2,3-BUTANEDIOL PRODUCTION FROM CELLOBIOSE BY ENGINEERED *SACCHAROMYCES CEREVISIAE*

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THESIS

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ABSTRACT

Production of renewable chemicals from cellulosic biomass is a critical step towards energy sustainability and reduced greenhouse gas emissions. Microbial cells have been engineered for producing fuels and chemicals from cellulosic sugars. Among these chemicals, 2,3-butanediol (2,3-BDO) is a compound of interest due to its diverse applications. While microbial production of 2,3-BDO with high yields and productivities has been reported, there are concerns with the use of potential pathogenic bacteria and inefficient utilization of cellulosic sugars. To address these problems, we engineered *Saccharomyces cerevisiae* to produce 2,3-BDO, especially from cellobiose which is a prevalent sugar in cellulosic hydrolyzates. Specifically, we overexpressed *alsS* and *alsD* from *Bacillus subtilis* to convert pyruvate to 2,3-BDO via α-acetolactate and acetoin in engineered *S. cerevisiae* capable of fermenting cellobiose directly. Under oxygen-limited conditions, the resulting strain was able to produce 2,3-BDO. Still, the majority of carbon flux in the strain went to ethanol, resulting in significant amounts of ethanol production. To enhance pyruvate flux to 2,3-BDO through elimination of the pyruvate decarboxylation (PDC) reaction, we employed a deletion mutant of both *PDC1* and *PDC5* for producing 2,3-BDO from cellobiose. The subsequent strain was able to produce only 2,3-BDO without ethanol production from cellobiose under oxygen-limited conditions. These results suggest the possibility of producing 2,3-BDO safely and sustainably from cellulosic hydrolyzates.

To my parents

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TABLE OF CONTENTS

CHAPTER 1: 2,3-BUTANEDIOL PRODUCTION FROM ENGINEERED *S. CEREVISIAE*

1.1 INTRODUCTION

Growing concerns of greenhouse gas emissions causing global climate changes have prompted efforts in producing fuels and chemicals from renewable biomass. 2,3-Butanediol (2,3-BDO) is a promising chemical that can be produced via microbial fermentation. 2,3-BDO is versatile as a chemical as it can be used in diverse applications in industry; common products utilizing 2,3-BDO as a precursor molecule are printing inks, perfumes, moistening and softening agents, explosives, plasticizers, foods, and pharmaceuticals (Garg and Jain, 1995; Syu, 2001). Most importantly, 2,3-BDO can be converted into the chemical butadiene, one of the seven major building blocks of the chemical industry (Christensen et al., 2008).

Current microbial fermentations for the production of 2,3-BDO have been optimized in bacterial species, particularly in *Klebsiella*, *Enterobacter*, and *Bacillus* species. *Klebsiella* species are especially known to produce 2,3-BDO efficiently without any genetic modifications. As such, most of the previous studies have focused primarily on optimizing fermentation conditions, such as aeration and pH, for enhanced production of 2,3-BDO. While *Klebsiella* species can utilize various sugars present in biomass, production of 2,3-BDO by these species generated a concern as they are potential human pathogens. Thus, it is desirable to use a nonpathogenic microorganism, like *Saccharomyces cerevisiae*, for future industrial scale production. *S. cerevisiae* is a popular host for many metabolic reactions as it has already been used to successfully produce ethanol and various building block chemicals. A previous effort to produce 2,3-BDO in *S. cerevisiae* utilized various combinations of deletions in pyruvate decarboxylase (*PDC1*), deletions alcohol dehydrogenases (*ADH1, ADH3, ADH5*), deletion of

aldehyde dehydrogenase (*ALD6*), and deletion of glycerol 3-phosphate dehydrogenase (*GPD2*) predicted through *in-silico* gene deletion algorithm and introduction of a bacterial 2,3-BDO pathway with *alsS (Bacillus subtilis), budA* (*Enterobacter aerogenes*), and *budC* (*Enterobacter aerogenes*) for engineered 2,3-BDO production (Ng et al., 2012). The 2,3-BDO yield achieved by an engineered strain (Δ*ADH1,* Δ*ADH3,* Δ*ADH5*) was 0.113 $g_{2,3\text{-BDO}}/g_{glucose}$ with a final titer of 2.29 g/L under anaerobic conditions. Introduction of *alsS* and *alsD* from *Bacillus subtilis* and overexpression of *BDH1* in a pyruvate decarboxylase deletion mutant resulted in an engineered *S. cerevisiae* capable of producing 2,3-BDO with a higher yield (0.36 $g_{2,3\text{-BDO}}/g_{glucose}$) and productivity (0.32 g/L·h) (Kim et al., 2013).

Production of fuels and chemicals from cellulosic sugars, especiFally from cellobiose and xylose is a sustainable route. Cellobiose fermentation by engineered *S. cerevisiae* has been demonstrated as a promising strategy for producing cellulosic fuels and chemicals (Galazka et al., 2010). During the process of producing lignocellulosic ethanol by simultaneous saccharification and fermentation, a cellulase mixture is used to hydrolyze cellulose and hemicellulose into mainly glucose and xylose. However, the amount of β-glucosidase in cellulase mixtures is inefficient for hydrolyzing cellulose to glucose. Thus, supplementation of additional β-glucosidase is necessary during simultaneous saccharification and fermentation for producing fuels and chemicals. Cellulolytic enzymes constitute a major proportion of the cost for producing bioethanol, so addition of β-glucosidase will increase production costs. By introducing a cellobiose utilizing pathway into *S. cerevisiae*, the extra cost of enzyme addition can be eliminated since the fermenting organism is able to hydrolyze cellobiose intracellularly (Lee et al., 2013). In this study, we constructed an engineered *S. cerevisiae* capable of producing 2,3-BDO as a major fermentation product from cellobiose. Specifically, a 2,3-BDO producing

pathway consisting of *alsS* and *alsD* genes and a cellobiose utilizing pathway consisting of CDT-1 and GH1-1 were introduced into a pyruvate decarboxylase deficient *S. cerevisiae*.

1.2 MATERIALS AND METHODS

1.2.1 *Strains and plasmids*

Escherichia coli TOP10 (Invitrogen, Carlsbad, CA, USA) was used for plasmid DNA amplification. D452-2 (*MATa, his3, leu2, ura3, can1*) was used for 2,3-BDO tolerance assay. *S. cerevisiae* EJ2 (D452-2: *Matα, his3, leu2::gh1-1, ura3::cdt-1, can1*) engineered cellobiose consuming strain was used as a host strain for producing 2,3-BDO from cellobiose. 2,3-BDO production genes, *alsS* and *alsD,* were constitutively expressed on pRS423plasmid with GPD (TDH3) promoter and CYC1 terminator (pRS423_alsS_alsD) in EJ2. *S. cerevisiae* SOS2 (D452-2: *Matα*, *leu2*, *his3*, *ura3*, *can1*, Δ*PDC1*, and Δ*PDC5*; Kim et al., 2013) was used as a host strain for 2,3-BDO production and cellobiose utilization without ethanol production. Plasmids used for the expression of 2,3-BDO production genes in SOS2 were derived from pRS423 (pRS423_alsS) and pRS426 (pRS426_alsS) with GPD promoter and CYC1 terminator. Cellobiose utilization genes, *cdt-1* and *gh1-1*, were expressed constitutively on pRS425 with PGK1 promoter and CYC1 terminator (pRS425-BT). A complete list of strains and plasmids can be found in Table 1.1.

1.2.2 *Culture conditions and yeast transformation*

Luria-Bertani (LB) medium (5 g/L yeast extract, 10 g/L tryptone and 10 g/L NaCl) with 100 μg/mL ampicillin was used for plasmid preparation from *E. coli*. Yeast extract peptone (YP) medium (10 g/L yeast extract and 20 g/L peptone) with 20 g/L glucose was used for cultivation of D452-2. Synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acids and 0.6 g/L complete supplement mixture without histadine, leucine, tryptophan, and uracil;

adjusted to pH 6.0) with 20 g/L ethanol or 20 g/L cellobiose and 20 g/L agar was used for transformant selection of EJ2-control, EJ2-B, SOS2-B, and SOS2-CB on agar plates with addition of necessary amino acid. Transformation of plasmid(s) into EJ2 and SOS2 was accomplished using the EZ-Transformation kit (BIO 101, Vista, CA). Yeast strains were cultivated in SC with 20 g/L ethanol or 20 g/L cellobiose with supplementation of necessary amino acid prior to inoculation into main fermentations. Potassium hydrogen phthalate (KHP) buffer was added into precultures of SOS2 with low cell density main fermentation inoculation.

