Metabolic and process engineering of *Pichia pastoris* for the production of value-added products

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Abstract

Motivated by the surging demand of recombinant proteins and biofuels derived from renewable substrates, increasing attention has been paid to the development of novel strains via metabolic engineering strategies. *Pichia pastoris* is a eukaryotic platform suitable for protein expression and potentially for biofuel production due to its advantageous traits over *Escherichia coli* or *Saccharomyces cerevisiae*. In this thesis, we constructed a xylanase-producing *P. pastoris* strain. The fungal xylanase Xyn11A was successfully overexpressed under the constitutive GAP promoter. Biochemical characterization of the xylanase revealed that Xyn11A is optimally active at 70 °C and pH 7.4. This xylanase was stable over a wide range of pH ranging from pH 2 to pH 11. Excellent thermal stability was observed at temperature 60 °C. Enhanced production of Xyn11A was achieved by investigating the effect of carbon source and feeding strategies. The highest xylanase activity was detected at 15000 U/mL using high cell density cultivation.

Production of optically pure (2R, 3R)-2, 3-BD was achieved by engineering *P. pastoris* with a heterologous pathway. The pathway genes consisting of *Bacillus subtilis alsS, alsD* and *S. cerevisiae* BDH1 were assembled and transformed into *P. pastoris*. Cultivation conditions were optimized and the highest titer of 2, 3-BD obtained using YPD media was 45 g/L in fed-batch cultivation. To enhance the economic viability of 2, 3-BD production in *P. pastoris*, statistical medium optimization was performed. It was found that 75 g/L of 2, 3-BD was produced using optimized media in fed-batch cultivation.

Résumé

Étant donné la croissance des marchés pour protéines recombinantes et pour biocarburants, de plus en plus d'efforts et de ressources sont dédiés au développement de nouvelles lignées de microorganismes par génie métabolique. Comparé à *Escherichia coli et Saccharomyces cerevisiae, Pichia pastoris* possède plusieurs traits désirables pour la production de ces protéines et ces biocarburants. Cette thèse décrit le développement d'une nouvelle lignée de *P. pastoris* qui exprime l'enzyme xylanase. La xylanase Xyn11A, qui provient d'une moissure, fut sur-exprimée sous le contrôle du promoteur constitutif GAP. La caractérisation subséquente de l'enzyme indique que son activité est maximale à 70°C et à pH 7.4, et qu'elle reste active entre pH 2 et pH 11. En changeant le mode d'alimentation de source de carbone, une activité enzymatique maximale de 15000 U/ml fut obtenue durant une cultivation à haute densité.

La production de (2R, 3R) 2,3-BD pur fut accomplie en développant une nouvelle voie métabolique dans *P. pastoris* formée par les gènes *alsS, alsD* de *Bacillus subtilis*, et le gène *BDH1* de *S. cerevisiae*. Les paramètres de cultivation furent optimisés et la meilleure production de 3-BD fut obtenue pour l'alimentation continuelle de YPD à un débit de 45 g/L dans le mélange de croissance. Afin d'améliorer la viabilité économique du procédé de production de 3-BD, la composition du milieu de croissance fut optimisée. Sous conditions optimales, 75 g/L de 2, 3-BD funent obtenu.

Statement of co-author contributions

All the work described in this thesis was performed by Zhiliang Yang and co-authors. Yaqing Liu performed the experiments in Chapter 5 and she will be listed as a co-author on publication resulting from that chapter. Jinxing Wei carried out the experiments in Chapter 7 and he will be listed as a co-author in publication derived from that chapter. Zhiiang Yang performed all the other research work including literature review, experimental design, conducting experiments, analysis of results, manuscript preparation and revision and thesis writing. Dr. Jason Zhang provided guidance and editorial comments throughout the whole thesis.

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Table of Contents

List of Figures

List of Tables

List of Acronyms

α-MF	α-mating factor
ANN	Artificial neural network
ADH	Alcohol dehydrogenase
AOX	Alcohol oxidase
ATP	Adenosine triphosphate
ATR	Attenuated total reflection
BDH	Butanediol dehydrogenase
BLAST	Basic Local Alignment Search Tool
BSM	Basal salt medium
CAI	Codon adaptation index
CAL	*Candida antarctica* lipase
CES	Carboxylesterase
CORVET	Core vacuole/endosome tethering
DCW	Dry cell weight
DHA	Dihydroxyacetone
DO	Dissolved oxygen
EC	Enzyme commission
EDTA	Ethylenediaminetetraacetic acid
EFT	Elapsed fermentation time
ER	Endoplasmic reticulum
FID	Flame ionization detector
FLD	Formaldehyde dehydrogenase

FSEOF	Flux Scanning based on Enforced objective Function
FTIR	Fourier transform mid-infrared
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GC	Gas chromatography
GEM	Genome-scale metabolic model
GFP	Green fluorescent protein
GRAS	Generally regarded as safe
HSA	Human serum albumin
hSOD	Human superoxide dismutase
HPLC	High performance liquid chromatography
IDH	Isocitrate dehydrogenase
IPTG	Isopropyl β-D-1-thiogalactopyranoside
MFA	Metabolic flux analysis
MIR	Mid-infrared
μ	Specific growth rate
MLR	Multiple linear regression
MUT	Methanol utilization
MWF	Multiple wavelengths fluorescence
NADH	Nicotinamide adenine dinucleotide
NCBI	National center for biotechnology information
NIS	Near infrared spectroscopy
OD_{600}	Optical density at 600 nm
OTR	Oxygen transfer rate
OUR	Oxygen uptake rate
PAGE	Polyacrylamide gel electrophoresis

