Master's Thesis of Injae Cho

Stereoselective production of *(S)*-acetoin in *Saccharomyces cerevisiae*

***Saccharomyces cerevisiae*의 입체선택적**

***(S)*-아세토인 생산**

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Abstract

Acetoin, a GRAS (Generally Recognized as Safe) substance which can be applied to a wide variety of fields, including food additive, cosmetics, detergents, and pharmaceuticals. It naturally exists in two stereoisomeric forms: *(R)*-acetoin and *(S)*-acetoin, which is separable by gas chromatography. Production of *(R)*-acetoin is rather well known, both by fermentative ways and biocatalytic ways. But production of *(S)*-acetoin, because of its complex metabolic production pathway including spontaneous non-enzymatic oxidation, is still on its development. Especially by fermentation starting from glucose, little is known. In this study, stereoselective fermentation of *(S)*-acetoin from glucose using *Saccharomyces cerevisiae* as a production platform strain will performed.

First, starting strains and *Bacillus subtilis* alsS expression responsible for the conversion of pyruvate into *(S)*-α-acetolactate, were optimized for primary experiments. JHY901 and JHY901-9 utilized in previous study with *(R)*-acetoin production were not suitable for *(S)*-acetoin production. Also, expression of alsS alone critically impacted cell growth, and expression via strong promoters such as *TDH3* or *TEF1* were not suitable as well. In this study, *ADH1* promoter was used for expressing alsS.

Next, *S. cerevisiae* endogenous *BDH1* gene responsible for the conversion of *(R)*-acetoin into *(R,R)*-2,3-butanediol and conversion of diacetyl into *(R)*-acetoin, was deleted from wild-type strain. Then, endogenous pyruvate decarboxylase *PDC* was deleted for the blockage of *(R)*-acetoin formation. *PDC* is known for its anomalous carboligase activity, which is responsible for conversion of pyruvate and acetaldehyde into acetoin, and two molecules of acetaldehyde into one molecule of acetoin. Previous studies regarding protein structure modification of

yeast *PDC* has shown that *PDC* catalyzes formation of acetoin with the *(R)*-acetoin optical ratio of 40~60 %. In this study, deletion of *PDC1* and *PDC5* genes blocked both *(R)* and *(S)*-acetoin formation in *S. cerevisiae* endogenous pathway. Acetoin was produced with the *(S)*-acetoin enantiomeric excess of 90 %, and 0.38 g/L of *(S)*-acetoin was produced in *bdh1Δ pdc1Δ pdc5Δ* (JHYA111) strain. Finally, deletion of endogenous *ORA1* gene responsible for pyruvate conversion into 2,3-dimethylglycerate, was deleted from JHYA111, resulting in JHYA200. JHYA200 produced acetoin with the *(S)*-acetoin enantiomeric excess of 88 %, and 0.66 g/L of *(S)*-acetoin was produced.

Keyword: *Saccharomyces cerevisiae*, *(S)*-acetoin, Bdh1 (butanediol dehydrogenase), Pdc (pyruvate decarboxylase), diacetyl reductase Ora1, stereoselective fermentation

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Chapter 1.

Introduction

1.1 Acetoin

Acetoin is a GRAS (Generally Recognized as Safe) substance used in various fields. Because of its pleasant buttery flavor, acetoin is used in food additive, cosmetics, electric cigarette additive, detergents, and plant growth promoter. [6], [7], [8], [9], [10] It is also used as a precursor for synthesis of various chemical, including acetylbutanediol, 2,3,5,6-tetramethylpyrazine (TTMP, herb), diacetyl, and so on. [11], [12], [13], [14] Acetoin is known to be a key metabolite during detoxification of acetaldehyde produced after ethanol ingestion as well. [15]

Because of its potential for various applications, many biotechnological routes for natural acetoin production have been developed. Acetoin is a C4-compound consisting of a single chiral center, and it exists as a racemic mixture naturally, *(R)*-acetoin and *(S)*-acetoin, respectively. [16] Synthesis of optically pure substance is crucial for the formation of liquid crystal materials, potential pharmaceuticals and α-hydroxyketone derivatives. In this study, stereoselective production of optically pure, chiral *(S)*-acetoin will be performed in yeast strain *Saccharomyces cerevisiae*, which is a safe and robust cell factory platform for industrial production of chemical goods. [1]

1.2. Previous studies with producing acetoin

Previous studies regarding the production of chiral acetoin have been taken place. Key strategy for the production of acetoin is to enhance substrate metabolism (glycolysis), block by-product production flux, and weaken the catabolism of acetoin. Measurement of chiral acetoin can be done by gas chromatography, since the two stereoisomers can only be separated by GC. Enantiomeric excess (e.e.), a parameter for optical purity, was calculated by the percentage of $([S]-[R])/([S]+[R]) \times 100$ (%). [6]

(R)-acetoin, the enantiomer of *(S)*-acetoin, is relatively easy to produce by enzymatic reactions of α-acetolactate synthase (ALS) and α-acetolactate decarbyxylase (ALDB). [5] *(R)*-acetoin was able to be produced by fermentation in various strains using glucose, sucrose, lactose, xylose, methanol as substrate. [6] Fermentative production of *(R)*-acetoin was performed in *B.subtilis* CGMCC 13141 with the titer of 83.7 g/L and enantiomeric excess (e.e.) of 98.8%, *S. cerevisiae* JHY617-SDN with titer of 100.1 g/L, *S. cerevisiae* JHY903-159, *Corynebacterium glutamicum* CGS11 with titer of 102.45 g/L and (e.e.) higher than 90%, *B. subtilis* F126-2 with titer of 60.48 g/L, and so on. [1], [3], [16], [21], [22] Biocatalytic *(R)*-acetoin production was taken place using 2,3-butanediol, pyruvate, glycerol, racemic lactate as well. [23], [24], [25], [26], [27]

For the production of *(S)*-acetoin, several studies of biocatalytic production model were proposed. Using diacetyl or meso-2,3-butanediol as substrate, enzymes were expressed by protein expression strains, and then treated with substrates. [28], [29] One research with culturing *K. pneumoniae* CICC 10011 and *B. subtilis* 168 resulted in a titer of 56.7 g/L and (e.e.) of 96.2. [20] Starting from glucose, *K. pneumoniae* CICC 10011 produced mixture of meso-2,3-butanediol and *(S,S)*-2,3-butanediol, and above the two substances meso-2,3-butanediol was again converted into *(S)*-acetoin by *BDH* in resting cells of *B. subtilis* 168.

As far as we know, there is a single study in which *(S)*-acetoin was produced by fermentation. The precursor for *(S)*-acetoin, diacetyl, can only be produced by spontaneous decarboxylation of *(S)*-α-acetolactate and can be accumulated to a low amount, and this is why *(S)*-acetoin production by fermentation is difficult. Liu and colleagues deleted ALDC in *L. lactis* for *(R)*-acetoin formation as well as by-product (lactate, acetate, and ethanol) pathway to decrease undesired product concentration. Diacetyl reductase from *E. cloacae* was expressed, 0.2 μg/mL hemin was added for cofactor balance, and ferric ion was added to increase spontaneous decarboxylation. The final recombinant strain *L. lactis* CS4701 resulted in a titer of 5.8 g/L. [5] However, in this study, *(S)*-acetoin was measured by high-pressure liquid chromatography (HPLC). There is no reference whether the product is optically pure, since acetoin is separable only be gas chromatography, to the best of our knowledge.

1.3. Production of *(S)*-acetoin in *Saccharomyces cerevisiae*

Production of optically pure acetoin is divided into three steps: 1) adjusting glucose consumption for glycolysis, 2) blocking by-product formation flux, 3) Strengthening the flux for chiral acetoin production.