Main fermentations with EJ2 derived strains were performed in 125 mL flasks containing 25 mL of SC medium with 20 g/L glucose or 20 g/L cellobiose adjusted to pH 6.0. Precultured cells were harvested at a cell concentration of 1.5 g/L and inoculated at a concentration of 0.3 g/L for the main fermentation and cultivated at 30 °C under microaerobic conditions with an agitation speed of 80 rpm. Main fermentations with SOS2 derived strain were performed in 125 mL flasks containing 25 mL of SC medium with 20 g/L cellobiose at pH 6. Precultured cells were harvested at a concentration of 1.5 g/L and inoculated at a concentration of 0.3 g/L for the main fermentation. Cultivation was performed at 30 °C under microaerobic conditions with an agitation speed of 80 rpm during the fermentation.

1.2.3 *2,3-BDO tolerance assay*

D452-2 was grown overnight in YP medium with 20 g/L of glucose. Cells were inoculated at a cell concentration of 0.003 g/L into 250 µL of YP with 20 g/L glucose in a 96-well plate. 2,3-BDO was added into wells at 0 %, 1 %, 3 %, and 5 %. Prior to cultivation at 30 ^{o}C, 50 µL of mineral oil was added on top of the media to prevent evaporation. The experiment was done in triplicates using a microplate reader with shaking to measure cell density every half hour.

1.2.4 *Growth of SOS2-B on glucose*

SOS2 harboring pRS423GPD (SOS2-control) and SOS2 harboring pRS423_alsD and pRS426_alsS (SOS2-CB) were cultured in SC medium with 20 g/L ethanol prior to harvesting cells. Cells were diluted 10-fold and spotted in triplicates onto an SC agar plate with 20 g/L glucose or 20 g/L ethanol and supplementation of necessary amino acid. The plate was incubated at 30 ^{o}C for seven days.

1.2.5 *Analytical methods*

Cell optical density was measured using a spectrophotometer (Biomate 5, Thermo, NY, USA) and converted to dry cell weight (DCW) by DCW (g/L) = OD_{600} * 0.3. Metabolite concentrations were determined by a high performance liquid chromatography (HPLC) system (Agilent Technologies 1200 Series) equipped with a Rezex ROA Organic Acid H^+ column (Phenomenex, Torrance, CA, USA) and a refractive index (RI) detector (Agilent, Palo Alto, CA, USA). The column was eluted with 0.005 N H_2SO_4 at a flow rate of 0.6 mL/min at 50 °C.

1.3 RESULTS

1.3.1 *S. cerevisiae tolerance to 2,3-BDO*

Prior to introducing the heterologous 2,3-BDO production pathway into *S. cerevisiae*, tolerance of *S. cerevisiae* to 2,3-BDO was assessed by measuring specific growth rate under different concentrations of 2,3-BDO. Laboratory strain D452-2 was grown in a 96-well plate containing YP medium with 20 g/L glucose and 0 %, 1 %, 3%, or 5 % 2,3-BDO. The 96-well plate was incubated at 30 ^{o}C for 14 h and cell density (OD_{600}) was measured at 30 min intervals. At 14 h, cell growth rate was measured for the four different conditions. Addition of 2,3-BDO in the medium caused a decrease in growth rate, albeit minimal. With the addition of 1 % 2,3-BDO, cell growth rate was reduced by 3 % (0.302 h^{-1} vs. 0.310 h^{-1}) compared to the control without 2,3-BDO addition (Figure 1.1). The highest amount of 2,3-BDO tested (5 %) caused a 20 %

reduction (0.264 h^{-1} vs. 0.310 h^{-1}) of growth rate. While the presence of 2,3-BDO in the medium reduces cell growth rate, *S. cerevisiae* tolerance to 2,3-BDO is high in comparison to several other four-carbon alcohols (González-Ramos et al., 2013). Thus, the natural high tolerance of *S. cerevisiae* to 2,3-BDO indicates that this microorganism is a good host for 2,3-BDO production.

1.3.2 *Production of 2,3-BDO from cellobiose*

As cellobiose is a major component in cellulosic biomass, 2,3-BDO production from cellobiose was investigated by overexpressing *B. subtilis* genes *alsS* and *alsD* in an engineered cellobiose utilizing *S. cerevisiae* strain, EJ2. The resulting strain EJ2-B and the EJ2-control strain were cultured in SC medium containing 20 g/L cellobiose or 20 g/L glucose under microaerobic condition. The EJ2-B and EJ2-control strains consumed all cellobiose or glucose and produced ethanol (Figure 1.2). Both EJ2-B and EJ2-control strains also produced ~1 g/L glycerol in addition to acetoin and 2,3-BDO.

Under glucose condition, the major product of fermentation was ethanol. Specifically, the ratio of ethanol to 2,3-BDO pathway intermediates (acetoin and 2,3-BDO) was 80 to 1. Flux of pyruvate to the 2,3-BDO pathway was improved by the introduction of *alsS* and *alsD* (EJ2-B), shifting more pyruvate to 2,3-BDO at a ratio of 1 to 11. These results demonstrate that introduction of a heterologous 2,3-BDO pathway could indeed shift pyruvate flux. Cellobiose fermentation with the EJ2-control strain resulted in production of ethanol without any detectable amounts of acetoin and 2,3-BDO. As expected, the EJ2-B strain grown with cellobiose was able to produce 2,3-BDO (Figure 1.2B). Surprisingly, the flux of pyruvate to the 2,3-BDO pathway was better under cellobiose condition than glucose with a ratio of 2.5 ethanol to 1 2,3-BDO. These results demonstrate that cellobiose is a better substrate for producing 2,3-BDO than glucose in *S. cerevisiae*. The final yield of ethanol in EJ-B under cellobiose condition was 0.25

$g_{ethanol}/g_{cellobiose}$ suggesting that 2,3-BDO yield was improved by the introduction of a heterologous 2,3-BDO pathway in *S. cerevisiae*, but flux of pyruvate to ethanol is still the most dominant. Thus, additional improvements to the 2,3-BDO production pathway need to be made in order to improve yield.

1.3.3 *Introduction of 2,3-BDO pathway rescues growth of PDC mutant on glucose*

Deletion of pyruvate decarboxylase (PDC) genes is suggested as a way to improve yield of pyruvate-requiring metabolites by shifting the flux of pyruvate from ethanol to other pathways (Adachi et al., 1998; Kondo et al., 2012; Zelle et al., 2008). Thus, a PDC mutant (Δ*PDC1* and Δ*PDC5*) *S. cerevisiae* SOS2 (Kim et al., 2013), which is unable to produce ethanol, was utilized as a host strain for 2,3-BDO production. Previous reports indicate that PDC deficient strains are unable to consume glucose as a sole carbon source (Hohmann and Cederberg, 1990) unless C_2 compounds are supplied (Flikweert et al., 1996; Pronk et al., 1996). As a possible method to alleviate glucose toxicity in SOS2, and simultaneously produce 2,3-BDO, *alsS* and *alsD* were expressed in the strain. As expected, the SOS2-control strain harboring empty plasmids was unable to grow in a 5 mL culture of SC medium with glucose as the sole carbon source. However, SOS2-B was able to grow in SC medium with glucose without the addition of C_2 compounds.