PAT	Process analysis technology
PCA	Principal component analysis
PCR	Polymerase chain reaction
PDI	Protein disulfide isomerase
PDH	Pyruvate dehydrogenase
pIFN	Porcine interferon
PIP	Porcine insulin precursor
PLS	Partial least square
PMSF	Phenylmethylsulfonyl fluoride
PPP	Pentose phosphate pathway
PTH	Human parathyroid
rHGH	Recombinant human growth hormone
rHuEPO	Recombinant human erythropoietin
RML	*Rhizomucor miehei* lipase
RID	Refractive index detector
ROL	*Rhizomucor oryzae* lipase
ROS	Reactive oxygen species
SDH	Sorbitol dehydrogenase
SDS	Sodium dodecyl sulfate
SIA	Sequential injection analysis
TCA	Tricarboxylic acid
TLL	*Thermomyces lanuginosus* lipase
UTR	Untranslated region
VPS	Vacuole protein sorting
VWD	Variable wavelength detector

YPL	*Yarrowia lipolytica* lipase
2, 3-BD	2, 3-butanediol

Chapter 1: Introduction

The methylotrophic yeast *Pichia pastoris* has been a hot research topic since its characterization in the 1970s. *P. pastoris* was originally used as a potential source of single cell protein (SCP) owing to its capability to grow on methanol as sole carbon source (Cereghino and Cregg, 2000). Two alcohol oxidase genes: *AOX1* and *AOX2* are responsible for the methanol metabolism in *P. pastoris*. AOX could account for 30% of the total soluble proteins when grown on methanol cultures (Macauley‐Patrick et al., 2005). The isolation of strong promoter of alcohol oxidase 1 (P_{AOX1}) paved the way for developing *P. pastoris* as a platform for heterologous protein expression. The subsequent identification of alternative promoters such as the strong constitutive promoter P_{GAP}, the nitrogen source-regulated P_{FLD1} and more recently, the vitamin-induced P_{THI11} promoter (Landes et al., 2016), and the development of novel methanol-free strains based on promoter engineering, combined with the protocols for strain development, cultivation strategies and downstream purification methods contributed to the popularity of using *P. pastoris* as an increasingly mature host for protein production. *P. pastoris* is a single-celled eukaryote with four chromosomes. The eukaryotic protein synthesis machinery enables corrected-folded protein production attributed to post-translational modifications such as disulfide bond formation and glycosylation (Potvin et al., 2012). This merit makes *P. pastoris* especially suitable for the expression of eukaryotic proteins.

Biorefinery of lignocellulosic materials to produce value-added products has been a desirable alternative to the petrochemical counterpart (Bozell, 2008). However, lignocellulose generally cannot be used as feedstock for fermentation due to its complex structure. Degradation of lignocellulose components such as cellulose and xylan into fermentable sugars (glucose and

xylose) is essential for its efficient utilization by bacteria or yeasts. Two engineering aspects could be explored towards efficient production of bulk chemical from xylan using engineered *P. pastoris*: Engineering of *P. pastoris* for enhanced utilization of lignocellulose; Conversion of xylose to value-added products through metabolic engineering. Xylanase is an enzyme essential for the degradation of xylan. Searching for xylanase with robust properties such as high thermal and pH-stability is industrially significant. Xylan-fermenting *S. cerevisiae* strains have been constructed by co-displaying a xylanase and xylosidase and introducing a heterologous xylose metabolic pathway (Katahira et al., 2004). Xylose-fermenting *P. pastoris* strain has been developed (Li et al., 2015). Development of engineered *Pichia* strains with high xylanase activity would contribute to the goal of constructing xylan-fermenting *P. pastoris* strains. To this end, we developed a high xylanase-secreting *P. pastoris* strain. Commodity chemicals such as 2, 3-butanediol (2, 3-BD) have enormous applications and are currently dependent on the petrochemical synthesis route. Microbial fermentation for the production of 2, 3-BD has been gaining great interest due to the high titer achieved but challenges such as the use of pathogenic microorganisms and low optical purity remain to be overcome. We explored the viability of producing 2, 3-BD in *P. pastoris*, which expands the scope of *P. pastoris* application commonly used as a workhorse for protein production. *P. pastoris* is a well-established host for recombinant protein expression. High level expression of heterologous pathway could be achieved using the strategies reported in literatures. As a yeast, *P. pastoris* is exceptionally tolerant to high titer of alcohols. Moreover, *P. pastoris* is generally regarded as safe (GRAS) and is ideal for industrial fermentation. Based on these merits, we selected *P. pastoris* for the production of 2, 3-BD. Despite glucose was used as feedstock for the production of 2, 3-BD, renewable substrate such

as xylan could replace glucose in the future given that a xylan-fermenting *P. pastoris* strain is constructed.

Process engineering is an indispensable part of biochemical engineering, aiming to achieve maximized titer and yield. Bioreactor fermentation offers distinctive advantages over shake flask cultivations by monitoring the process parameters such as pH, temperature, dissolved oxygen (DO) and substrate concentration. In this thesis, once the recombinant *P. pastoris* strains were developed, scale-up of production were conducted in bioreactor cultivations. Cultivation strategies were designed and optimized specifically to the target products. The overall objective of this thesis is to use metabolic and process engineering strategies to achieve cost-effective production of valuable products in *P. pastoris*.

