
NCU-for	5'-CAAAAAGTTTTTTTAATTTTAATCAAAAATGTCTGACG CAATCGCCTCCTTCATCCCC-3'
NCU-for	5'-CTACTCCTTCCACGCCCTGTACAGGATCTTTTTGCGAACACTTTTATTAATTCATGATC-3'
AEP Knockout	
YHRKO-for	5'-CATTATGTTACTGCAATAAGTAAATTCACCTAAAAAGCCATTCGTACGCTGCAGGTCGAC-3'
YHRKO-rev	5'-AATTTATAGAAAAATAATGCGACAGTGCAAAGTAAACAGCATAGGCCACTAGTGGATCTG-3'
GALKO-for	5'-GAAAATTCAATATAAATGACAGCTCAGTTACAAAGTGAAATTCGTACGCTGCAGGTCGAC-3'
GALKO-rev	5'-GTAAGCTGGCAAATCAGGAAAATCTGTAGACAATCTTGGCATAGGCCACTAGTGGATCTG-3'
AEP KO confirmation	
YHRKOCONF-for	5'-CCACCAAATTACAAGAATAT-3'
YHRKOCONF-rev	5'-ACGAGCTCAAAGAAATCTGT-3'
GALKOCONF-for	5'-ATATGGATATGTATATGGTG-3'
GALKOCONF-rev	5'-AAAATAGCATAAAATTCGTT -3'

Table 3.3 List of YKO strains

Strain Name	Genotype
BY4741 Δ YHR	BY4741; Mat a; his31; leu20; met150; ura30; YHR210c::kanMX4
BY4741 Δ YNR	BY4741; Mat a; his31; leu20; met150; ura30; YNR071c::kanMX4
BY4741 Δ GAL	BY4741; Mat a; his31; leu20; met150; ura30; YBR019c::kanMX4
BY4742 Δ YHR	BY4742; Mat α ; his31; leu20; met150; ura30; YHR210c::kanMX4
BY4742 Δ YNR	BY4742; Mat α ; his31; leu20; met150; ura30; YNR071c::kanMX4
BY4742 Δ GAL	BY4742; Mat α ; his31; leu20; met150; ura30; YBR019c::kanMX4

3.7 Figures

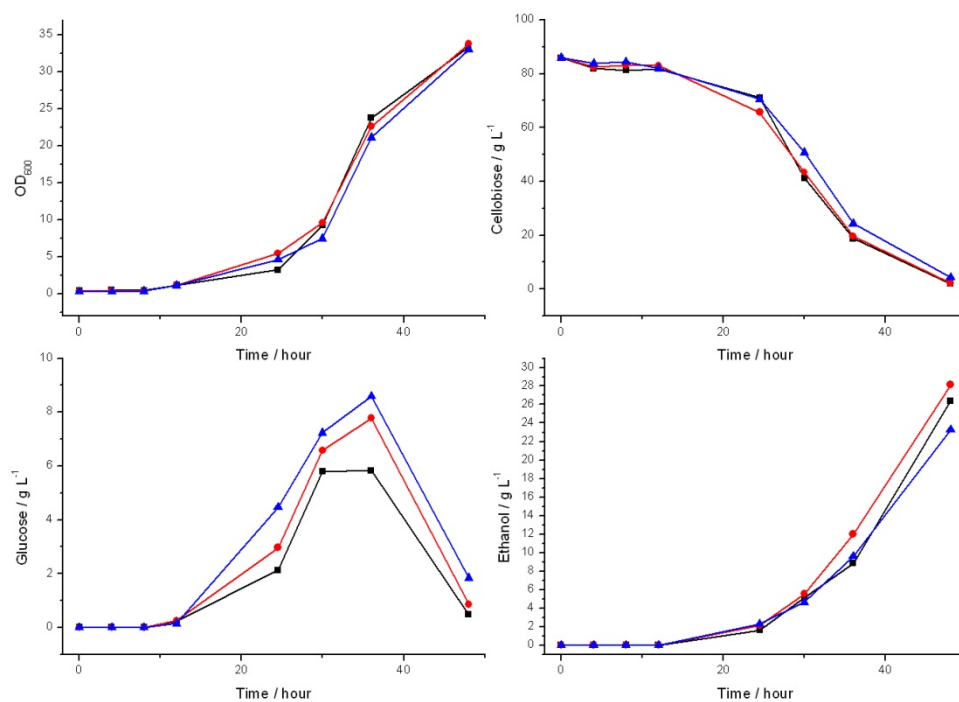


Figure 3.1 Concentrations of cellobiose, glucose, ethanol, and OD₆₀₀ in the fermentation of 80 g L⁻¹ cellobiose of AEP1 (♦), AEP2 (■), and AEP0 (▲) strains, plotted as a function of time.