To confirm this observation, SOS2-B and SOS2-control strains were cultured and serially spotted on an SC agar plate with 20 g/L glucose. After seven days of incubation, colonies of SOS2-B were observed for all dilutions (Figure 1.3). However, no colonies of SOS2-control formed on the SC agar plate with 20 g/L glucose (Figure 1.3). Prolonged incubation of SOS2-control did not improve colony formation. To eliminate the possibility that increased mutation rate of the SOS2-B strain led to growth on glucose, SOS2-control and SOS2-B were also spotted serially on an SC agar plate with ethanol. After 14 days of incubation, both strains were able to

grow on the ethanol plate, with the control strain growing faster (data not shown). These results suggest that introduction of a 2,3-BDO pathway in a PDC mutant *S. cerevisiae* strain can rescue the lethality of the mutant grown in SC medium with glucose as the sole carbon source.

1.3.4 *Production of 2,3-BDO from cellobiose in a PDC mutant*

As co-production of 2,3-BDO and ethanol from cellobiose was observed, a pyruvate decarboxylase mutant was used as a host for producing 2,3-BDO from cellobiose. To this end, two pathways (cellobiose utilization and 2,3-BDO production) were introduced into a *PDC1* and *PDC5* deletion mutant, SOS2. When the resulting strain SOS2-CB was cultured in SC medium with 20 g/L cellobiose, consumption of cellobiose along with production of 2,3-BDO without the production of ethanol was observed (Figure 1.4A). In addition, ~1.5 g/L acetoin, ~1.5 g/L glycerol, and ~1 g/L cellodextrin were produced. Even though the final 2,3-BDO yield was 0.26 $g_{2,3\text{-BDO}}/g_{\text{cellobiose}}$, the fermentation took over 200 h to complete. In addition, cellobiose was not completely consumed and the final pH reached 3.

While 2,3-BDO production from cellobiose using a pyruvate decarboxylase mutant resulted in improved yield, the rate of fermentation was limiting. To improve the rate of fermentation, a high cell concentration inoculum was used. Fermentation with a high cell concentration inoculum resulted in complete consumption of cellobiose in 60 h (Figure 1.4B) and minimal accumulation of cellodextrin (<0.5 g/L). The SOS2-CB strain accumulated acetoin (~3.0 g/L) and glycerol (~1.7 g/L). The yield of 2,3-BDO yield at the end of fermentation was 0.19 $g_{2,3\text{-BDO}}/g_{\text{cellobiose}}$. An improvement of 2,3-BDO productivity was observed with a high cell density inoculation (0.02 g/L·h to 0.06 g/L·h). This result demonstrated that increasing initial cell density can improve the rate of 2,3-BDO fermentation without significant loss in 2,3-BDO yield.

Lastly, a high cell concentration fermentation with YP medium containing 20 g/L cellobiose was used to further improve 2,3-BDO productivity in SOS2-CB. Within 24 h, almost all cellobiose was consumed with production of 5.29 g/L 2,3-BDO (Figure 1.4C). The 2,3-BDO productivity at 24 h was 0.22 g/L·h. Cellodextrin (~1.9 g/L), glycerol (~2.7 g/L), and acetoin (~2.2 g/L) also accumulated during the fermentation.

1.4 DISCUSSION

While *S. cerevisiae* is able to synthesize 2,3-BDO without the introduction of heterologous genes, natural production capability is limited. The low affinity of native *ILV2* for pyruvate and inability to control spontaneous conversion of α-acetolactate to diacetyl make the innate 2,3-BDO pathway difficult to improve. Thus, *B. subtilis* genes *alsS* and *alsD* were introduced in *S. cerevisiae* for enhanced 2,3-BDO production ability. To synthesize 2,3-BDO in a sustainable and environmentally friendly manner, an engineered cellobiose utilizing *S. cerevisiae* was used as a host strain.

Successful production of 2,3-BDO from cellobiose was achieved from an evolved cellobiose consuming *S. cerevisiae* strain overexpressing *alsS* and *alsD* (EJ2-B). Interestingly, the EJ-B strain was able to produce more 2,3-BDO with cellobiose than glucose as a substrate. The apparent difference between glucose utilization and cellobiose utilization in *S. cerevisiae* is transport of the two molecules and cleavage of cellobiose into two molecules of glucose (Galazka et al., 2010). However, fermentation rate and/or signal transduction by these two substrates may cause a difference in 2,3-BDO yield. Future studies may try to elucidate regulation of the pyruvate branch point in the presence of different substrates.

The limiting factor in 2,3-BDO production in the EJ2-B strain was the production of ethanol as a major byproduct. Inherent strong flux of pyruvate to the ethanol production pathway

by means of PDC genes reduces the availability of pyruvate for other pyruvate utilizing reactions. As 2,3-BDO synthesis requires pyruvate as a precursor, improving pyruvate flux to the 2,3-BDO pathway can improve product yield. Accordingly, *alsS* and *alsD* were overexpressed in SOS2, a *PDC1* and *PDC5* deletion *S. cerevisiae* strain, generating SOS2-B. PDC deletion mutants have slow growth rate and cannot consume glucose as a sole carbon source in defined mineral medium due to their inability to synthesize acetyl-CoA (Flikweert et al., 1996; Pronk et al., 1996). While lack of acetyl-CoA may be the main reason why PDC mutants cannot grow on glucose, redox imbalance from the mutants' inability to regenerate NAD^+ may also contribute to lack of growth on glucose (van Maris et al., 2004). Thus, observed growth of SOS2-B strain on SC agar plates with glucose indicate that introduction of a 2,3-BDO pathway may rescue toxicity to glucose. The 2,3-BDO pathway is NAD^+ regenerating, conferring PDC mutants a more balanced redox state than wild type PDC mutants. More importantly, acetoin can be cleaved into acetaldehyde (López et al., 1975), thus allowing synthesis of cytosolic acetyl-CoA which is required for growth on glucose (Flikweert et al., 1996; Pronk et al., 1996) *B. subtilis* gene *alsS* is also known to encode an enzyme with pyruvate decarboxylase activity (Atsumi et al., 2009) so it is likely that both AlsS and AlsD can catalyze nonspecific decarboxylation of pyruvate to acetaldehyde, thus producing transient amounts of acetaldehyde for acetyl-CoA synthesis. In this way, introduction of *alsS* and *alsD* for 2,3-BDO production in SOS2 eliminated lethality of the strain to glucose as a sole carbon source.

To produce 2,3-BDO from cellobiose in SOS2-B, *Neurospora. crassa* cellodextrin transporter (*cdt-1*) and β-glucosidase (*gh1-1*) were expressed, resulting in the strain SOS2-CB. Production of 2,3-BDO from cellobiose in SOS2-CB resulted in a higher 2,3-BDO yield than in EJ2-B, mainly due to improved flux of pyruvate to the 2,3-BDO pathway. Slow growth rate of

SOS2-CB in SC medium with cellobiose can be attributed to metabolic burden of PDC deletions. Additionally, a decrease in pH occurred during the fermentation which also contributed to slow growth rate. Pyruvic acid is known to be secreted in strains containing deletions in PDC (Flikweert et al., 1996; Kim et al., 2013; Pronk et al., 1996) which may partially explain the observed pH decrease. Production of other organic acids may have contributed to the decreased pH as well.

Since producing 2,3-BDO through the engineered pathway only regenerates one mole of NAD^+, cell growth may stop due to insufficient cofactor regeneration. Glycerol production may be an efficient method for cells to regenerate NAD^+, thus explaining the accumulation of glycerol during fermentation with SOS2-CB. Acetoin also accumulated during fermentations with SOS2-CB. Since acetoin is the intermediate directly before 2,3-BDO, improved conversion of acetoin to 2,3-BDO is necessary for improved 2,3-BDO yields. This can be accomplished by overexpressing native *S. cerevisiae* Bdh1p responsible for reduction of acetoin to 2,3-BDO (Kim et al., 2013). In fermentations with SOS2-CB in SC medium, cellodextrin accumulation was also observed, reducing 2,3-BDO productivity as cellodextrin transporter CDT-1 is less efficient in transporting cellodextrin compared to cellobiose (Ha et al., 2011). To improve 2,3-BDO productivity, overexpression of cellobiose phosphorylase (cep94A) may be considered as less glucose secretion occurs (Ha et al., 2012).