This thesis contained 8 chapters. Chapter 1 is the introduction, giving an overview of the content of the whole thesis and objective of the research. Chapter 2 is a literature review (in revision) of the recent progress on the engineering strategies for enhancing protein production in *P. pastoris*. Strategies on two levels: genetic and process engineering were thoroughly discussed. Identification of novel promoter and advances in promoter engineering was also included. Chapter 3 is a literature review on the state of the art advances on the production of 2, 3-BD in engineered microbes. Metabolic engineering strategies for various 2, 3-BD producers including native or engineered microorganisms were summarized. The efforts of using renewable feedstocks to achieve economical production of 2, 3-BD were also discussed. Both reviews were relevant to the scope of this thesis, which could aid the understanding of the experimental work described in other chapters.

Chapter 4 describes the overexpression and characterization of a fungal xylanase in *P. pastoris*. Strain development, enzymatic characterization including optimal pH, optimal temperature, pH stability and thermal stability were carried out. Kinetic parameters were determined. Fed-batch cultivation for large scale production of xylanase was performed.

Chapter 5 is an extension of Chapter 4, focusing on the process optimization of the xylanase-producing strain using batch and fed-batch fermentations. Feeding strategies of glucose and glycerol were investigated.

Chapter 6 describes the production of optically pure (2R, 3R)-2, 3-BD by engineering *P. pastoris*. Strain development, characterization and fermentation were performed. Optimization of fermentation conditions including pH, agitation speeds and aeration rates were carried out. Fed-batch cultivation was conducted to achieve maximized production of 2, 3-BD.

Chapter 7 describes the optimization of medium composition for enhanced production of 2, 3-BD using engineered strains constructed in Chapter 6. Statistical approaches such as Plackett-Burman and Box-Behnken design were employed.

Chapter 8 is the conclusions and recommendations, giving a summary of the work described in this thesis. Possible strategies for improvement in future work were also provided.

References

Bozell JJ. Feedstocks for the future–biorefinery production of chemicals from renewable carbon. CLEAN–Soil, Air, Water. 2008;36:641-7.

Cereghino JL, Cregg JM. Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS microbiology reviews. 2000;24:45-66.

Katahira S, Fujita Y, Mizuike A, Fukuda H, Kondo A. Construction of a xylan-fermenting yeast strain through codisplay of xylanolytic enzymes on the surface of xylose-utilizing *Saccharomyces cerevisiae* cells. Applied and environmental microbiology. 2004;70:5407-14.

Landes N, Gasser B, Vorauer-Uhl K, Lhota G, Mattanovich D, Maurer M. The vitamin-sensitive promoter PTHI11 enables pre-defined autonomous induction of recombinant protein production in *Pichia pastoris*. Biotechnology and bioengineering. 2016;113:2633-43.

Li P, Sun H, Chen Z, Li Y, Zhu T. Construction of efficient xylose utilizing *Pichia pastoris* for industrial enzyme production. Microbial cell factories. 2015;14:22.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. Yeast. 2005;22:249-70.

Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal. 2012;64:91-105.

Chapter 2: Engineering strategies for enhanced production of protein and bio-products in *Pichia pastoris:* A review

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2.1 Abstract

Pichia pastoris has been recognized as one of the most industrially important hosts for heterologous protein production. Despite its high protein productivity, the optimization of P. pastoris cultivation is still imperative due to strain- and product-specific challenges such as promoter strength, methanol utilization type and oxygen demand. To address the issues, strategies involving genetic and process engineering have been employed. Optimization of codon usage and gene dosage, as well as engineering of promoters, protein secretion pathways and methanol metabolic pathways have proved beneficial to innate protein expression levels. Large-scale production of proteins via high cell density fermentation additionally relies on the optimization of process parameters including methanol feed rate, induction temperature and specific growth rate. Recent progress related to the enhanced production of proteins in P. pastoris via various genetic engineering and cultivation strategies are reviewed. Insight into the regulation of the P. pastoris alcohol oxidase 1 (AOX1) promoter and the development of methanol-free systems are highlighted. Novel cultivation strategies such as mixed substrate feeding are discussed. Recent advances regarding substrate and product monitoring techniques are also summarized. Application of P. pastoris to the production of other value-added products via metabolic engineering was also reviewed. P. pastoris is becoming an indispensable platform through the use of these combined engineering strategies.

Keywords: *Pichia pastoris*, Fermentation, Fed-batch cultivation, Yeast promoters, Process monitoring, Metabolic engineering

2.2 Introduction

The methylotrophic yeast *Pichia pastoris* has been established as a successful protein production platform, especially in the sector of industrial enzymes and the biopharmaceutical industry. As a "generally regarded as safe" (GRAS) microorganism, it has been used for the production of over 500 pharmaceutical proteins and more than 1000 recombinant proteins as of 2009 (Fickers, 2014). Driven by increasing demands in the food and feed industries, *P. pastoris* has also become an important host to produce enzymes such as xylanase and phytase, which are relevant to these sectors (Spohner et al., 2015). Recently, *P. pastoris* has also been favoured in the expression of eukaryotic membrane proteins, facilitating advances in structural biology (Byrne, 2015, Goncalves, 2013). Using cell surface display techniques, *P. pastoris* has been used to synthesize biofuels and other chemicals (Tanaka et al., 2012). The success of *P. pastoris* as such a versatile system is mainly attributed to its ability to grow to a high biomass concentration on defined media, its capacity to perform complex post-translational modifications which include correct protein folding, disulfide bond formation as well as glycosylation, its high secretion efficiency and its repertoire of both inducible and constitutive promoters.