The metabolic pathway of *S. cerevisiae* to produce acetoin and 2,3-butanediol is shown in Figure 1. [1] Starting from glucose uptake, glucose is converted into two molecules of pyruvate by glycolysis. Pyruvate is then converted into acetaldehyde with the existence of pyruvate decarboxylase *PDC* 1,5,6, and again converted into ethanol by alcohol dehydrogenase ADH 1-5. [2]

PDC, possessing the role of decarboxylation of pyruvate, is known to have anomalous carboligase (decarboxylation and ligation) activity to condensate pyruvate and acetaldehyde into one acetoin or two acetaldehyde molecules into one acetoin. [17], [18] Studies have shown that mutating yeast pyruvate decarboxylase (YPDC) to form E477Q, D28A, and D28N could help synthesizing more *(R)*-acetoin and *(S)*-α-acetolactate than wild-type *PDC*, and that the major form of acetoin in this particular carboligation is *(R)*-form. [19] Other metabolic pathway that pyruvate undergoes is where *(S)*-α-acetolactate is formed with the aid of endogenous Ilv2 and Ilv6. This enzymatic reaction is taken place in mitochondria. Pyruvate can be converted into *(S)*-α-acetolactate with the expression of *B. subtilis* alsS (α-acetolactate synthase) as well.

(S)-α-acetolactate can be converted into *(R)*-acetoin with the expression of *B. subtilis* alsD (α-acetolactate decarboxylase), resulting in relatively stable pathway. [3] However, *(S)*-acetoin can be produced in a rather complex way, by converting *(S)*-α-acetolactate by spontaneous decarboxylation with aeration to form diacetyl, and expression of diacetyl reductase (*YPR1*, *ARA1*, and *ORA1*) to convert diacetyl into *(S)*-acetoin. Previous studies have shown that among *S. cerevisiae* oxidoreductases, only Ypr1, Ara1, and Ora1 were shown to have affinity to the

substrate. [1] Properties regarding the affinity of each diacetyl reductases to the substrates are shown.

Blocking the formation of by-products can increase acetoin production. Previous studies with deletion of *S. cerevisiae ADH1-5* and *GPD1,2* blocked ethanol and glycerol formation. Deletion of such by-product-forming enzymes can yield serious growth defect, causing cofactor imbalance, but expressing *L. lactis* noxE is shown to be beneficial for growth recovery. [2] Deletion of endogenous 2,3-butanediol dehydrogenase, *BDH1*, decreased 2,3-butanediol formation. [3] It was also shown that 2,3-dimethylglycerate can be formed with the presence of endogenous *ORA1*, and deletion of this gene can lead to blocking 2,3-dimethylglycerate formation. [1]

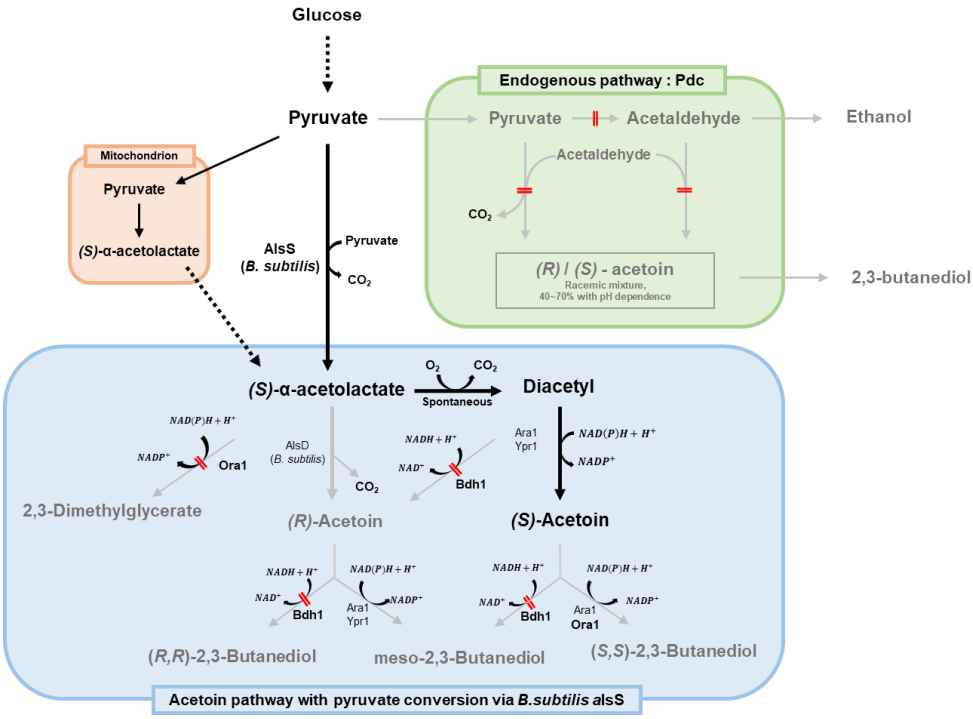

Figure 1. Acetoin production pathway in *Saccharomyces cerevisiae*

Figure above represents the metabolic pathway of *S. cerevisiae* to produce *(S)*-acetoin. *Bacillus subtilis (S)*-α-acetolactate synthase alsS is introduced, and endogenous formation *(R)*-acetoin is blocked by deletion of pyruvate decarboxylase *PDC.* By-product (2,3-butanediol and 2,3-dimethylglycerate) forming pathways are blocked by deletion of 2,3-butanediol dehydrogenase (*BDH1*), and *ORA1*, respectively.
Red lines represent endogenous pathways that were blocked by deletion of endogenous genes (bold), and thick arrows indicate pathway to be reinforced.

1.4. Research goals

The purpose of this study is to produce optically pure *(S)*-form of acetoin in GRAS strain, *Saccharomyces cerevisiae*. Previous studies have found that *S. cerevisiae* alone produces acetoin with the presence of pyruvate decarboxylase *PDC* with the enantiomeric excess of 40~70% of *(R)*-acetoin depending on pH, [19] so the key strategy is to delete the following gene to block *(R)*-acetoin formation. Among three *PDC* genes, *PDC1* and *PDC5* are deleted for the construction of *(S)*-acetoin producing strain. Acetoin production pathway is reinforced with the expression of *B. subtilis* alsS. Formation of by-product, especially 2,3-butanediol, is blocked by deletion of endogenous butanediol dehydrogenase *BDH1*. Also, in order to block 2,3-dimethylglycerate formation, endogenous *ORA1*, a diacetyl reductase, is deleted as well.

Chapter 2.

Materials and Methods

2.1 Strains and culture conditions

S. cerevisiae strains used in this study derived from CEN.PK2-1C strain are shown in Table 1. JHYA100, *BDH1* deleted strain from CEN.PK2-1C, was constructed via CRISPR/*Cas9*-mediated genome editing system. The plasmid coex416-*Cas9*-*BDH1* gRNA consisting of *Cas9* and *BDH1* single guide RNA was transformed with donor DNA. For curing plasmid, cells were cultivated in YPD media, 2 days. For the construction of JHY101, JHY102, JHY103, JHY111, JHY112, and JHY113, *Cre/LoxP* mediated homologous recombination system was used with the proper donor DNA. Donor DNA was constructed in the form of plasmid, consisting of up/down 300 bp of the target gene, and *LoxP*-*Ura*^{R}-*LoxP* cassette between. Plasmids were treated with restriction enzyme SmiI for 1 hour, and then inactivated in 65℃ for 15 minutes. Donor DNA was transformed and cells were spread on Sc-Ura plate, and incubated for 4 days. Again, the cells were transformed with pRS413TEF-Cre, and curing of plasmid was performed in YPD media for 2 days. Integration of alsS to *ADH1* site and deletion of *ORA1* were performed via *Cre/LoxP* mediated homologous recombination system as well, resulting in JHYA121, JHY122, JHY123, and JHYA200, respectively. Other strains, JHY901 and JHY901-9, were obtained from laboratory storage unit.