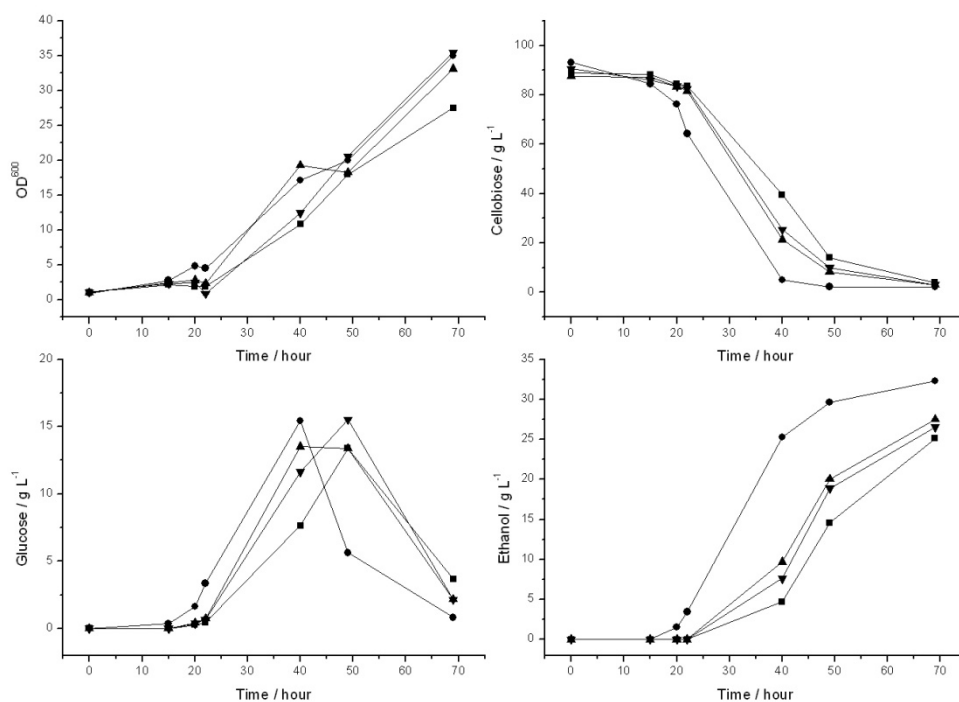


Figure 3.2 Concentrations of cellobiose, glucose, ethanol, and OD₆₀₀ in the fermentation of 80 g L⁻¹ cellobiose of ΔYHR (◆), ΔGAL (■), ΔHG (▲) and SL01 (●), plotted as a function of time.