The successful development of high-yield yeast strains is imposed with strain- and product-specific challenges. To overcome these challenges, engineering strategies comprising genetic and process engineering approaches have been employed (Fig. 2-1). Extensive progress has been made for protein expression in *P. pastoris*. In this review, we will focus on recent progress related to the production of proteins and other bio-products, aiming to update our previously published review (Potvin et al., 2012) from an engineering perspective. Strategies involving genetic and bioprocess engineering will be discussed. State-of-the-art monitoring techniques for substrates and products will also be briefly summarized.

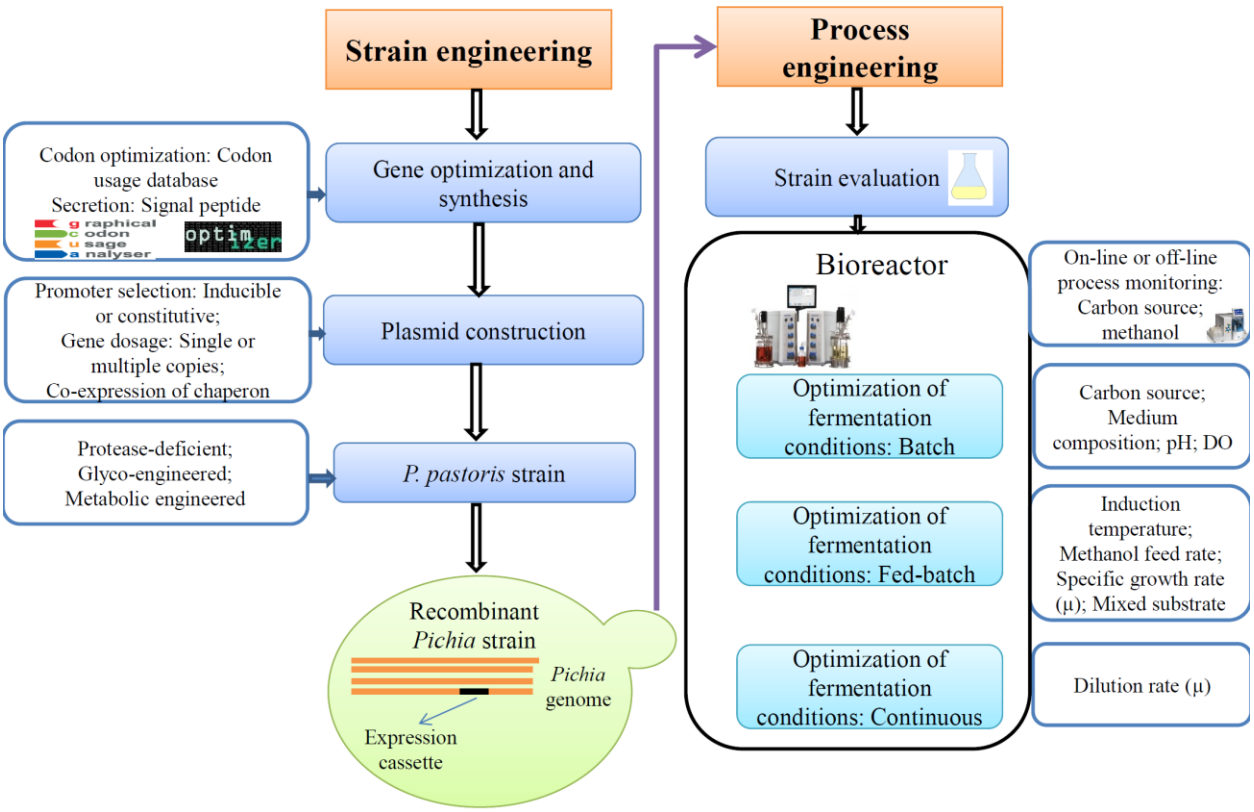

Fig. 2-1 General strategies for enhanced production of recombinant proteins in *P. pastoris*. Strategies are divided into strain engineering wise and process engineering wise. Considerations were indicated for each stage. Single or combined strategy could be used for product-specific purposes**.**

2.3 Genetic engineering strategies for improved protein production

2.3.1 Promoters

2.3.1.1 Advances of AOX1 promoter regulation

The alcohol oxidase 1 (AOX1) promoter regulates the metabolism of methanol and catalyzes the first step of methanol assimilation, converting methanol to formaldehyde. It is widely used to drive heterologous protein expression due to its tight regulation and strong inducibility when methanol is used as the sole carbon source. Although it is the most widely studied promoter in *P.*

pastoris, the mechanisms for the regulation of P_{AOX1} are still gaining great attention. This is because insights into its regulation profile could facilitate the fine-tuning of P_{AOX1} and the development of methanol-free expression systems. The regulation of AOX1 expression mainly occurs at the transcriptional level. It has been recognized that the AOX1 promoter is strongly induced by methanol and repressed by glucose, glycerol and ethanol (Vogl and Glieder, 2013). Kim and coworkers investigated the regulation of AOX1 under methanol-limited and oxygen-limited conditions by visualizing the localization and expression of green fluorescent protein (GFP)-fused proteins (Kim et al., 2013). Their results confirmed that the rate of methanol consumption plays a crucial role in the final maximal protein yield. Methanol induction is regulated through the interaction between cis- or trans-acting elements. Through deletion and insertion analysis, it was determined that a region, D, which is located between positions -638 and -530 (relative to the transcription start site) of the AOX1 promoter is a cis-acting element (Xuan et al., 2009). The expression level was enhanced to 157% of that of the wild type when three copies of this region were inserted into a promoter lacking region D. The 5'-untranslated region (5'-UTR) of the AOX1 promoter consists of both positive and negative cis-acting elements which, according to a systematic analysis of this region, affect the translational efficiency of the AOX1 promoter (Staley et al., 2012). Mxr1 is a key transcription factor which regulates the activation of methanol utilization. Mxr1 contains a region which allows the binding of 14-3-3 proteins in response to various carbon sources (Parua et al., 2012). More recently, transcription factor 1 (Mit1) was identified and found to be critical to the activation of P_{AOX1} (Wang et al., 2016c). The study also provided a regulatory model for P_{AOX1} to elucidate the transduction of the methanol induction signal from the cytoplasm to the nucleus. The response to

methanol induction is accomplished through a cascade of Mit1, Mxr1 and Prm1. Furthermore, the tight regulation of P_{AOX1} is attributed to the repression of methanol utilization genes.