Cultures with cells harboring pRS413-derived vectors were taken place in Sc media (20 g/L glucose, 6.7 yeast nitrogen base without amino acids, and 1.4 g/L amino acids lacking His, Trp, Leu, and Ura) supplemented with auxotrophic nutrient His. Cells were cultured in 30℃ shaking incubator with the rpm of 170.

Table 1. Strains used in this study

Strain	Genotype and description	Reference
CEN.PK2-1C	*MATa ura3-52 trp1-289 leu2-3 his3Δ::loxp1 MAL2-8C SUC2*	[1]
JHY901	CEN.PK2-1C *adh1Δ::loxp adh2Δ::loxp adh3Δ::loxp adh4Δ::loxp adh5Δ::loxp gpd1Δ::loxp gpd2Δ::loxp bdh1Δ::loxp*	[1]
JHY901-9	JHY901 *ora1Δ*	[1]
JHYA100	CEN.PK2-1C *bdh1Δ*	This study
JHYA101	JHYA100 *pdc1Δ*::*loxp*	This study
JHYA102	JHYA100 *pdc5Δ*::*loxp*	This study
JHYA103	JHYA100 *pdc6Δ*::*loxp*	This study
JHYA111	JHYA102 *pdc1Δ*::*loxp*	This study
JHYA112	JHYA103 *pdc1Δ*::*loxp*	This study
JHYA113	JHYA103 *pdc5Δ*::*loxp*	This study
JHYA121	JHYA111 *adh1Δ*::*loxp*::alsS-*loxp*	This study
JHYA122	JHYA112 *adh1Δ*::*loxp*::alsS-*loxp*	This study
JHYA123	JHYA113 *adh1Δ*::*loxp*::alsS-*loxp*	This study
JHYA200	JHYA111 *ora1Δ*::*loxp*	This study
JHYA100-O	JHYA100 harboring pRS413ADH	This study
JHYA101-O	JHYA101 harboring pRS413ADH	This study
JHYA102-O	JHYA102 harboring pRS413ADH	This study
JHYA103-O	JHYA103 harboring pRS413ADH	This study
JHYA111-O	JHYA111 harboring pRS413ADH	This study
JHYA112-O	JHYA112 harboring pRS413ADH	This study
JHYA113-O	JHYA113 harboring pRS413ADH	This study
JHYA100-S	JHYA100 harboring pRS413ADH-alsS	This study
JHYA101-S	JHYA101 harboring pRS413ADH-alsS	This study
JHYA102-S	JHYA102 harboring pRS413ADH-alsS	This study
JHYA103-S	JHYA103 harboring pRS413ADH-alsS	This study
JHYA111-S	JHYA111 harboring pRS413ADH-alsS	This study
JHYA112-S	JHYA112 harboring pRS413ADH-alsS	This study
JHYA113-S	JHYA113 harboring pRS413ADH-alsS	This study
JHYA111-Y	JHYA111 harboring pRS413TEF-YPR1	This study
JHYA111-SY	JHYA111 harboring pRS413_pADH1-alsS_pTEF-YPR1	This study
JHYA121-Y	JHYA121 harboring pRS413TEF-YPR1	This study
JHYA121-A	JHYA121 harboring pRS413TDH3-ARA1	This study
JHYA121-YA	JHYA121 harboring pRS413TEF-YPR1_pTDH3-ARA1	This study
JHYA200-O	JHYA200 harboring pRS413ADH	This study
JHYA200-S	JHYA200 harboring pRS413ADH-alsS	This study
JHYA200-SY	JHYA200 harboring pRS413ADH-alsS_pTEF-YPR1	This study

2.2 Plasmid construction

Plasmids and primers used in this study are shown in Table 2 and Table 3. The empty vectors pRS413ADH, pRS413GPD, and pRS413TEF were acquired from laboratory storage unit. The alsS gene from *B.subtilis* was obtained from pRS413GPD-alsS, amplified by PCR with the appropriate primers. alsS sequence was cloned to empty vectors between BamHI and XhoI site.

BDH1 disruption was performed with coex416-*Cas9*-*BDH1* gRNA. Plasmid was generated from coex416-*Cas9*-HXK2 gRNA previously constructed, by replacing 20-bp of *HXK2* targeting gRNA sequence with *BDH1*-specific gRNA sequence using DpnI mediated site-directed PCR mutagenesis. Donor DNA was obtained by PCR with primers in length of 50-bp and 20-bp overlapping each other, resulting in up 40-down 40.

For the construction of PDC, *ADH1*, *ORA1* disruption donor plasmids, up/downstream 300 base pair of the target gene were used as homologous arms, and *LoxP*-*Ura*^{R}-*LoxP* cassette was inserted in between. Upstream sequence, *LoxP*-*Ura*^{R}-*LoxP*, and downstream sequence were obtained by PCR and cloned to co-expression vector with SacI, XhoI, NotI, and PfoI, respectively. For alsS integration donor plasmid, alsS sequence was inserted between up sequence and *LoxP*-*Ura*^{R}-*LoxP* sequence with BamHI and XhoI. Plasmids were treated with SmiI for 1 hr in 37°C incubator, and inactivated in 65°C for transformation.