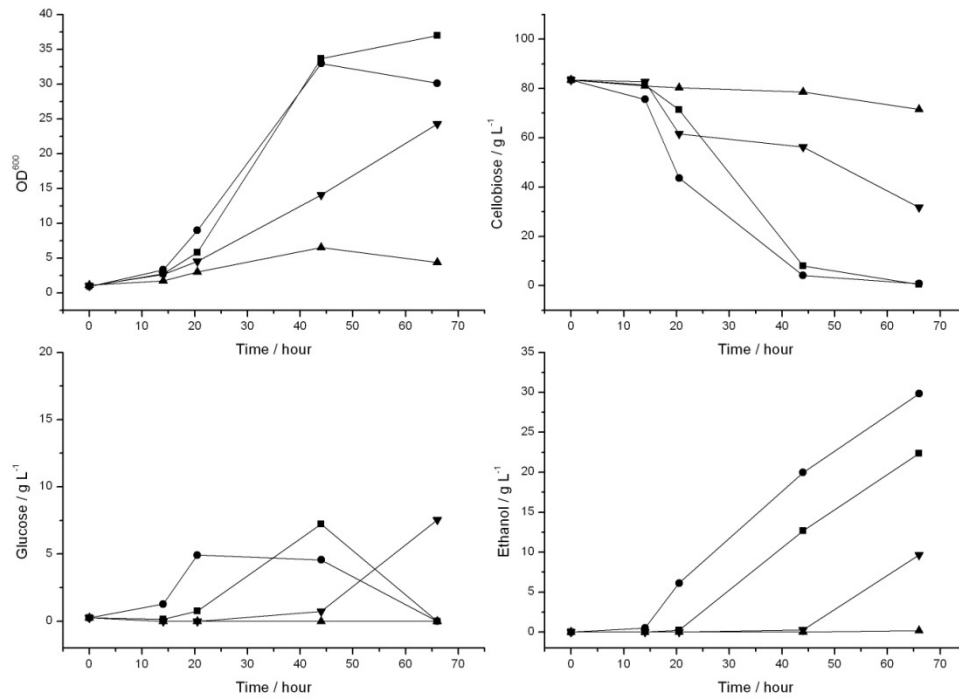


Figure 3.3 Concentrations of cellobiose, glucose, ethanol, and OD₆₀₀ in the fermentation of 80 g L⁻¹ cellobiose of BY4741 Δ YHR (◆), Δ YNR(■), Δ GAL(▲) and wild type(●), plotted as a function of time.

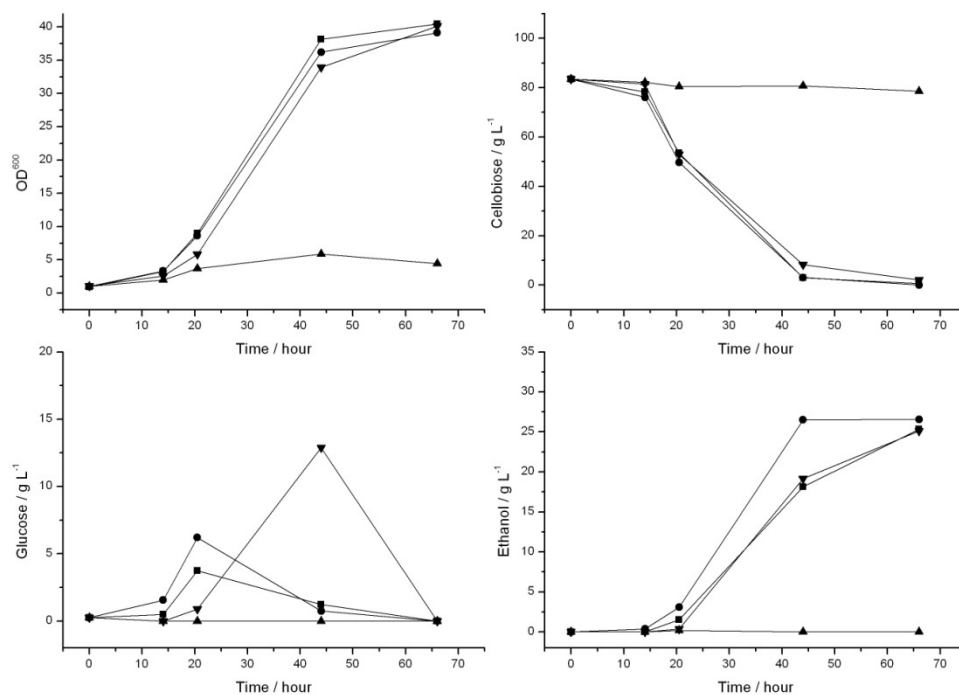


Figure 3.4 Concentrations of cellobiose, glucose, ethanol, and OD₆₀₀ in the fermentation of 80 g L⁻¹ cellobiose of BY4742 Δ YHR (◆), Δ YNR (■), Δ GAL (▲) and wild type (●), plotted as a function of time.