This work attempted to produce 2,3-BDO from cellobiose by introducing a heterologous 2,3-BDO pathway in an evolved cellobiose utilizing strain. 2,3-BDO was produced from cellobiose at a higher yield than from glucose, however ethanol yields were still high. Instead, a PDC deletion mutant of S. cerevisiae was used as a host strain for introducing the cellobiose utilization and 2,3-BDO production pathway. As a result of fermentation under YP medium with

cellobiose, the resulting strain was able to produce 5.29 g/L 2,3-BDO in 24 h. The results of this study demonstrate the ability to efficiently produce 2,3-BDO from cellobiose in engineered S. cerevisiae.

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1.6 TABLE

Table 1.1. List of strains and plasmids used in this study.

Strain or plasmid	Description	Reference
Strain		
D452-2	*MATα*, *leu2*, *his3*, *ura3*, and *can1*	Hosaka et al., 1992
EJ2	*MATα*, *leu2::LEU2* pRS405_gh1-1, *his3*, *ura3::URA3* pRS406_cdt-1, and *can1*	Oh et al.; in preparation
SOS2	*MATα*, *leu2*, *his3*, *ura3*, *can1*, *PDC1Δ*, *PDC5Δ*	Kim et al.; submitted
EJ2-control	EJ2/pRS423GPD	This study
EJ2-B	EJ2/pRS423_alsS_alsD	This study
SOS2-control	SOS2/pRS426GPD/pRS423GPD	This study
SOS2-B	SOS2/pRS426_alsS/pRS423_alsD	This study
SOS2-CB	SOS2/pRS426_alsS/pRS423_alsD/pRS425_cdt-1_gh1-1	This study
Plasmid		
pRS426GPD	*URA3*, P_{GPD}-T_{CYC1}, 2μ, ampR	ATCC® 87361™
pRS423GPD	*HIS3*, P_{GPD}-T_{CYC1}, 2μ, ampR	ATCC® 87355™
pRS426_alsS	*URA3*, P_{GPD}-*alsS*-T_{CYC1}, 2μ, ampR	Kim et al., submitted
pRS423_alsD	*HIS3*, P_{GPD}-*alsD*-T_{CYC1}, 2μ, ampR	Kim et al., submitted
pRS423_alsS_alsD	*HIS3*, P_{GPD}-*alsS*-T_{CYC1}-P_{GPD}-*alsD*-T_{CYC1}, 2μ, ampR	This study
pRS425-BT	*LEU2*, P_{PGK1}-*cdt-1*-T_{CYC1}-P_{PGK1}-*gh1-1*-T_{CYC1}, 2μ, ampR	Oh et al., 2013

1.7 FIGURES

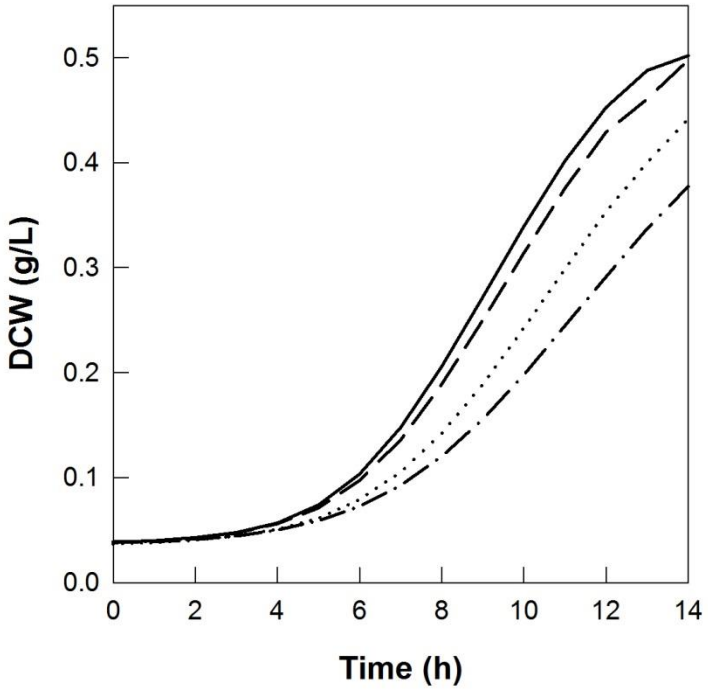


Figure 1.1. Tolerance of *S. cerevisiae* D452-2 strain to 2,3-BDO. Symbols: 0 % 2,3-BDO (──), 1 % 2,3-BDO (─ ─), 3 % 2,3-BDO (· · · ·), and 5% 2,3-BDO (─ · ─). Results are the average of triplicate experiments with error bars.

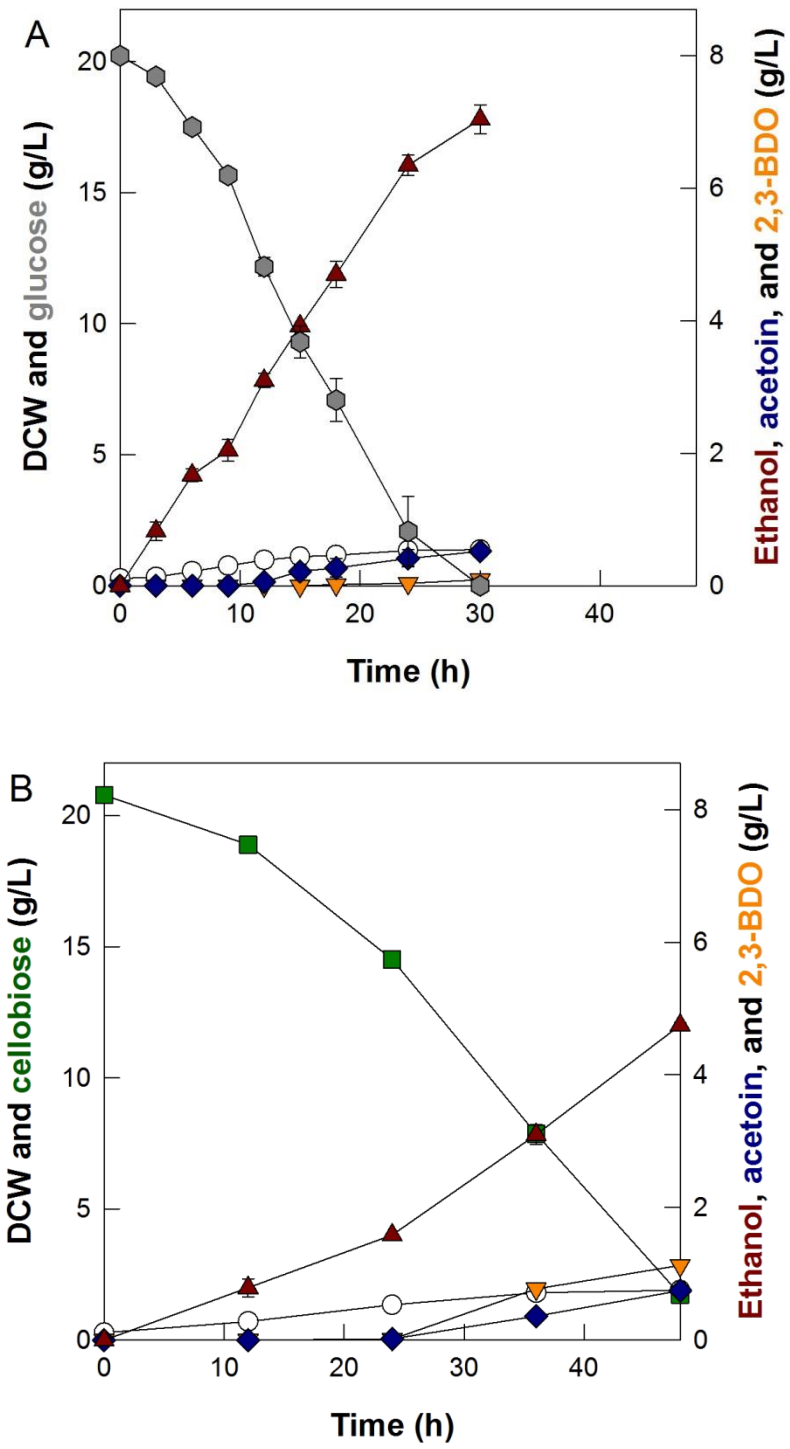


Figure 1.2. Fermentation profile of EJ2-B from SC medium with glucose (A) and EJ2-B from SC medium with cellobiose (B). Symbols: DCW (open circle), glucose (gray hexagon), cellobiose (green square), ethanol (red triangle up), acetoin (blue diamond), and 2,3-BDO (orange triangle down). Results are the averages of duplicate experiments with error bars.