Table 2. Primers used in this study

Primer	Sequence	Reference
alsS BamHI F	tcgaGGATCCATGACAAAAGCAACAAAAGAA	This study
alsS XhoI R	tcgaCTCGAGtcaGAGAGCTTTCGTTTTCATGA	This study
URA-LoxP XhoI F	gcgcCTCGAGATAACTTCGTATAGCATACATTATACGAA GTTATTTCACACCGCATAGGGTAATAACTG	This study
URA-LoxP NotI R	gcatGCGGCCGCATAACTTCGTATAATGTATGCTATACG AAGTTATCCATACCACAGCTTTTCAATTCAATTCAT	This study
PDC1 up SacI-SmiI F	tcgaGAGCTCATTTAAATCCCGTTTTTCTGTTAGACGG	This study
PDC1 up XhoI R	tcgaCTCGAGTGACTGTGTTATTTTGCGTG	This study
PDC1 down NotI F	tcgaGCGGCCGCGGTTCATATTATTACTGCACTG	This study
PDC1 down SmiI-PfoI R	tcgaTCCNGGAATTTAAATTCGAATTCGCTGGCAGTTTT	This study
PDC5 up SacI-SmiI F	tcgaGAGCTCATTTAAATTCCAAAGGTCGCGTTTCTTTTA	This study
PDC5 up XhoI R	tcgaCTCGAGGTATTGTGTTGTTCTCTTTGAG	This study
PDC5 down NotI F	tcgaGCGGCCGCCATGATTCAACGTTTGTGTA	This study
PDC5 down SmiI-PfoI R	tcgaTCCNGGAATTTAAATGGTTAAAGATCACACCACCC	This study
PDC6 up SacI-SmiI F	tcgaGAGCTCATTTAAATGTGACAGAAAAAGCCCACAA	This study
PDC6 up XhoI R	tcgaCTCGAGGTTGGCAATATGTTTTTGCT	This study
PDC6 down NotI F	tcgaGCGGCCGCGCCATTAGTAGTGTACTCAAAA	This study
PDC6 down SmiI-PfoI R	tcgaTCCNGGAATTTAAATGCGGCTGCGGAATTTTATAA	This study
ORA1 up SacI-SmiI F	tcgaGAGCTCATTTAAATGCTGAACTAAAACGTAAGCA	This study
ORA1 up XhoI R	tcgaCTCGAGTATCAAAATACGTTCTCAATGTTC	This study
ORA1 down NotI F	tcgaGCGGCCGCACCTAGCTAAACTAAGTAAATCTG	This study
ORA1 down SmiI-PfoI R	tcgaTCCNGGAATTTAAATTTTTTGTCCTTGTAGGTTACT	This study
ADH1 up SacI-SmiI F	tcgaGAGCTCATTTAAATACAGCACCAACAGATGTCGT	This study
ADH1 up BamHI R	tcgaGGATCCTGTATATGAGATAGTTGATTGTATGCT	This study
ADH1 down NotI F	tcgaGCGGCCGCGCGAATTTCTTATGATTTATGATTTTT	This study
ADH1 down SmiI-PfoI R	tcgaTCCcGGAATTTAAATTTGTCCTCTGAGGACATAAAA TACA	This study
BDH1 gRNA F	GGTAAGCAAAGGATTGAGGAGTTTTAGAGCTAGAAAT AGCAAGTTAAAATAAGGC	This study
BDH1 gRNA R	TCCTCAATCCTTTGCTTACCGATCATTTATCTTTCACTG CGGAGAAG	This study
coex, MauBI F	gactCGCGCGCGGGAACAAAAGCTGgagctC	This study
coex, AscI-NotI-MluI, R	GACTACGCGTGCGGCCGCTAATGGCGCGCCATAGGG CGAATTGGGTACC	This study
BDH1 donor F	CCAAGTCAGAAAAAAAAAAAAAAAAGGAACTAAAAAAAG TTTTAATTAATTTGACAAAATA	This study
BDH1 donor R	TCTTAGATACTACAAATGAGCCGCGAGGGGCCCCAAA TATTATTTTGTCAAATTAATTAA	This study
PDC1 up confirm F	CATGAGGGTAACAACATGCG	This study
PDC5 up confirm F	GAATTCCTTCAACAAAGGCCAA	This study
PDC6 up confirm F	AGCAAAAACATATTGCCAAC	This study
ORA1 up confirm F	TCGGTGATGAGGTGGATCAAGA	This study
alsS confirm F	ACCGGGAGAAAAAGTGGTTTCTGTCTCTGG	This study
URA3 confirm R	GGAATTACTGGAGTTAGTTGAAGCATTAGGTCCC	This study

Table 3. Vectors used in this study

Vector	Description	Reference
pRS413ADH	CEN/ARS plasmid, *HIS*3, P_{ADH1}-T_{CYC1}	[1]
pRS413GPD	CEN/ARS plasmid, *HIS*3, P_{TDH3}-T_{CYC1}	This study
pRS413TEF	CEN/ARS plasmid, *HIS*3, P_{TEF1}-T_{CYC1}	This study
pRS413ADH-alsS	CEN/ARS plasmid, *HIS*3, P_{ADH1}-alsS-T_{CYC1}	This study
pRS413GPD-alsS	CEN/ARS plasmid, *HIS*3, P_{TDH3}-alsS-T_{CYC1}	This study
pRS413TEF-alsS	CEN/ARS plasmid, *HIS*3, P_{TEF1}-alsS-T_{CYC1}	This study
coex-PDC1up-loxp-UraR-loxp-PDC1down	CEN/ARS plasmid, *URA*3, *PDC1* up 300 b.p–*loxp-URA^R-loxp-PDC1* down 300 b.p	This study
coex-PDC5up-loxp-UraR-loxp-PDC5down	CEN/ARS plasmid, *URA*3, *PDC5* up 300 b.p–*loxp-URA^R-loxp-PDC5* down 300 b.p	This study
coex-PDC6up-loxp-UraR-loxp-PDC6down	CEN/ARS plasmid, *URA*3, *PDC6* up 300 b.p–*loxp-URA^R-loxp-PDC6* down 300 b.p	This study
coex-ORA1up-loxp-UraR-loxp-ORA16down	CEN/ARS plasmid, *URA*3, *ORA1* up 500 b.p–*loxp-URA^R-loxp-ORA1* down 500 b.p	This study
coex-ADH1up-alsS-loxp-UraR-loxp-ADH1down	CEN/ARS plasmid, *URA*3, *ADH1* up 300 b.p-alsS–*loxp-URA^R-loxp-ADH1* down 300 b.p	This study
coex416-Cas9-BDH1 gRNA	CEN/ARS plasmid, *URA*3, P_{TDH3}-*Cas9*-T_{TPI1}, P_{SNR52}-*BDH1* gRNA-T_{GPM1}	This study
pRS413TEF-Cre	CEN/ARS plasmid, *HIS*3, P_{TEF1}-*Cre*-T_{CYC1}	This study
pRS413TEF-YPR1	CEN/ARS plasmid, *HIS*3, P_{TEF1}-*YPR1*-T_{GPM1}	This study
pRS413GPD-ARA1	CEN/ARS plasmid, *HIS*3, P_{TDH3}-*ARA1*-T_{CYC1}	This study
coex413_YA	CEN/ARS plasmid, *HIS*3, P_{TEF1}-*YPR1*-T_{GPM1}, P_{TDH3}-*ARA1*-T_{CYC1}	This study
coex413_SY	CEN/ARS plasmid, *HIS*3, P_{ADH1}-alsS-T_{CYC1}, P_{TEF1}-*YPR1*-T_{GPM1}	This study

2.3 Analytical methods

Cell density was measured by optical density at 600 nm. For the measurement of concentration of metabolites such as ethanol, glycerol, glucose, and acetoin, 850 μL of culture supernatant was collected and filtered through 0.22 μm filter. Filtered samples were measured by HPLC using UltiMate 3000 HPLC system (Thermo fishers scientific) at a flow rate of 0.6 mL/min with 5 mM using a BioRad Aminex HPX-87H (300 mm × 7.8 mm, 5 μm, BioRad) at 60 ℃ and refractive index (RI) detector at 35 ℃. For the separation of *(R)* and *(S)*-acetoin, samples were measured by gas chromatography as well. β- 120 column (30 m × 0.25 μm film thickness, Supelco) with isotherm at 75 ℃ for 8 min and increasing of temperature 2 ℃ a minute to reach 85 ℃, and then isotherm at the set temperature (85 ℃) for 14 min. and flame ionization detector (FID) with the temperature of 275 ℃, were equipped. Acetoin standard solution was obtained from Supelco with the form of racemic mixture, and used for calibration curve.

Chapter 3.

Results and Discussion

3.1 Selection of starting strain

Primarily, JHY901 and JHY901-9, *ADH1-5*, *GPD1-2* and *BDH1* (and *ORA1* for JHY901-9) deleted strains constructed from previous study regarding production of *(R)*-acetoin, were tested for the production of *(S)*-acetoin. JHY901 and JHY901-9 were transformed with pRS413ADH-alsS to compare the enantiomeric excess of acetoin production.

Results showed that JHY901 and JHY901-9 with alsS vector expressed possess the *(R)*-acetoin enantiomeric excess of 21.8 % and 2.9 %, with the *(S)*-acetoin accumulative titer of 0.43 g/L and 0.66 g/L. JHY901-9 showed higher levels of enantiomeric excess and titer compared to JHY901, but neither of the strains were able to produce *(S)*-acetoin selectively since *(R)*-acetoin was produced more in both strains. Other attempts such as changing promoters of alsS to strong promoters (*TDH3* and *TEF1*) and expressing L. lactis noxE, didn't work as well. Moreover, JHY901 and JHY901-9 strains had already been through many genetic manipulations, so further manipulations were hard to be done. Key strategy of this study is to delete *PDC* to block endogenous *(R)*-acetoin production, so selecting new starting strain for this research was required.