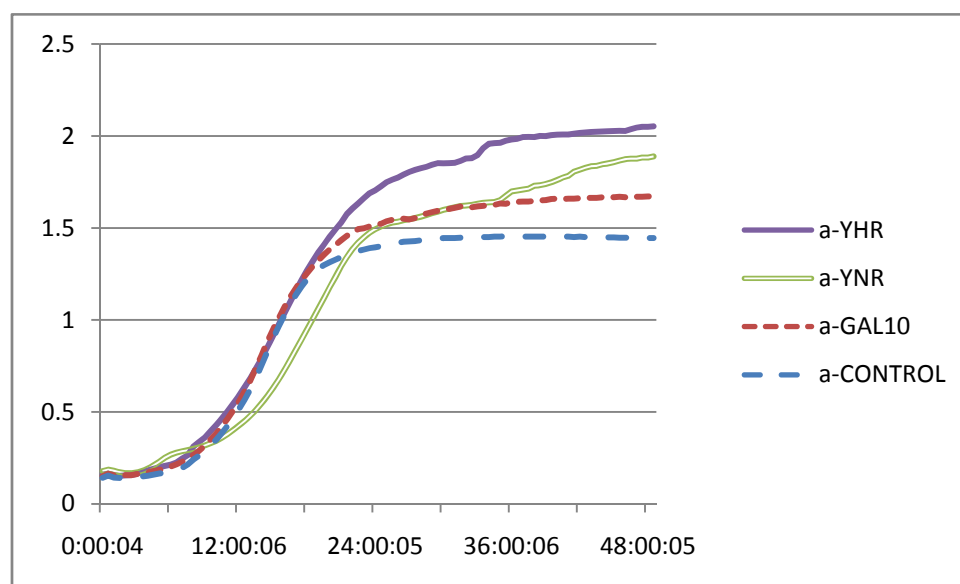


Figure 3.5 Micro-culture assay in the BY4741 AEP disrupted strains, 80 g L⁻¹ cellobiose as a sole carbon source, plotted as a function of time.

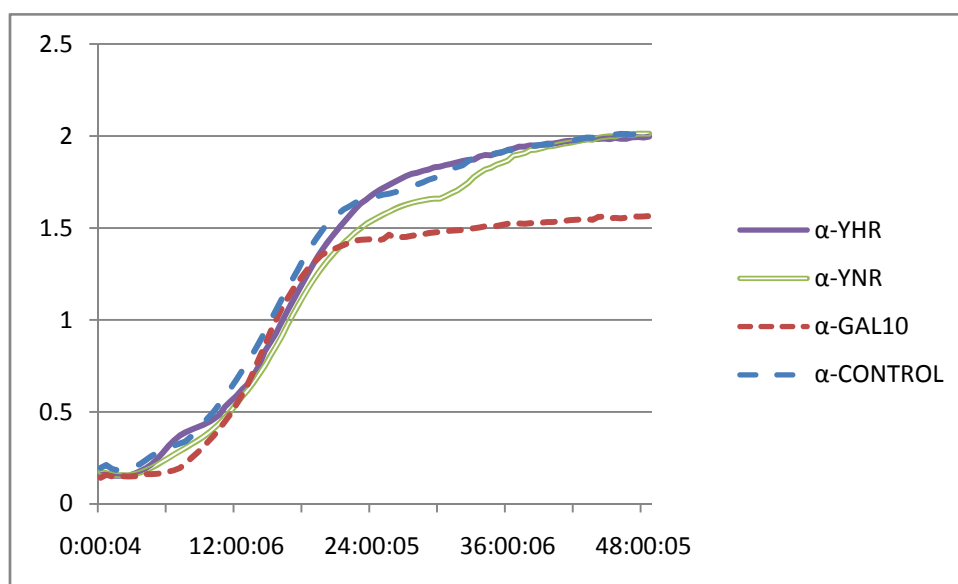


Figure 3.6 Micro-culture assay in the BY4742 AEP disrupted strains, 80 g L⁻¹ cellobiose as a sole carbon source, plotted as a function of time.