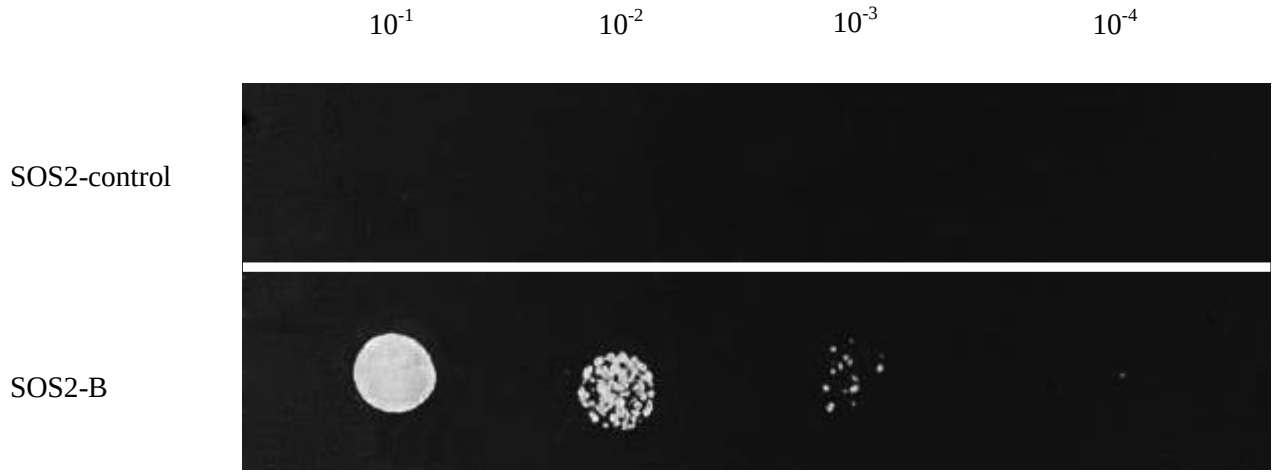


Figure 1.3. Rescue of SOS2 growth on glucose by introduction of 2,3-BDO pathway. Four serial dilutions were prepared and spotted in triplicates on SC agar plates with 20 g/L glucose.

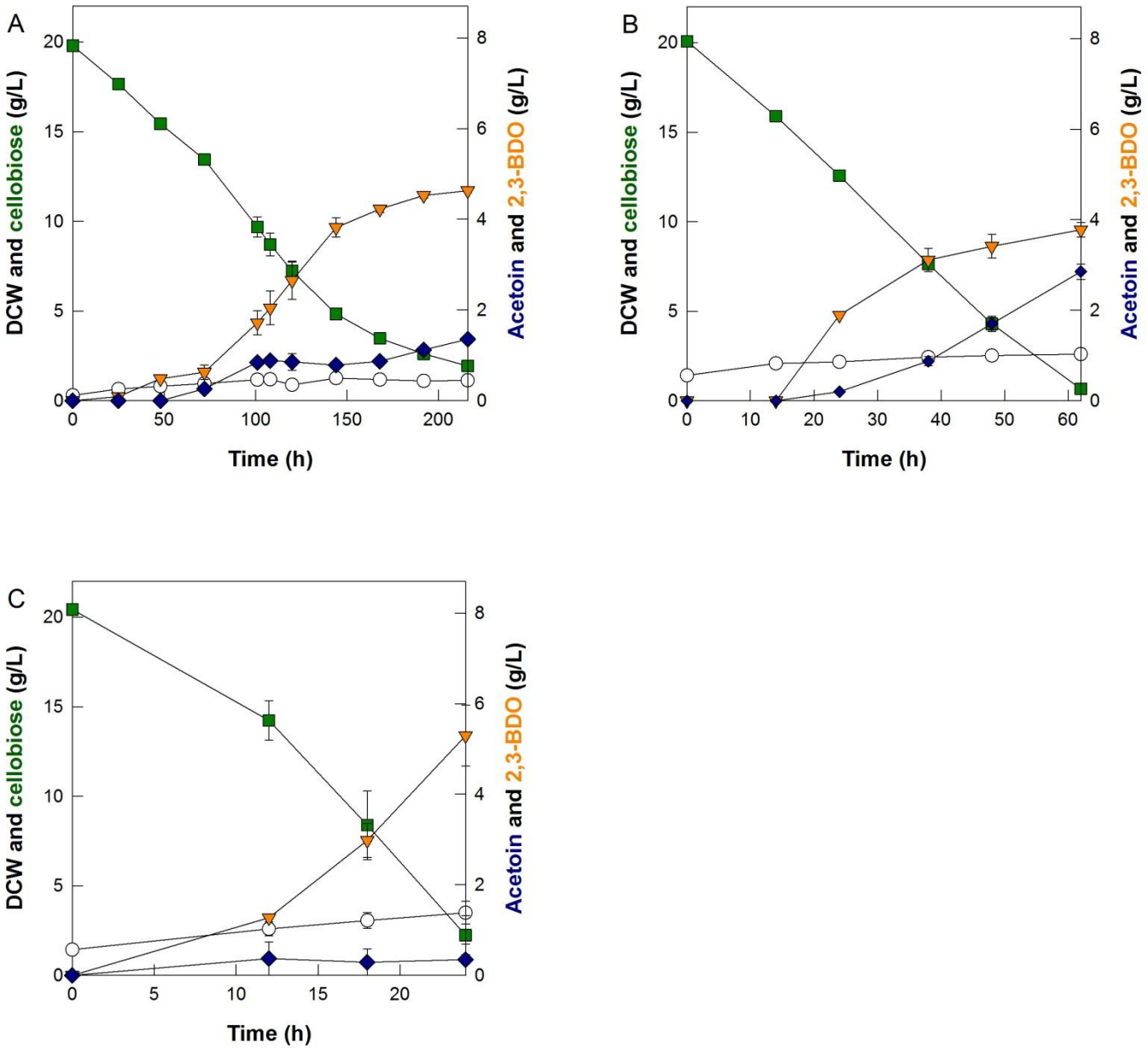


Figure 1.4. Fermentation profile of SOS2-CB from cellobiose. SOS2-CB fermentation from SC medium, low cell concentration inoculation (A), SOS2-CB fermentation from SC medium, high cell concentration inoculation (B), and SOS2-CB fermentation from YP medium, high cell concentration inoculation (C). Symbols: DCW (open circle), cellobiose (green square), acetoin (blue diamond), and 2,3-BDO (orange triangle down). Results are the averages of duplicate experiments with error bars.

CHAPTER 2: CREATING INTEGRATION PLASMIDS FOR GENE EXPRESSION

2.1 INTRODUCTION

Vectors are important genetic tools for the cloning, expression, and transport of genetic materials in microorganisms. In *S. cerevisiae*, a system of high copy number shuttle vectors (pRS420 series) was constructed for gene overexpression (Christianson, 1992). The pRS420 series contains multiple restriction enzyme sites (MCS) for cloning and in addition, yeast and bacterial origins of replication. Also, four autotrophic markers are available for selection in yeast and ampicillin is available for selection in bacteria. Due to the importance of gene expression in metabolic engineering, the pRS420 series provides a good tool for overexpressing heterologous genes. Additionally, a series of plasmids (pRS400 series) were constructed for the stable integration of genes into the yeast chromosome at auxotrophic m Overexpression of *cdt-1*, *gh1-1*, *alsS*, and *alsD* was accomplished using the pRS420 arker loci (Stratagene). Integration vectors allow the maintenance of genes without concern of plasmid loss during further manipulations of cells.

series of plasmids. While the maintenance and selection of overexpression plasmids is done by cell culture in minimal media with addition of appropriate amino acids, we noticed plasmid instability even during fermentations with SC medium selection pressure. To maintain heterologous gene expression stably, we proposed to integrate the cellobiose and 2,3-BDO pathway genes. Single gene integration vectors were constructed by traditional cloning techniques (restriction enzyme digestion and ligation). However, with the availability of only three (*HIS*, *LEU*, and *URA*) auxotrophic markers in SOS2 and necessary single gene integration of four genes using three integration sites is not feasible. Thus, construction of integration vectors containing two genes (*alsS* and *alsD* or *CDT-1* and *GH1-1*) integration was needed. A

method of constructing plasmids with multiple cassettes (promoter, gene, and terminator) in one step was utilized for the construction of cellobiose and 2,3-BDO integration plasmids.