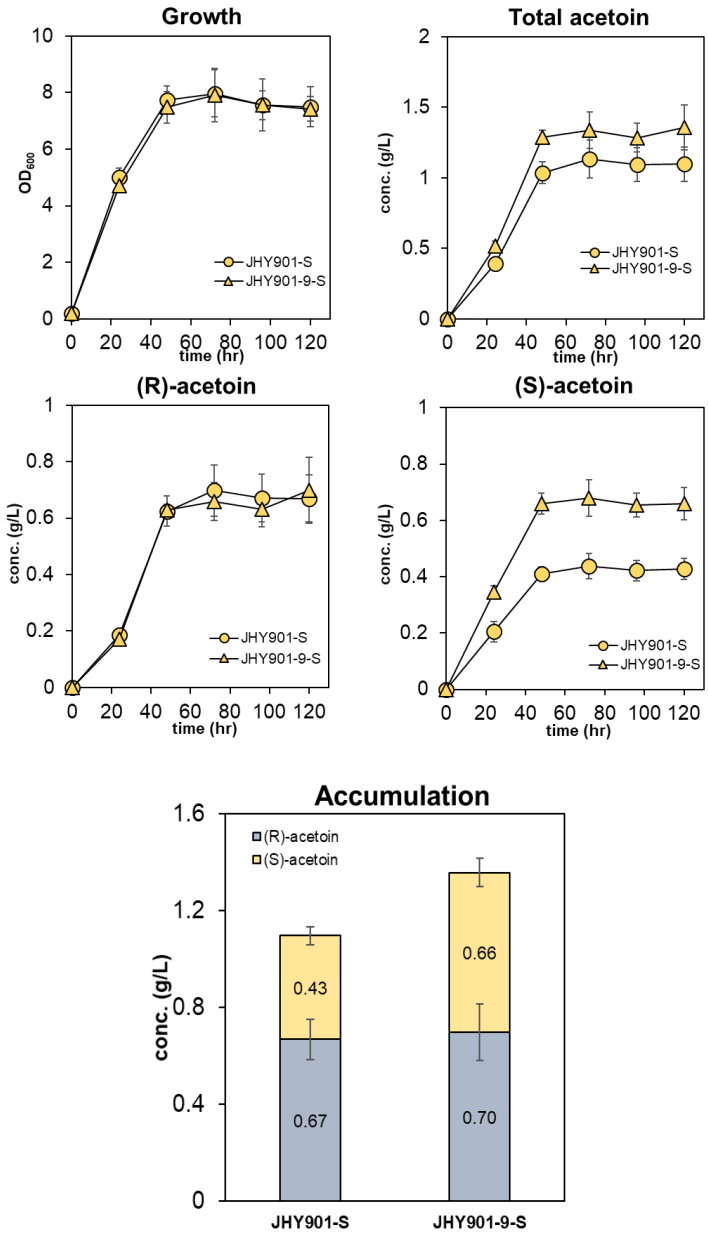

Figure 2. Acetoin production model in JHY901 and JHY901-9

JHY901 and JHY901-9, a (R)-acetoin production platform strain from previous study, was analyzed for (S)-acetoin production. Two strains were transformed with pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 120 h. Accumulation shows the accumulative acetoin measured after cultivating 120 h. Each value indicates the average ± SD of triplicate experiments.

Therefore, starting from *S. cerevisiae* CEN.PK2-1C wild-type strain, genetic manipulations were taken place for new starting strain. First, endogenous *BDH1* gene responsible for the formation of *(R)*-acetoin from diacetyl and *(R,R)*-2,3-butanediol from *(R)*-acetoin, was deleted resulting in JHYA100. JHYA100 was again tested with pRS413ADH empty vector and alsS vector transformed.

Results showed that deletion of *BDH1* blocked 2,3-butanediol formation. Empty vector transformant of JHYA100 (JHYA100-O) produced accumulative acetoin with *(R)*:*(S)* ratio of 31:69, whereas alsS vector expression transformant (JHYA100-S) produced acetoin with the accumulative ratio of 43:57. A slight growth effect was observed when alsS is expressed. Compared to JHY901 and JHY901-9, JHYA100 was able to produce acetoin with *(S)*-form of acetoin dominantly, but still with a low enantiomeric excess of 14 %. Production pathway flux of *(S)*-acetoin is relatively weak due to expression of alsS with *ADH1* promoter, so endogenous *(R)*-acetoin formation affected enantiomeric excess to a critical level.

JHYA100 was selected as a starting strain for *(S)*-acetoin production, and genetically manipulated for further experiments.

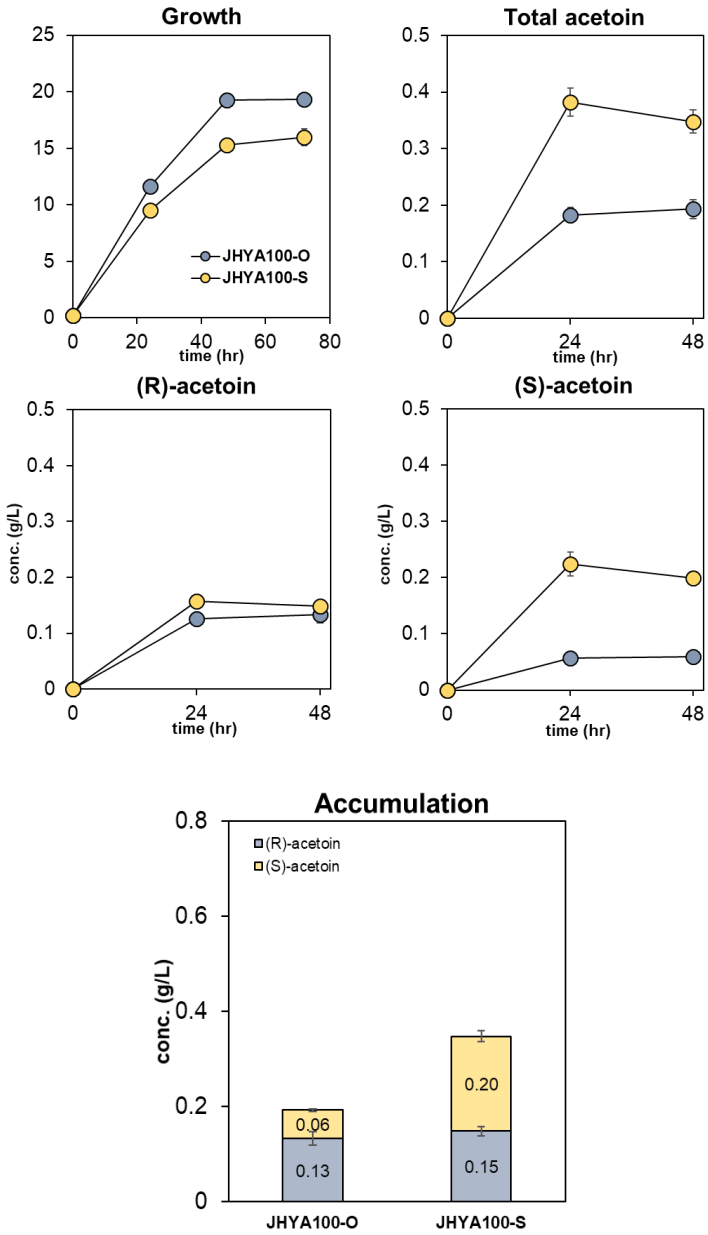

Figure 3. Acetoin production model in JHYA100 (*bdh1Δ*)

JHYA100, a *BDH1* deleted strain from wild-type *S. cerevisiae*, was analyzed for the starting strain of this study. JHYA100 was transformed with pRS413ADH empty vector and pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 48 h. Accumulation shows the accumulative acetoin measured after cultivating 48 h. Each value indicates the average ± SD of triplicate experiments.