2.3.1.2 Development of methanol-free P_{AOX1} systems

To address the issues associated with the use of methanol such as the toxicity and storage of large amount of inflammable methanol, expression systems based on non-methanol-induced P_{AOX1} may be established by activation of the methanol activation pathway or by inactivation of the catabolite repression pathway. A methanol-free, P_{AOX1}-based strain, MF1, was recently developed (Wang et al., 2017). This strain was constructed by deleting three transcription repressors associated with catabolite repression and by overexpressing the transcription activator Mit1, as previously described. The activity of P_{AOX1} in glycerol cell cultures reached 36% that of the wild type cultured in methanol. A fermentation strategy termed "Glucose-glycerol-shift" was designed based on the fact that MF1 is repressed by high concentrations of glucose, while repression is removed by glycerol or low concentrations of glucose (Wang et al., 2017). This strategy consists of batch and fed-batch phases to support biomass growth and P_{AOX1} repression, followed by a glycerol induction phase. An insulin precursor was shown to accumulate to a titer of 2.46 g/L, reaching 58.6% that of the wild type strain. Oxygen demand was also significantly reduced, making this strain a promising methanol-free system for protein production. Shen and coworkers screened 92 kinase mutants to identify two candidates: Δ*gut1* and Δ*dak* showed high AOX1 activities when cultured in non-methanol carbon sources (Shen et al., 2016). The Δ*gut1* mutant was introduced with a glycerol dehydrogenase from *Hansenula polymopha* to obtain Δ*gut1*-HpGCY1, a new strain that may be induced by glycerol. The Δ*dak* mutant on the other hand, can be induced by dihydroxyacetone (DHA). Both strains reached better expression levels than the glyceraldehyde-3-phosphate dehydrogenase (GAP) promoter and 50-60% that of P_{AOX1},

indicating the potential of those strains as novel methanol-free systems. Lately, a transcriptional repressor PpNrg1 was found to repress transcription of the AOX1 gene by binding directly to five sites on P_{AOX1}, two of which overlap the binding site of the key activator, Mxr1 (Wang et al., 2016b). The repression of AOX1 was partially released through the deletion of a hexose transporter, HXT1. This suggests that this transporter may be involved in the catabolite repression of P_{AOX1}. The HXT1-deficient *P. pastoris* strain has the potential for further engineering as a methanol-free induction strain (Zhang et al., 2010).

2.3.1.3 GAP promoter

The GAP promoter offers an alternative to P_{AOX1} when the toxicity of methanol is of concern, such as in the food industry. It is featured by a high-level constitutive expression and is more suitable for continuous cultivation due to simpler process controls. The P_{GAP} system can achieve titers of grams per liter scales of expression, which is comparable to the P_{AOX1} system (Zhang et al., 2009). The P_{GAP} system avoids the use of methanol and a shift from the biomass growth phase to the methanol induction phase, which is favored in continuous fermentation processes. The GAP promoter has been extensively used to drive protein expression, providing that the product is nontoxic to the host. Yang et al. reported that the expression of an alkaline phytase under the control of P_{GAP} is eight-fold higher than that under the P_{AOX1}-based system (Yang et al., 2015b). Constitutive-inducible expression has been achieved through the sequential transformation of target genes under P_{GAP} and P_{AOX1} into the same yeast strain. This strategy has shown to improve the yield of a *Rhizomucor miehei* lipase (RML) by six-fold (He et al., 2015). This dual promoter strategy has also proven to be useful in enhancing the production of acidic α-amylase and phytase (Parashar and Satyanarayana, 2016) as well as Thermomyces lanuginosus lipase (TLL) (Fang et al., 2014).

2.3.1.4 Alternative promoters

The choice of promoters for protein expression in *P. pastoris* is mostly limited to either P_{AOX1} or P_{GAP}. Persistent efforts have been made to identify novel regulatory sequences with the potential to replace the routinely used promoters. In order to identify alternative methanol inducible promoters, the transcriptional response of *P. pastoris* on microarray chips was observed under various carbon source conditions including glucose-repressed, derepressed and methanol-induced (Vogl et al., 2016). The authors tested a total of 45 promoters involved in the methanol utilization (MUT) pathway. Fifteen methanol-regulated promoters with varying strengths were identified. Notably, P_{CAT1}, involved in defenses against reactive oxygen species (ROS), showed a strong methanol induction and a high level of derepression. It was also found that P_{CAT1} can be induced by oleic acid at a level similar to methanol. Thus, P_{CAT1} could be used as a promising alternative derepressed, methanol-free promoter if its regulation mechanism was to be further elucidated.