DNA assembler is a method of combining multiple fragments of DNA using *in vivo* homologous recombination in *S. cerevisiae* (Shao, 2008). The assembly of a complete biochemical pathway (~19kb) on a single plasmid using one yeast transformation procedure is possible at a very high success rate (70-100% efficiency) (Shao, 2008). To assemble multiple cassettes on one plasmid, the cassettes are first amplified by PCR with flanking homology regions to the plasmid backbone or next cassette in the pathway. Then, a single copy plasmid (pRS410 series, Stratagene) was linearized and transformed along with the amplified PCR products into *S. cerevisiae*. Assembled plasmids in *S. cerevisiae* are amplified in *E. coli* and confirmed for correct constructs (Figure 2.1). Due to a lack of restriction enzymes sites and prior low success rate of traditional cloning, the DNA assembler method was used for the construction of vectors for cellobiose and 2,3-BDO pathway integration in *S. cerevisiae*.

2.2 MATERIALS AND METHODS

2.2.1 *Strains and plasmids*

S. cerevisiae CEN.PK2-1C (*MATα, leu2-3,112 ura3-52 trp1-289 his3-Δ1 MAL2-8c*) was used as the host strain for *in vivo* DNA assembly. The pRS410 series plasmids were used as backbones for DNA assembler and pRS400 series plasmids were used for constructing integration vectors (Euroscarf, Frankfurt, Germany).

2.2.2 *Culture conditions*

CEN.PK was grown in yeast extract peptone (YP) medium (10 g/L yeast extract, 20 g/L Bacto peptone, and 20 g/L glucose, pH 6.0) overnight prior to use in transformations procedures. Synthetic complete (SC) medium (6.7 g/L yeast nitrogen base without amino acid, 20 g/L

cellobiose or glucose and 0.625 mg/L CSM without His, Leu, Trp and Ura, pH 6) with appropriate nucleotides and amino acids was used for selection of transformants and cultivation of CEN.PK after transformation.

2.2.3 *DNA manipulation*

Genes of interest were PCR amplified by primers with 20 bp annealing sequence and ~40 bp homology sequence to amplify entire cassettes (promoter, gene, and terminator) for the 2,3-BDO pathway assembly. The promoter and gene were amplified together and the terminator was amplified separately in the cellobiose pathway DNA assembly. Overexpression plasmids (pRS420 series) containing *alsS*, *alsD*, *cdt-1*, and *gh1-1* were used as templates for PCR. The TEF terminator was amplified from the genomic DNA of *S. cerevisiae* D452-2 strain. Amplified PCR products were PCR purified or gel purified prior to transformation.

Backbone plasmid DNA was digested with restriction enzymes for linearization. pRS413 was linearized by XhoI and XbaI and pRS415 was linearized by HindIII and SpeI. Both digested plasmids were gel purified.

High efficiency yeast transformation

Yeast transformations were done by the lithium acetate/single stranded DNA/PEG method (Gietz, 2007). CEN.PK was cultured overnight and inoculated in a 125 mL flask with 25mL of YP with 20 g/L glucose at OD_{600}=0.1. Once reaching OD_{600}=0.8, the cells were harvested and washed with LiAc. After washing, a transformation mix consisting of PEG, LiAc, ssDNA, and desired DNA was added to the yeast cells and incubated at 42^{o}C for 40 min. The transformation mix was then removed and the cells were resuspended with water. Cells were plated on SC plates and incubated at 30^{o}C for two days.

2.2.4 *Construct confirmation*

After preparation of assembled DNA from yeast and amplification in *E. coli*, PCR and restriction enzymes digests were used for construct confirmation. PCR primers were designed to amplify the entire assembled product consisting of two cassettes. A combination of single cut restriction enzyme sites were used for additional confirmation.

2.2.5 *Construction of integration vectors*

The *alsS* and *alsD* insert and pRS403 backbone were digested by SacI and SalI. After digestion and appropriate CIP treatment and purification of DNA products, the DNA fragments were ligated and transformed into *E. coli*. Several *E. coli* colonies were cultured for isolation of plasmid DNA for confirmation of proper ligation. The *cdt-1* and *gh1-1* inserts and pRS405 backbone were digested by HindIII and XmaI. Other procedures remained the same.

2.3 RESULTS

2.3.1 *Assembly of 2,3-BDO pathway integration vector*

In our DNA assembler plasmid construct design, we expressed *alsS* followed by *alsD* (Figure 2.2A). All primers used for the amplification of genes typically have a 20 bp annealing region with a 40 bp homology region and restriction enzyme sites added. After amplifying *alsS* and *alsD* from their corresponding multicopy plasmids, PCR products were PCR purified and confirmed by gel electrophoresis (Figure 2.3A). The plasmid (pRS413) used for assembly was linearized and gel purified. Subsequently, purified PCR products and plasmid were transformed into *S. cerevisiae* CEN.PK using high-efficiency yeast transformation. After colonies formed, 16 random transformants were selected for confirmation of correct assembly. The cells were cultured to extract plasmid DNA for transformation into *E. coli* for amplification of the assembled plasmid. Plasmid was then isolated from *E. coli* for PCR and restriction enzyme digest confirmations. From the 16 transformants, only one harbored the correctly assembled

construct. The correctly assembled plasmid was then digested to release the *alsS* and *alsD* cassettes, which were then gel purified. The integration plasmid pRS403 was digested with the same restriction sites flanking the *alsS* and *alsD* cassettes. Ligation and transformation of the pRS403-alsS-alsD plasmid followed. Confirmation of successful ligation was done by PCR and restriction enzyme digestion.

2.3.2 *Assembly of cellobiose utilization pathway integration vector*

The procedure for cellobiose pathway integration vector design (Figure 2.2B) and assembly were done in a similar fashion as the 2,3-BDO pathway assembly. Primers were designed to amplify *cdt-1* and *gh1-1* along with the promoter with flanking homology regions and added restriction enzyme sites. For this assembly, primers were also designed to amplify the terminators (*cdt1-1* with TEF and *gh1-1* with CYC) separately and assemble them to the ends of the genes to remove unwanted restriction enzyme sites. In addition, a 40 base pair linker sequence was added after the terminator of *cdt-1* and before the promoter of *gh1-1*. After preparing all necessary DNA and gel electrophoresis confirmation of fragment size (Figure 2.3B), the PCR fragments and digested plasmid (pRS415) were transformed into CEN.PK. For screening of the correct construct, 5 colonies were randomly picked. From the 5 colonies, all plasmids were correctly assembled. One of the correctly assembled plasmids was chosen for digestion and construction of a pRS405-cdt1-gh1-1 integration vector.

2.4 DISCUSSION

As restriction sites and low success rates of traditional cloning hindered construction of integration vectors for cellobiose and 2,3-BDO pathways, DNA assembler was identified as a method to overcome these concerns. Though the successful isolation of correctly assembled cellobiose and 2,3-BDO pathway integration plasmids was accomplished, there were many road

blocks throughout the process. Initially, our design for the 2,3-BDO integration plasmid was to use the same promoter and terminator for *alsS* and *alsD* expression. However, we found that this caused inherent problems with our primer design. Due to the amount of sequence similarity with using two of the same promoters and terminators, our primers either dimerized or annealed to an incorrect location on the template. To alleviate this issue, 30 bp of the *alsS* terminator was removed at the 3' end. With this simple change, primer design with successful amplification was possible. In order to avoid this problem for the cellobiose pathway plasmid construction, we decided to change the terminator for *cdt-1* to avoid difficulty with primer design. In addition, we added a 40 base pair linker sequence between the terminator of *cdt-1* and promoter of *gh1-1*. This linker sequence was not found elsewhere in our DNA assembler fragments and thus allowed additional accuracy for assembler success.