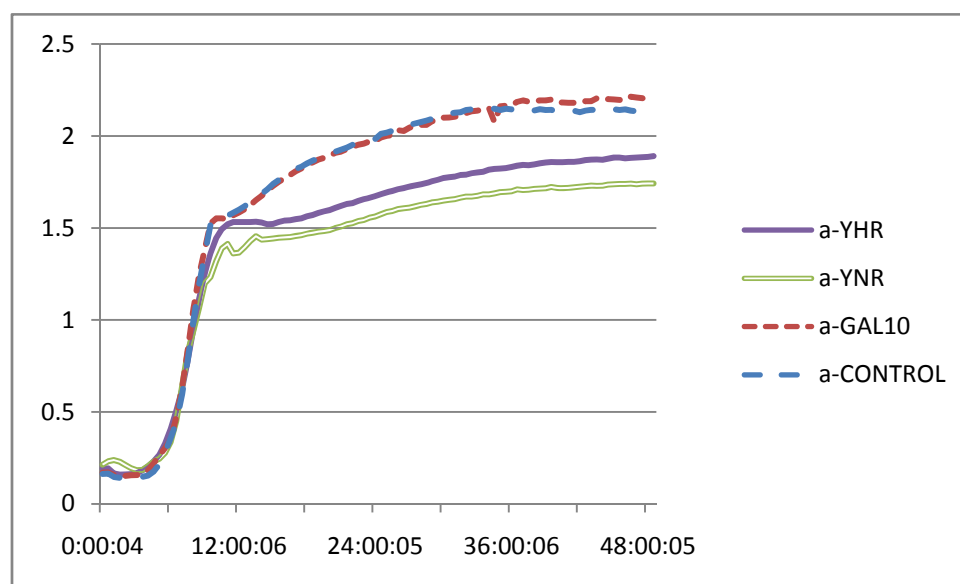


Figure 3.7 Micro-culture assay in the BY4741 AEP disrupted strains, 20 g L⁻¹ glucose as a sole carbon source, plotted as a function of time.

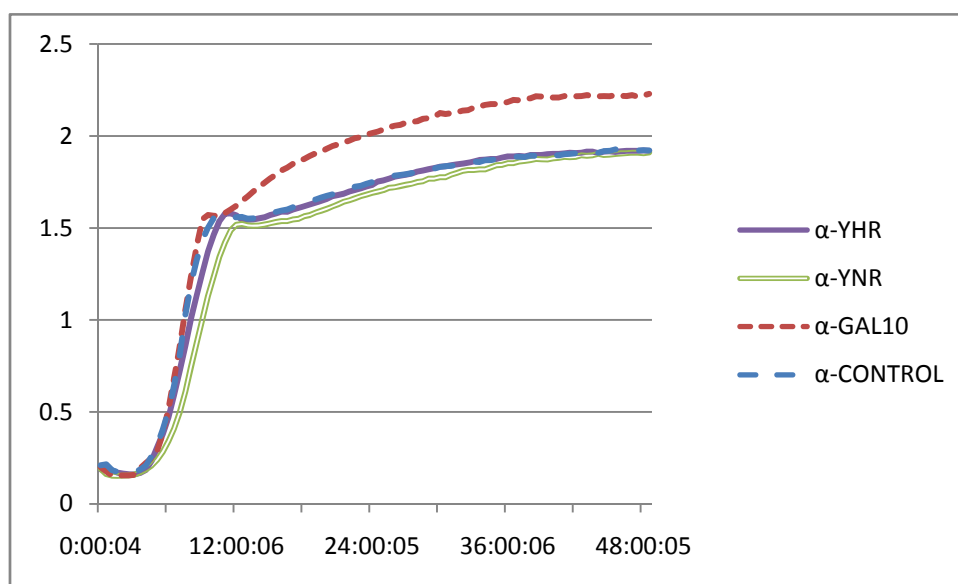


Figure 3.8 Micro-culture assay in BY4742 AEP disrupted strains, 20 g L⁻¹ glucose as a sole carbon source, plotted as a function of time.