Six novel, glucose-limited promoters were isolated and characterized by Prielhofer *et al*. using DNA microarray analysis (Prielhofer et al., 2013). These promoters were found to be repressed in glycerol batch cultivations and activated in the glucose feeding phase. Candidate P_{G1} is responsible for the regulation of a high-affinity glucose transporter, GTH1. When tested with two copies of the human serum albumin gene, P_{G1} achieved a two-fold greater expression than P_{GAP}.

An effective inducible promoter using formaldehyde, P_{FLD1}, is induced by methanol and requires methylamine as the sole nitrogen source. This promoter has been used to produce a *Yarrowia lipolytica* lipase (YPL), and expression levels were found to be comparable to those of the P_{AOX1}

system (Wang et al., 2012a). Co-expression of two proteins in the same vector under the control of P_{AOX1} and P_{FLD1} was achieved by methanol induction (Duan et al., 2009).

Another promoter, P_{PGK1}, was derived from the 3-phosphoglycerate kinase gene, and its essential fragment was identified by deletion analysis. Expression vectors incorporating P_{PGK1} have also been developed (Arruda et al., 2016). Optimization of *Candida antarctica* lipase (CAL) production from crude glycerol using P_{PGK1} was performed (Robert et al., 2017). This promoter could support a similar specific growth rate to that of P_{GAP}, indicating that P_{PGK1} could be used as an efficient alternative promoter. The promoter derived from the GCW14 gene in *P. pastoris* was isolated (Liang et al., 2013) and its regulatory sequences were characterized (Zhang et al., 2013). This promoter enables stronger constitutive expression than P_{GAP} and P_{TEF1} when enhanced green fluorescent protein (eGFP) is used as a reporter. A 20% improvement in promoter activity was obtained by screening a mutant library. The promoter regulating the sorbitol dehydrogenase gene (SDH) was reported to have a comparable activity to P_{GAP} for the expression of human serum albumin (HAS) (Periyasamy et al., 2013). The gene ADH3, responsible for ethanol utilization in *P. pastoris*, has also been characterized (Karaoglan et al., 2016). The capability of its promoter, P_{ADH3}, to drive protein expression was compared to those of P_{AOX1} and P_{GAP}. Fed-batch cultivation using glycerol as the carbon source and ethanol as an inducer resulted in higher enzymatic activity and biomass concentration than those obtained by P_{AOX1} and P_{GAP} systems. Other carbon source-dependent promoters including P_{ICL1}, P_{DAS1} and P_{PEX8} were repressed by glucose and activated by ethanol, methanol and oleate methanol, respectively (Vogl and Glieder, 2013).

P. pastoris can utilize L-rhamnose as the sole carbon source. The genes responsible for L-rhamnose metabolism were recently characterized. Two promoters associated with L-rhamnose

catabolism, P_{LRA3} and P_{LRA4}, were found to be strictly regulated by L-rhamnose and are able to drive efficient protein expression (Liu et al., 2016a). Further characterization indicated that P_{LRA3} has an intermediate strength and that P_{LRA4} is a weak promoter.

Promoters regulated by non-carbon source such as phosphate represent potential alternatives to both P_{AOX1} and other carbon source limited promoters. The promoter of a phosphate symporter gene, *PHO89*, was isolated and characterized (Ahn et al., 2009). Expression of a lipase under the control of P_{PHO89} was achieved upon the depletion of phosphate. Cell growth was affected but not ceased when phosphate starvation was needed for the activation of P_{PHO89}, which allowed continued protein production in the stationary phase. It was found that a 7.4-fold and 14.8-fold higher specific productivity of lipase under P_{PHO89} was observed compared with those of P_{TEF1} and P_{GAP}, respectively. The tight regulation and high transcriptional activation of P_{PHO89} by phosphate made this promoter a potential candidate for cost-effective production of proteins by optimizing initial phosphate concentration in *P. pastoris*. More recently characterized was P_{THI11}, which is derived from a gene involved in thiamine biosynthesis (Landes et al., 2016). Expression based on P_{THI11} was found to be carbon source- and growth-independent. In this case, protein expression may be controlled by the addition of thiamine to the medium. This promoter may represent a novel, methanol-free system and is worthy of further research. Most constitutive promoters are involved in carbohydrate metabolism, so further characterization is needed before they can be commercially viable for protein production.

2.3.1.5 Promoter engineering

Precise and predictive control of gene expression is critical to achieve maximal production. Endogenous promoters are subject to inherent levels of regulation at the transcriptional level. To alter promoter strength and to fine-tune gene expression, promoter engineering is used to fulfill

the capacity of a certain promoter. The use of libraries containing promoters of various strengths is useful as a tool to fine-tune expression levels and to identify novel cis-acting elements. Error-prone techniques such as polymerase chain reaction (PCR) and site-directed mutagenesis are frequently utilized to generate these promoter libraries. In this manner, a P_{AOX1} library containing promoters of varying activities ranging from 6-160% was developed, in which more than 12 cis-acting elements were identified (Hartner et al., 2008). Screening promoter variants should eliminate the effects of gene dosage and glucose repression. To circumvent this, Berg *et al*. developed a novel episomal plasmid selection strategy (Berg et al., 2013). Mutants of P_{AOX1} obtained by random mutagenesis were inserted into a vector containing a *P. pastoris* autonomous replication sequence and were screened in a *P. pastoris* GS115 strain with its glucose repression abolished. In these screening experiments, either Zeocin or luciferase was used as a reporter. Random mutagenesis was used to create a P_{GAP} library with activities between 0.006-fold to 19.6-fold (Qin et al., 2011). To obtain a deeper understanding of the transcriptional regulation of P_{GAP}, a synthetic library of variants containing duplicated or deleted putative transcriptional factor binding sites were constructed and characterized recently (Ata et al., 2017). The expression level was determined to be 0.35-3.1 fold and 1.6-2.4 fold that of the wild type P_{GAP} using GFP and human growth hormone as a reporter, respectively. Several transcription factors were investigated, among which duplicated GAL4-like binding sites or overexpression of GAL4-like transcription factor were found to boost protein expression in all tested conditions. The expression level of GFP was enhanced by 3.1-fold using a promoter P_9 containing duplicated GAL4-like binding sites and a strain overexpressing GAL4-like transcription factor. Similar results were observed for the expression of human growth hormone, indicating that GAL4-like transcription factor might play a critical role in the regulation of P_{GAP}. Synthetic promoters