Due to the innate amount of restriction enzymes present within *alsS*, *alsD*, *cdt-1*, *gh1-1*, promoters, and terminators and undesirable multicopy plasmid design (lack of removing many MCS restriction enzyme sites), few if any restriction sites were available for traditional cloning. Especially, the use of a single restriction site in traditional cloning disallows the possibility for selecting directionality of the inserted fragment. Thus, DNA assembler allowed us to simultaneously assemble two cassettes and thus required only two unique restriction sites for later cloning instead of three restriction sites for traditional cloning of each cassette sequentially. Restriction enzyme sites are easily introduced in the primer design for DNA assembler. Once a correctly assembled plasmid is isolated, the cassettes can be digested out and cloned into other pRS series plasmids.

DNA assembler was also a useful method for the construction of an integration plasmid for cellobiose utilization. The multicopy plasmids used for the amplification of *cdt-1* and *gh1-1*

cassettes contained the remaining restriction sites from the original multipcopy plasmid construction process. As a result, there were no restriction sites available for any type of cloning procedure. As the remaining restriction sites were present between the gene and terminator, it was possible to remove them by assembling the terminator to the end of the gene, instead of prior amplification of the entire cassette. With this method, we were able to amplify the promoter and gene together and then assemble the terminator, making more restriction sites available for cloning of the entire two cassette construct.

As Shao et al. noted during the DNA assembler process, high success rates of correct assembly could be easily achieved (2008). In the case of our 2,3-BDO pathway assembly, we were only able to achieve a success rate of 6%. Unlike Shao et al., we did not use different promoters and terminators for the expression of genes. Consequently, homology regions were present in our design and hindered desired assembly. In the cellobiose pathway assembly, we achieved a 100% success rate for correct assembly, thus indicating the importance of reducing the amount of homology regions.

In order maintain the expression of cellobiose and 2,3-BDO pathway genes more stability, we constructed plasmids for integration of these pathways into the genome of *S. cerevisiae*. The DNA assembler method was used to do a one-step construction of a two gene integration plasmid using *in vivo* homologous recombination in *S. cerevisiae*. Utilization of different promoters and terminators is recommended for a higher success rate of correct assembly. With the correctly assembled product, we are able digest and clone the construct into any pRS series plasmid. Once the constructed integration plasmid is completely finished, integration of these genes into the *S. cerevisiae* genome will allow for more stable cellobiose consumption and 2,3-BDO production in SOS2. Also, the integration of genes will allow future evolutionary

engineering of cells in order to develop a faster cellobiose consuming and possibly a better 2,3-BDO producing engineered *S. cerevisiae* strain for industrial scale production of 2,3-BDO.

2.5 REFERENCES

Christianson, T. W., Sikorski, R. S., Dante, M., Shero, J. H., & Hieter, P. (1992) Multifunctional yeast high-copy-number shuttle vectors. Gene, 110(1), 119-122.

Gietz, R. D., & Schiestl, R. H. (2007) High-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. Nature Protocols, 2(1), 31-34.

Shao, Z., Zhao, H., & Zhao, H. (2009) DNA assembler, an in vivo genetic method for rapid construction of biochemical pathways. Nucleic Acids Research, 37(2).

2.6 TABLE

Table 2.1 List of primers used in this study.

Target DNA	Primer name	Sequence	Restriction Enzyme	Template
alsS	*Jin971*	GCAATTAACCCTCACTAAAGGGAACAAAAGCTGGAGCTCAGTTTATCATTATCAATACTC	*SacI*	pRS426-GPD-alsS-CYC1
	Jin972	TATTCTTTGAAATGGCGAGTATTGATAATGATAAACTAAACCTTCTCAAGCAAGGTTTTC	---	
alsD	*Jin973*	CATGTAACATTATACTGAAAACCTTGCTTGAGAAGGTTTAGTTTATCATTATCAATACTC	---	pRS423-GPD-alsD-CYC1
	Jin974	GCGCGCGTAATACGACTCACTATAGGGCGAATTGGGTACCGTCGACGGCCGCAAATTAAAGCCTTCGAGCG	*SalI*	
CDT-1	*Jin1070*	CCGCGGTGGCGGCCGCTCTAGAACTAGTGGATCCCCCGGGGTGAGTAAGGAAAGAGTGAG	*Xma*	pRS426-PGK1-CDT-1-CYC1
	Jin1071	GTATATAAAAATATTATATGGAAGCAATAATTATTACTCTCAGCAACGATAGCTTCGGAC	---	
TEF-term	*Jin1072*	TCCAGGCCGACGGCCATGTGTCCGAAGCTATCGTTGCTGAGAGTAATAATTATTGCTTCC	---	*S. cerevisiae* D452-2
	Jin1073	CGTACCTCGCTGGATGAATTGCCGCAGTTTATCAATGTGCCGCTGACATGGTTTCTTTAG	---	
GH1-1	*Jin1074*	GCACATTGATAAACTGCGGCAATTCATCCAGCGAGGTACGGTGAGTAAGGAAAGAGTGAG	---	pRS425-PGK1-GH1-1-CYC1
	Jin1075	GGAGGGCGTGAATGTAAGCGTGACATAACTAATTACATGATTAGTCCTTCTTGATCAAAG	---	
CYC1-term	*Jin1076*	TTGAAGCCGCTCTTTGACTCTTTGATCAAGAAGGACTAATCATGTAATTAGTTATGTCAC	---	pRS425-PGK1-GH1-1-CYC1
	Jin1077	TACCGGGCCCCCCCTCGAGGTCGACGGTATCGATAAGCTTGGCCGCAAATTAAAGCCTTC	*HindIII*	
alsS-alsD	*Jin1032*	CCGGCTCCTATGTTGTGTGG	---	pRS423-alsS-alsD
	Jin1039	GGGGGATGTGCTGCAAGGCG	---	
CDT-1-GH1-1	*Jin1032*	CCGGCTCCTATGTTGTGTGG	---	pRS415-CDT-1-GH1-1
	Jin1039	GGGGGATGTGCTGCAAGGCG	---	