3.2 Endogenous pyruvate decarboxylase *PDC* deletion

3.2.1 Single deletion

Endogenous pyruvate decarboxylase, responsible for the conversion of pyruvate into acetaldehyde (and also for endogenous acetoin production pathway) was deleted. Three *PDC* genes, *PDC1*, *PDC5*, and *PDC6*, are known to be responsible and *PDC1*, 5 are known to be major genes.

Deleting single *PDC1,5,6* gene from JHYA100, resulting in JHYA101, JHYA102, and JHYA103. The three strains were transformed with pRS413ADH empty vector and pRS413ADH-alsS. JHYA101 and JHYA103 were selected for observation.

Results showed that JHYA101 produced accumulative acetoin with (S):(R) accumulative ratio of 61:39, whereas JHYA103 produced acetoin of 60:40 resulting in *(S)*-acetoin enantiomeric excess 22 % and 20 %, respectively. Compared to starting strain JHYA100, neither of the two single deletion strains show dramatic increase of *(S)*-acetoin ratio nor the titer of *(S)*-acetoin.

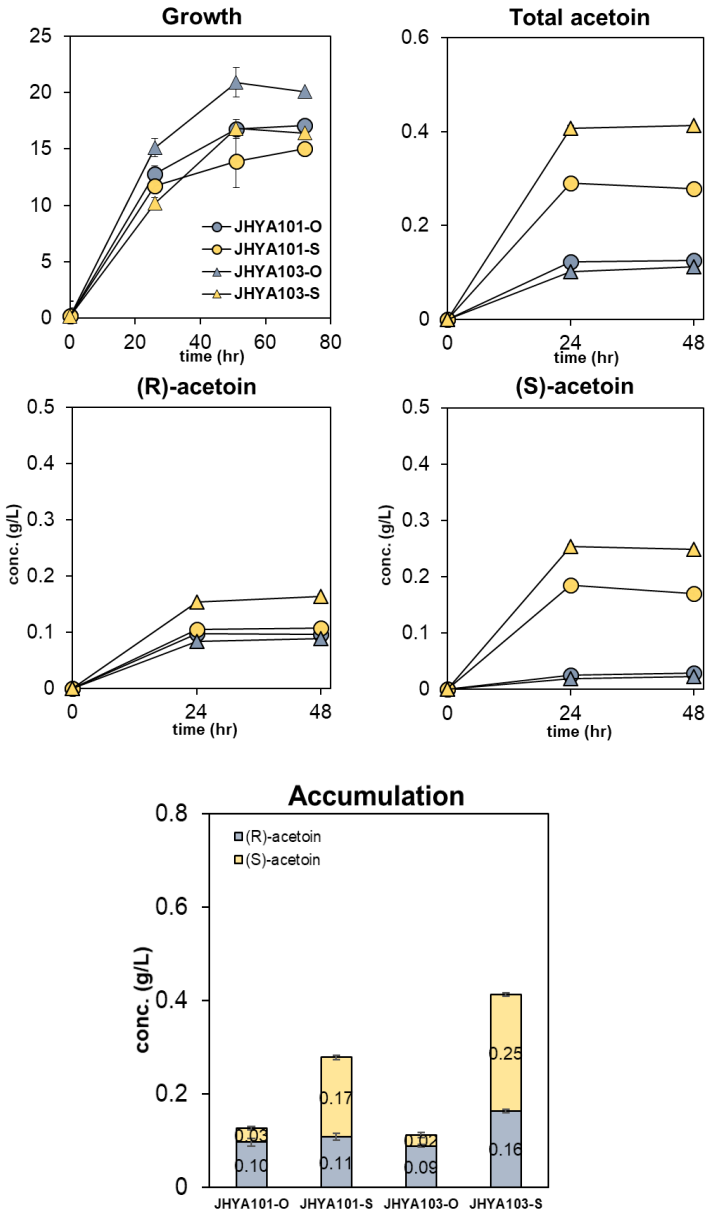

Figure 4. Acetoin production in JHYA101 and JHYA103

JHYA101 and JHYA103, *PDC1* and *PDC6* deleted strain from JHYA100, was analyzed. Each two strains were transformed with pRS413ADH empty vector and pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 48 h. Accumulation shows the accumulative acetoin measured after cultivating 48 h. Each value indicates the average ± SD of triplicate experiments.

3.2.2 Double deletion

Two *PDC* genes were deleted from JHYA100 for further analysis, resulting in JHYA111 (*bdh1*Δ *pdc1,5*Δ), JHYA112 (*bdh1*Δ *pdc1,6*Δ), and JHYA113 (*bdh1*Δ *pdc5*,6Δ). Three strains were transformed with empty vector and alsS-expressing vector. First, JHYA112 and JHYA113 were primarily tested for observation. Growth slightly decreased in JHYA112-S, which produced accumulative *(S)*-acetoin with the enantiomeric excess of 38 %. Other double deletion strain, JHYA113, did not show dramatic increase of *(S)*-acetoin ratio nor the titer of *(S)*-acetoin. It was concluded that deletion of *PDC1,6* or *PDC5,6* does not significantly block endogenous acetoin production.

Then, JHYA111, a *bdh1*Δ *pdc1,5*Δ strain, was observed with the control strain JHYA100. Empty vector transformants showed that in JHYA111, neither *(R)* nor *(S)*-acetoin was formed, compared to JHYA100 control sample. Also, alsS expression vector transformants showed that in JHYA111-S, *(S)*-acetoin was selectively produced with the enantiomeric excess of 90 % and titer of 0.38 g/L. Deletion of two major *PDC* genes, *PDC1* and *PDC5*, significantly changed the acetoin ratio.