combining both basal promoters and cis-acting elements can be used in DNA circuit designs and to improve protein expression. Vogl *et al*. reported the design of a fully synthetic core promoter and a 5′-UTR for *P. pastoris* (Vogl et al., 2014). The consensus sequence of the core promoter was identified by alignment with a set of promoters. Transcription factor binding sites were incorporated into the consensus to obtain a core promoter. When fused to the upstream sequence of P_{AOX1}, synthetic variant promoters with 10-117% activity compared to that of the wild-type P_{AOX1} were observed using GFP as a reporter protein. Despite limited improvements, synthetic promoters provide a module for further promoter engineering based on rational design with a deeper understanding of the regulation mechanisms of target promoters.

2.3.2 Protein secretion

Use of the secretion pathway is one of the major advantages of *P. pastoris* over other expression systems such as *E. coli* or *S. cerevisiae*. The downstream purification process is therefore significantly simplified due to this feature. Protein secretion can be directed by either the native signal peptides of certain proteins such as that of fungal xylanase or that of the commonly used α-mating factor (MF) available in many commercial vectors. Fine-tuning of α-MF by site-directed mutagenesis was performed to further improve the secretion of proteins (Lin-Cereghino et al., 2013). Deletion of amino acids 57-70 of α-MF resulted in a 50% increase in protein secretion. Another 18-amino acid secretion sequence from a *P. pastoris* protein was recently identified by Govindappa *et al*. This signal peptide serves as an efficient alternative when the target protein contains the internal cleavage sites of the α-MF (Govindappa et al., 2014). The efficiency of protein secretion may also be improved by modifying the N-terminus (Wang et al., 2014a) or by removing extra linker peptides (Chang et al., 2011). The selection of a specific

signal peptide is protein-specific. In another case, native signal peptides were found to outperform the α-MF, in which grams-per-liter titers of human calreticulin were obtained (Ciplys et al., 2015). The use of native signal peptides from *Candida antartica* lipase (CAL) B led to an increased secretion of three reporter proteins compared to that of α-MF (Vadhana et al., 2013). The native signal peptide was reported to be critical to the expression of *Trichoderma reesei* Cel61A, leading to a high yield and correct processing of the N-terminus (Tanghe et al., 2015). Endo protease Kex2 targets peptides at sites with pairs of dibasic amino acids, and is responsible for the cleavage of α-MF. Yang *et al*. examined the impact that the first amino acid residue downstream from the Kex2 site has on the cleavage efficiency (Yang et al., 2013b). Their study suggests that cleavage efficiency may be enhanced by optimizing the context of the Kex2 cleavage target sites and that by increasing the number of copies of Kex2 in the genome, secretion efficiency could be improved. Protein secretion is also related to the amino acid sequence at the N-terminus, the endoplasmic reticulum retention signal at the C-terminus and the acidic stability. The secretion of a methyl parathion hydrolase was enhanced 195-fold by combining the optimization of factors mentioned above (Wang et al., 2015b).

Protein folding in *P. pastoris* occurs in the endoplasmic reticulum (ER). Co-expression of chaperone genes facilitating protein folding was also found to be beneficial to protein secretion. Enzymatic yields of lipase r27RCL, which contains 3 disulfide bonds, were increased 2.74-fold by co-expressing protein disulfide isomerase (PDI) (Sha et al., 2013). Enhanced production of alkaline β-mannanase was observed in strains co-expressed with PDI or with *S. cerevisiae* Hac1p (Zhu et al., 2014). Azoun and coworkers tested the co-expression of five endogenous helper proteins which are involved in oxidative protein folding with rabies virus glycoprotein (Ben Azoun et al., 2016b). Notably, protein expression levels were enhanced 15-fold in a clone with

six copies of PDI. *Vitreoscilla* hemoglobin is an oxygen carrier with a high oxygen dissociation rate constant. Co-expression with *Vitreoscilla* hemoglobin in *P. pastoris* led to improved biomass and enzyme production due to alleviated oxygen limitations (Wang et al., 2012b) (Wang et al., 2016a). Higher secretions were also achieved by overexpressing a transcription factor, AFT1, which is involved in the regulation of secretion-related and carbon-responsive genes (Ruth et al., 2014).