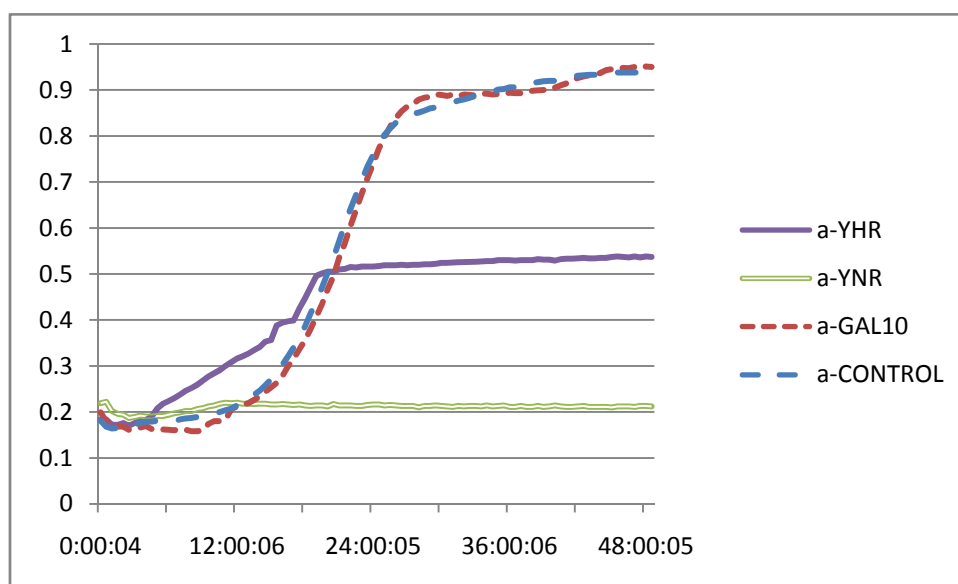


Figure 3.9 Micro-culture assay in the BY4741 AEP disrupted strains, 20 g L⁻¹ PNPD as a sole carbon source, plotted as a function of time.

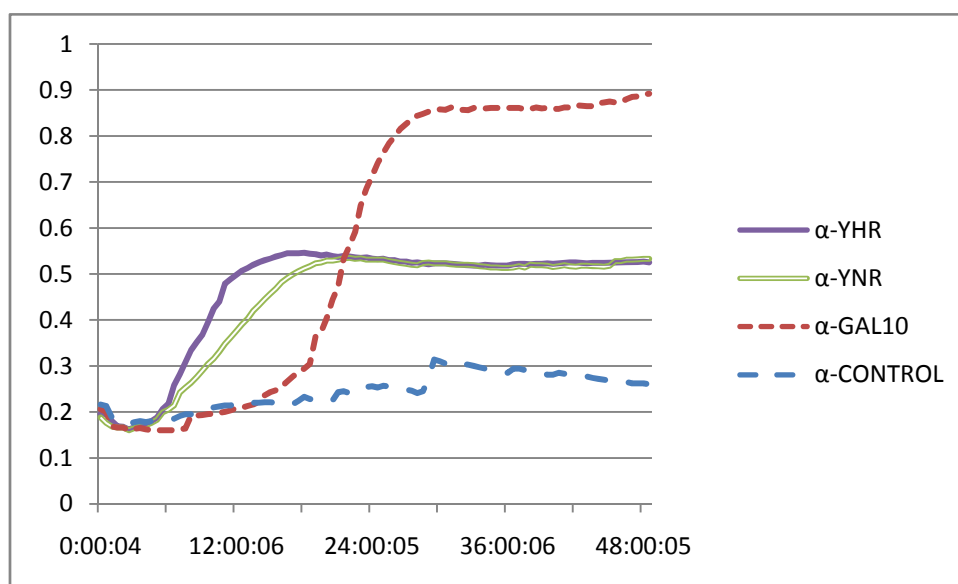


Figure 3.10 Micro-culture assay in the BY4742 AEP disrupted strains, 20 g L⁻¹ PNPD as a sole carbon source, plotted as a function of time.