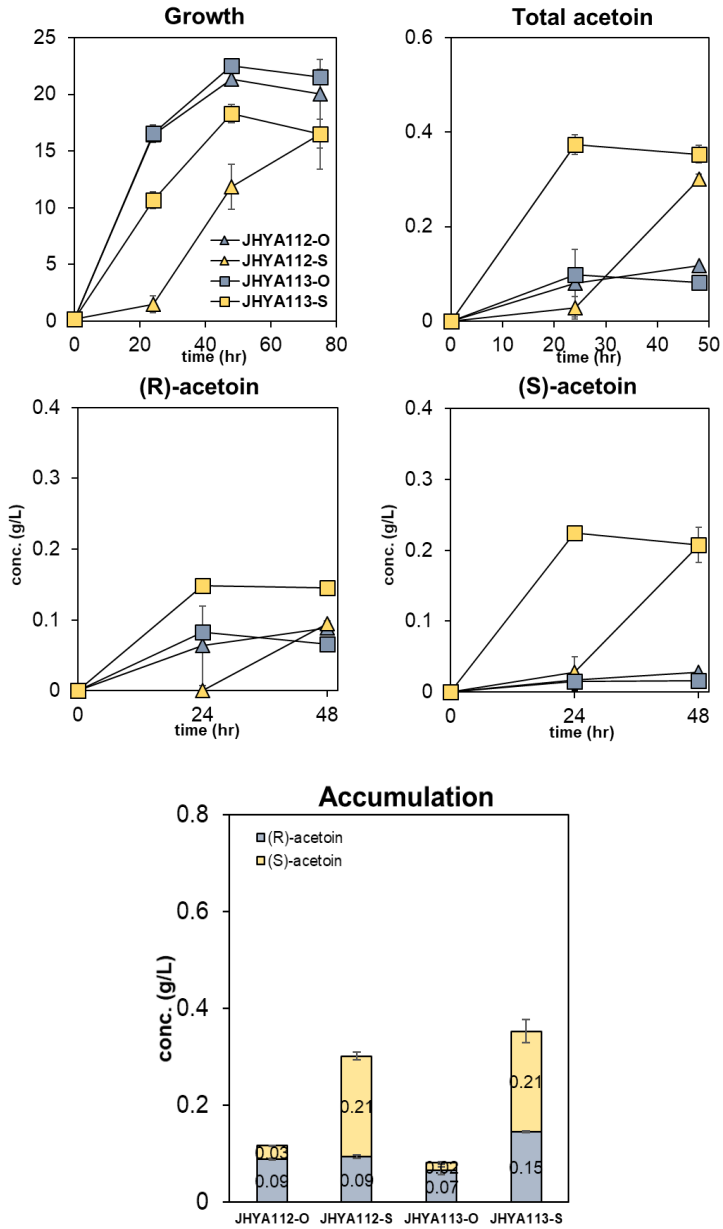

Figure 5. Acetoin production in JHYA112 and JHYA113

JHYA112 and JHYA113, *PDC1,6* and *PDC5,6* deleted strain from JHYA100, were analyzed. Each two strains were transformed with pRS413ADH empty vector and pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 48 h. Accumulation shows the accumulative acetoin measured after cultivating 48 h. Each value indicates the average ± SD of triplicate experiments.

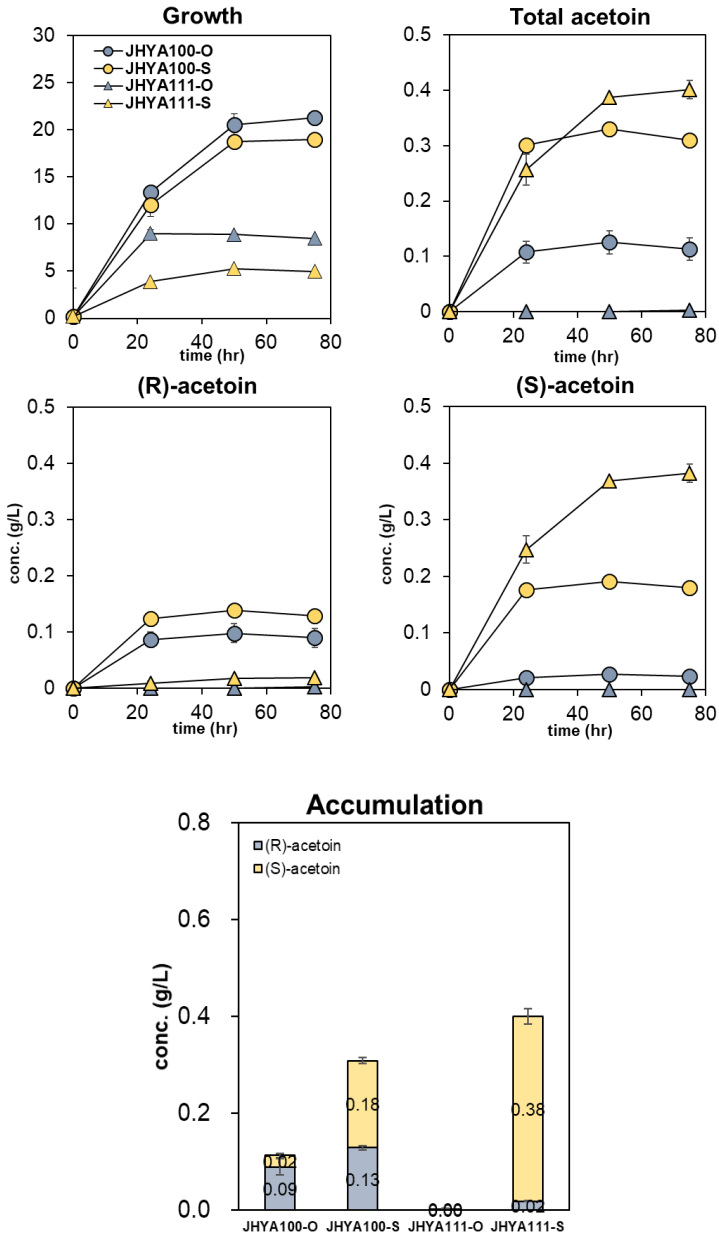

Figure 6. Acetoin production in JHYA111

JHYA111, a *PDC1,5* deleted strain from JHYA100, was analyzed with control strain JHYA100. Each two strains were transformed with pRS413ADH empty vector and pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 75 h. Accumulation shows the accumulative acetoin measured after cultivating 75 h. Each value indicates the average ± SD of triplicate experiments.

3.3 Diacetyl reductase *ORA1* deletion

Previous experiment regarding the comparison of acetoin production between JHY901 and JHY901-9 revealed that deletion of endogenous diacetyl reductase *ORA1* increased *(S)*-acetoin production titer and stereoselective ratio. So, deletion of *ORA1* to JHYA111 strain for further study was required.

ORA1 deletion was performed, resulting in JHYA200. JHYA200 was transformed with two vectors above, and cultivated in Sc-His media. Empty vector transformants showed that in JHYA200, neither *(R)* nor *(S)*-acetoin was formed, which is similar to the result of JHYA111. Also, alsS expression vector transformants showed that in JHYA200-S, *(S)*-acetoin was selectively produced with the enantiomeric excess of 88 % and titer of 0.66 g/L. It was concluded that deletion of endogenous *ORA1* resulted in the increased titer of *(S)*-acetoin by 73.7 % compared to JHYA111, while maintaining its stereoselective production capacity as shown by the rate of enantiomeric excess.