2.7 FIGURES

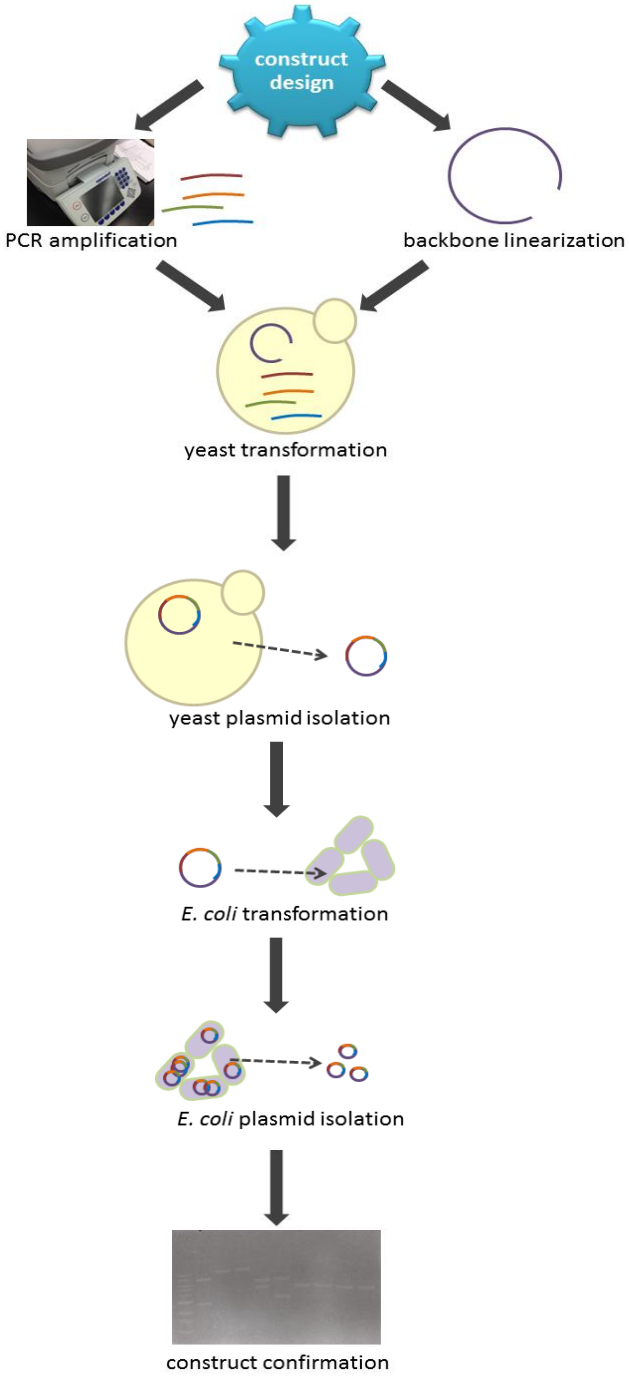
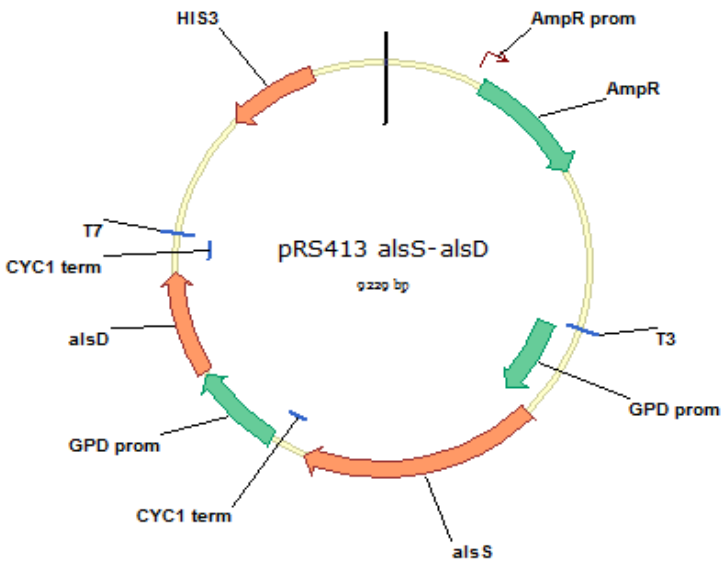


Figure 2.1 Diagram of DNA assembler process.

A



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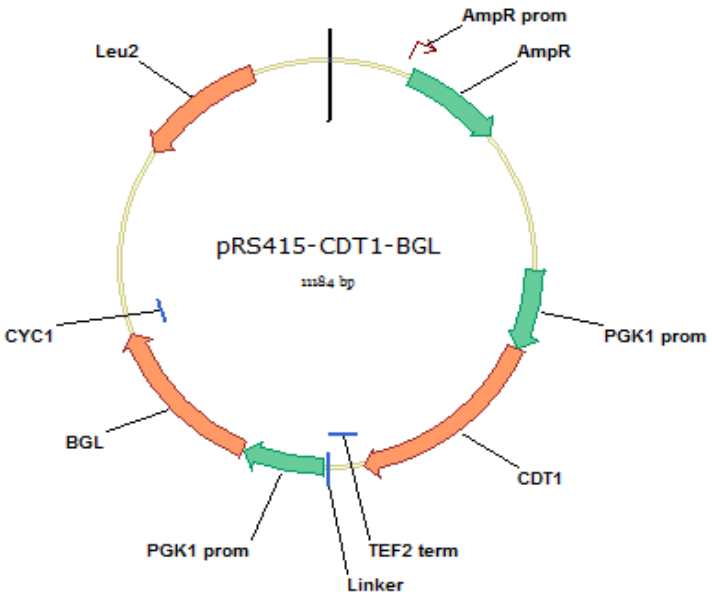
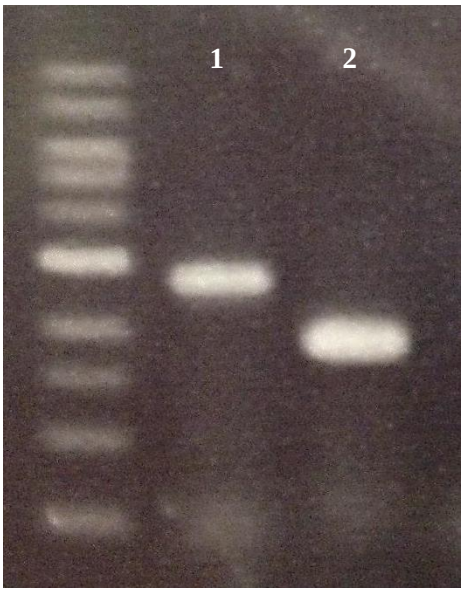


Figure 2.2. Map of assembled 2,3-BDO pathway on pRS413 (A). Map of assembled cellobiose pathway on pRS415 (B).

A



B

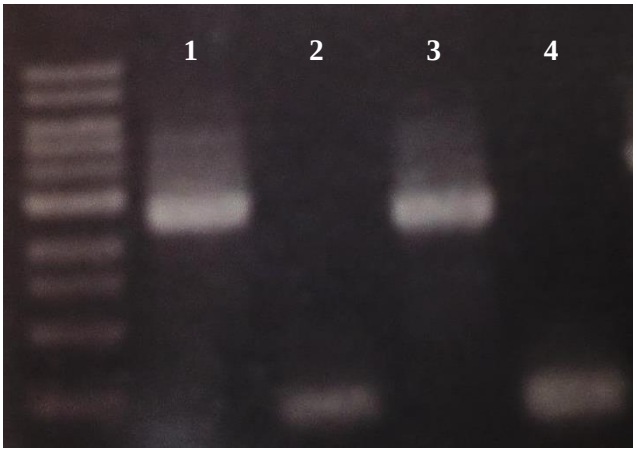


Figure 2.3. Agarose gel electrophoresis of PCR amplified alsS cassette (lane 1) and alsD cassette (lane 2) (A). Agarose gel electrophoresis of PCR amplified PGK1-CDT-1 (lane 1), TEF2 (lane 2), PGK1-GH1-1 (lane 3), and CYC1 (lane 4) (B).

CHAPTER 3: CONCLUSION

As a result of environmental concerns on greenhouse gas emissions, the ability to synthesize fuels and chemicals from renewable resources is vital. 2,3-BDO is emerging as a valuable chemical due to its diverse applications. However, current microbial production of 2,3-BDO from renewable materials utilizes potential human pathogens. In order to alleviate this problem, we proposed to engineer *S. cerevisiae* for producing 2,3-BDO from cellobiose, a component of cellulosic hydrolysates. We first introduced a 2,3-BDO production pathway in an engineered cellobiose utilizing strain. Two heterologous genes were introduced to allow the improved production of 2,3-BDO in *S. cerevisiae*. Due to accumulation of ethanol during the fermentation, it was proposed to utilize a strain lacking ethanol production as the host strain. Accordingly, we introduced the cellobiose and 2,3-BDO pathway into a *S. cerevisiae* strain unable to produce ethanol. Growth rate of the ethanol deficient producer was slow, but served as a better host for 2,3-BDO production. In order to further engineer our strains for improved 2,3-BDO production, we created integration plasmids for the stable maintenance of 2,3-BDO production and cellobiose utilization genes using the DNA assembler method.

Future studies include the integration of 2,3-BDO and cellobiose genes into the genome of an ethanol negative strain to improve 2,3-BDO production through the process of evolutionary engineering. Other methods of eliminating or decreasing ethanol production in *S. cerevisiae* may be investigated to produce a host strain with less growth rate effects. However, as 2,3-BDO production can only generate one reducing equivalent, production of only 2,3-BDO may not be feasible without engineering of enzymes for cofactor requirements.