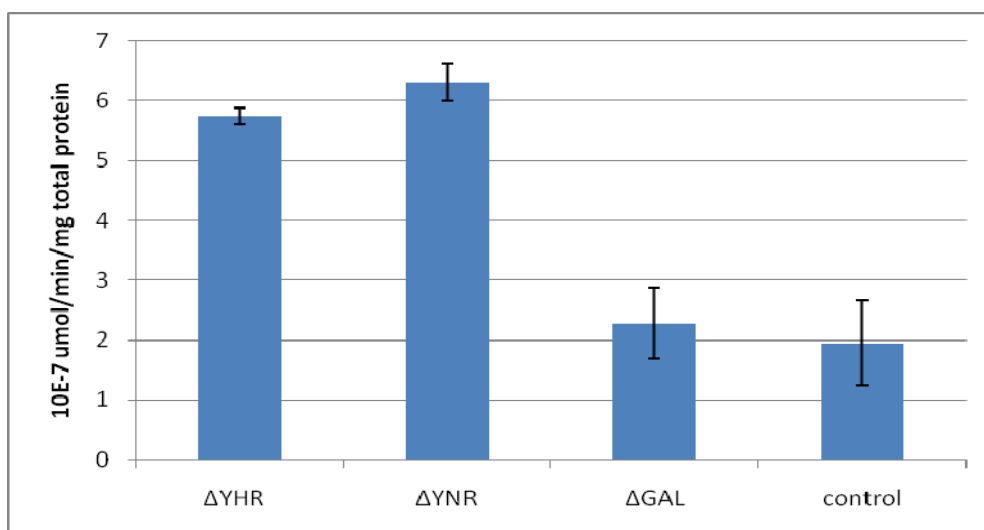


Figure 3.11 Specific mutarotase activity in the BY4742 AEP disrupted strains, 20 g L⁻¹ glucose as a sole carbon source. Data were from duplicate experiments.

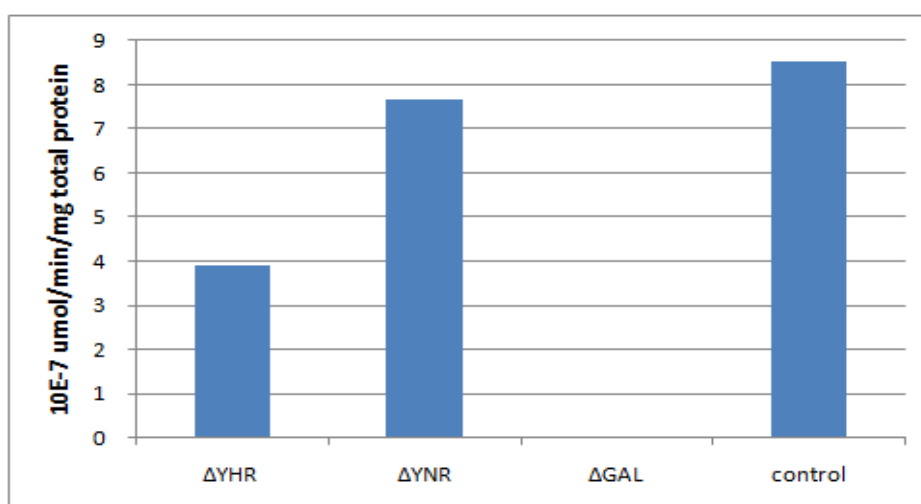


Figure 3.12 Specific mutarotase activity in the BY4742 AEP disrupted strains, 80 g L⁻¹ cellobiose as a sole carbon source.

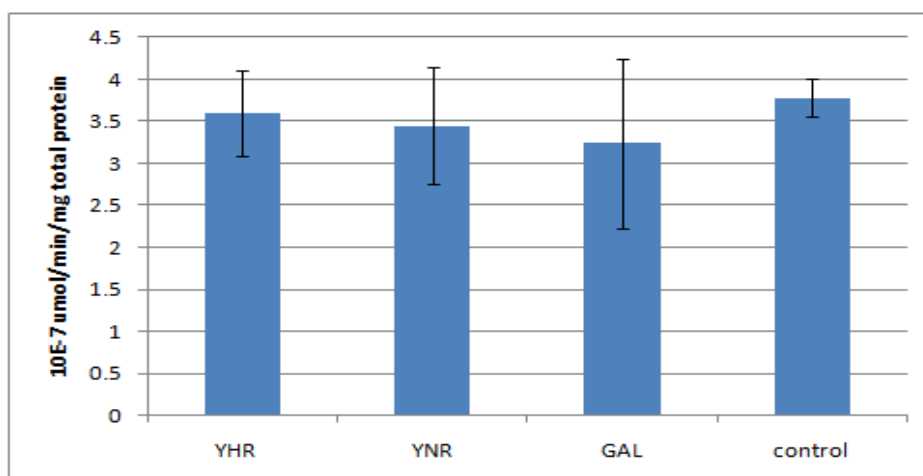


Figure 3.13 Specific mutarotase activity in the BY4742 AEP overexpression strains, 20 g L⁻¹ glucose as a sole carbon source. Data were from duplicate experiments.

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