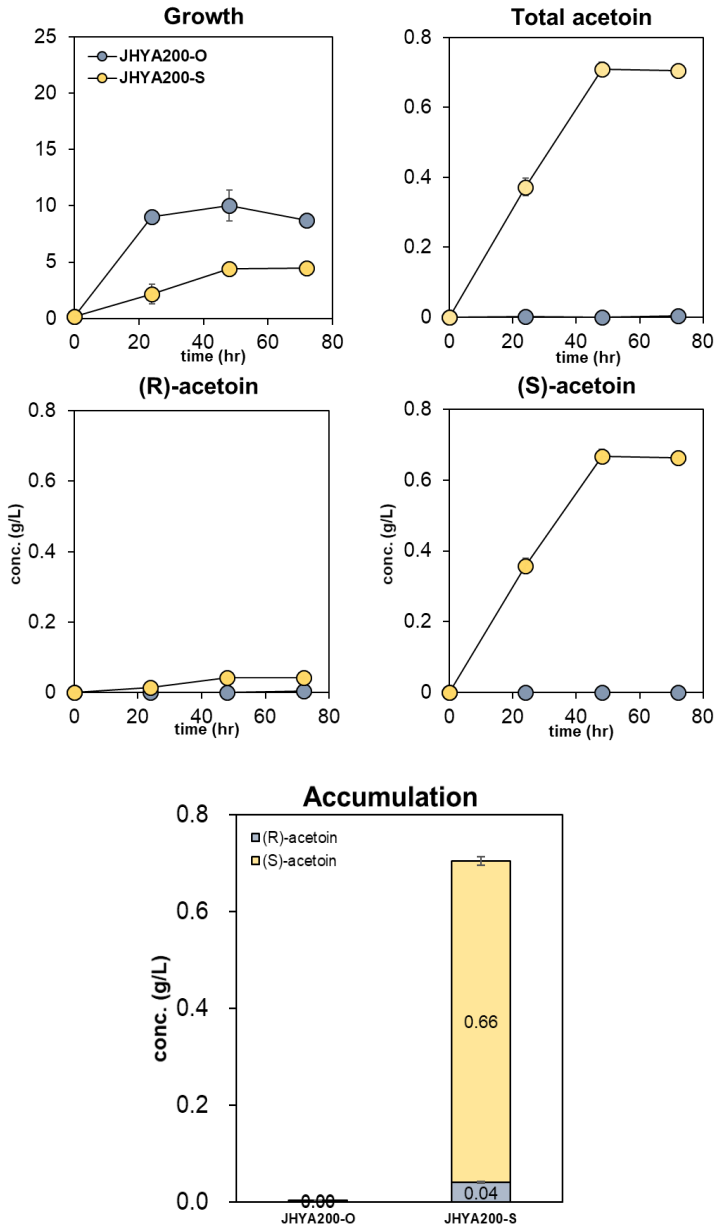

Figure 7. Acetoin production in JHYA200

JHYA200, *ORA1* deleted strain from JHYA100, was analyzed. JHYA200 was transformed with pRS413ADH empty vector and pRS413ADH-alsS, and cultivated in 10 mL Sc-His media (20 g/L glucose) for 72 h. Accumulation shows the accumulative acetoin measured after cultivating 72 h. Each value indicates the average ± SD of triplicate experiments.

Chapter 4.

Conclusions

In this study, production of optically pure *(S)*-acetoin was accomplished via deletion of *S. cerevisiae* endogenous pyruvate decarboxylase, *PDC*. Pdc is thought to be responsible for production of *(R)*-acetoin *in vivo*, through its carboligase activity to condense pyruvate and acetaldehyde, or two acetaldehyde molecules into one acetoin. Previous study has shown that Pdc catalyzes acetoin formation with its optical ratio ranging from 40 % to 70 % depending on pH, and empty vector transformant in JHYA100 shows that *S. cerevisiae* produces acetoin with the *(R)*:*(S)* ratio of 31:69 endogenously. The key result of this study was that deleting two major *PDC* genes, 1 and 5, can significantly decrease *(R)*-acetoin formation when alsS, which catalyzes *(S)*-α-acetolactate formation from pyruvate, is expressed. Also, formation of by-products and catabolism of acetoin is blocked by deleting *BDH1* and *ORA1*, blocking formation of 2,3-butanediol and 2,3-dimethylglycerate, respectively.

One step of fermentative *(S)*-acetoin is completed which is producing chiral *(S)*-acetoin and blocking formation of *(R)*-acetoin. But still the enantiomeric excess is 90 %, so deletion of minor *PDC*, *PDC6*, will still have to be performed in order to achieve high enantiomeric excess. Another significant step of this study is to obtain *(S)*-acetoin with high titer, since the final titer of the target product is only 0.66 g/L. This step is thought to be achieved by reinforcing the conversion of pyruvate into *(S)*-α-acetolactate by expression of *B. subtilis* alsS, *(S)*-α-acetolactate conversion into diacetyl by spontaneous decarboxylation, and diacetyl conversion into *(S)*-acetoin with the presence of diacetyl reductase, while also considering cell growth defect. Previous attempts to reinforce alsS expression using strong promoters significantly decreased cell growth, so optimizing downstream flux will be necessary. Spontaneous decarboxylation can be activated by adding oxidative reagents into the medium, ferric ion for instance. Further studies should also contain results of blocking ethanol and glycerol formation, which can be accomplished by deleting endogenous *ADH1*-5 and *GPD1,2*, as shown in previous

studies. Finally, selecting appropriate endogenous diacetyl reductase will be necessary since diacetyl reductase can also catalyze *(S)*-acetoin conversion into meso or *(S,S)*-2,3-butanediol *in vivo*. Introduction of foreign diacetyl reductase will also be possible.

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초록

식품첨가물, 화장품, 세제, 의약품 등 다양한 분야에 적용될 수 있는 GRAS(General Recognized as Safe) 물질인 아세토인은 자연적으로 *(R)*-아세토인과 *(S)*-아세토인의 두 가지 입체이성질체 형태로 존재하며, 가스 크로마토그래피에 의해 분리될 수 있다. *(R)*-아세토인의 생성은 발효 방식과 생물 촉매 방식을 통해 이루어질 수 있다. 그러나 자발적인 비효소적 산화를 포함한 복잡한 대사 생성 경로 때문에 *(S)*-아세토인의 생산은 아직 개발 중에 있다. 특히 포도당으로부터 시작되는 발효에 의한 연구는 거의 알려져 있지 않다. 본 연구에서는 생산 플랫폼 균주로 *Saccharomyces cerevisiae*를 사용하여 포도당으로부터 *(S)*-아세토인의 입체선택적 생산을 그 목표로 한다.

먼저, 시작 균주 선정 및 피루브산을 *(S)*-α-acetolactate로 전환시키는 데 관여하는 *Bacillus subtilis* 유래 alsS의 발현이 최적화되었다. 선행연구에서 *(R)*-아세토인 생산에 사용된 JHY901 및 JHY901-9는 *(S)*-아세토인 생성에 적합하지 않았다. 또한, alsS의 발현만으로도 세포 성장에 결정적인 영향을 미쳤으며, *TDH3* 또는 *TEF1*과 같은 강한 promoter를 통한 발현 또한 적합하지 않았다. 본 연구에서는 *ADH1* promoter를 이용하여 alsS를 발현하였다.

다음으로, *(R)*-아세토인의 *(R,R)*-2,3-부탄다이올로의 전환 및 diacetyl의 *(R)*-아세토인으로의 전환을 담당하는 *S. cerevisiae BDH1* 유전자가 wild-type 균주에서 결손되었다. 그 후, *(R)*-아세토인 형성을 차단하기 위해 pyruvate decarboxylase *PDC*가 결손되었다. *PDC*는 피루브산과 아세트알데하이드를 아세토인으로, 아세트알데하이드 2분자를 하나의 아세토인으로 전환시키는 역할을 하는 anomalous carboligase 활성으로 알려져 있다. 효모 *PDC*의 단백질 구조 변형에 관한 이전 연구는 *PDC*가 *(R)*-아세토인의 enantiomeric excess가 40~60%인 아세토인의 형성을 촉매한다는 것을 보여주었다. 본 연구에서 *PDC1* 및 *PDC5* 유전자를 deletion하였고, *S. cerevisiae* endogenous pathway에서 *(R)* 및 *(S)*-아세토인

형성을 모두 차단한 것을 확인할 수 있었다. *bdh1*Δ *pdc1*Δ *pdc5*Δ (JHYA111) 균주에서 아세토인은 *(S)*-아세토인이 90 %의 enatiomeric excess로 생산되었으며, 0.38 g/L의 *(S)*-아세토인이 생산되었다. 마지막으로 피루브산이 선행연구를 통해 알려진 2,3-dimethylglycerate로 전환되는 것을 담당하는 *ORA1* 유전자의 deletion이 JHYA111에서 진행되어 JHYA200 균주를 얻을 수 있었다. JHYA200은 *(S)*-아세토인이 88 %의 enatiomeric excess로 생산되었으며, 0.66 g/L의 *(S)*-아세토인이 생산되었다.

핵심 용어: *Saccharomyces cerevisiae*, *(S)*-아세토인, *BDH1*, *PDC*, *ORA1*, 입체선택적 생산

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