Use of a protease-deficient strain could significantly reduce the rate of protein degradation. More than 80% of secreted human serum albumin (HSA) and human parathyroid (PTH) fusion protein in a YPS1, PEP4 double mutant remained intact compared to only 30% in the wild-type strain (Wu et al., 2013). Other strategies to reduce proteolytic degradation include the addition of protease inhibitors such as phenylmethylsulfonyl fluoride (PMSF) to the methanol feed solution. Kaushik reported that the titer of dengue virus serotype-3 envelope domain III increased 9-fold through the addition of 1% casamino acid (Kaushik et al., 2016). Besides extracellular proteolytic degradation, intracellular protein degradation mediated by vacuole should be minimized as well. It was found that vacuole protein sorting (Vps) encoding genes are responsible for the possible mis-sorting of heterologous proteins. The deletion of *vps*8 or *vps*21 involved in class C core vacuole/endosome tethering (CORVET) aiming to limit mis-sorting was found to result in less extracellular of heterologous carboxylesterase (CES) due to high serine proteinase level in supernatant, as was confirmed by liquid chromatography-mass spectrometry and protease inhibitor assay (Marsalek et al., 2017). Inactivation of Prb1, a major cellular serine protease, was found to enhance the protein yield by 56% and 80% compared with parental strain in the *vps*8 mutant and *vps*21 mutant, respectively. This study highlighted the significance of

protease deletion in CORVET-engineered strains. A thorough review of the metabolic engineering of the secretion pathway was reported in literature (Idiris et al., 2010).

2.3.3 Codon optimization

Codon usage varies among different organisms, and so replacing rarely occurring codons according to the preferred codon usage of *P. pastoris* is imperative to achieve high levels of expression. The *P. pastoris* genome has an average GC content of 41% based on the published genome sequence. To balance the GC distribution in a given gene sequence, synonymous codons may be used. Other factors including the secondary structure of messenger RNA, the codon adaption index (CAI) and the transfer RNA pool should also be taken into account when performing codon optimization. The services of codon optimization are provided by companies such as Genscript (USA) at no additional costs to the client. A summary of expression of codon-optimized genes was listed in Table 2-1.

Table 2-1 Expression of codon-optimized genes in *P. pastoris*

Gene name	Gene product	Promoter	Gene optimization method	Highest protein yield	Reference
xylA	*T. thermophilus* glucose isomerase	P_{AOX1}	Genscript, OPTIMIZER, Codon usage database	137 U/gDCW	(Ata et al., 2015)
GCSF	Human granulocyte colony-stimulating factor	P_{GAP} or P_{AOX1}	Premier Biosoft	0.66 mg/gDCW	(Maity et al., 2016)
Irisin	Human hormone irisin	P_{AOX1}	Codon usage database	77.98 mg/L	(Duan et al., 2015)
rml	*R. miehei* lipase	P_{GAP} and P_{AOX1}	NA[a]	175 U/mL	(He et al., 2015)
Cel6A	*T. reesei* cellulase	P_{AOX1} or P_{GAP}	DNA2.0	2 g/L	(Sun et al., 2016)
Egl1s	*T. reesei* endoglucanase	P_{AOX1}	GeneArt	NA	(Akcapinar et al., 2011)
Cap	Porcine circovirus type 2 capsid protein	P_{AOX1}	DNASTAR	174 mg/L	(Tu et al., 2013)
RABV-G	Rabies virus	P_{GAP}		150 ng/mL	(Ben Azoun

	glycoprotein				et al., 2016a)
Xyn11A	*Corynascus thermophilus* xylanase	P_{GAP}	Genscript	2200 U/mL	(Yang and Zhang, 2017)
Pmp-01867 Pmo-02916 Pmo-03328 Pmo-08760	*Neurospora crassa* polysaccharide monooxygenases	P_{AOX1}	NA	1.57 g/L 2.76 g/L 1.82 g/L 1.33 g/L	(Kittl et al., 2012)
ROL PhyA	*R. oryzae* lipase *A. niger* phytase	P_{AOX1}	Replacing rare codons, keeping GC content 45-60%, RNAfold	2.7 g/L 2.2 g/L	(Yang et al., 2012)
BlAmy	*B. licheniformis α*-amylase	P_{AOX1}	Graphical codon usage analyzer, DNA2.0	12.2 g/L	(Wang et al., 2015a)
Lip2	*A. niger* lipase	P_{AOX1}	DNA2.0	154 mg/L	(Yang and Liu, 2010)
IL25	Human interleukin-25	P_{AOX1}	Codon usage database	100 mg/L	(Liu et al., 2013)
hIFNγ	Human interferon gamma	P_{AOX1}	Invitrogen, GeneArt	16 μg/L	(Razaghi et al., 2017)
CALB	*C. antarctica* lipase B	P_{AOX1}	DNA2.0, codon usage database, Graphical codon usage 2.0	3 g/L	(Yang et al., 2013a)
pap	*A.oryzae* prolyl aminopeptidase	P_{AOX1}	Genscript Rare codon analysis tool	61.26 U/mL	(Yang et al., 2016)
Pgp	P-glycoprotein	P_{AOX1}	Entelechon software, Leto	NA	(Bai et al., 2011)
Lip2	Y. lipolytica lipase	P_{AOX1}	DNA2.0, RNAfold, Graphical codon usage 2.0	2.82 g/L	(Zhou et al., 2015)
KerA	*B. licheniformis* keratinase	P_{AOX1}	Synonymous codon replacement	324 U/mL	(Hu et al., 2013)
Sptk	*T. koningii* serine protease	P_{AOX1}	DNAWorks	3.2 g/L	(Shu et al., 2016)
PPL	Porcine pancreatic lipase		Codon optimization of 537 bp of 5-terminal	146 mg/L	(Zhao et al., 2014)
Srcam602 Or-7 E-760 L-1077	Broad antimicrobial spectrum bacteriocins	P_{AOX1}	GeneArt life technologies	NA	(Arbulu et al., 2015)
Endo-PGase	*Pectobacterium carotovorum* endo-polygalacturonase	P_{GAP}	Genscript	NA	(Rafique et al., 2016)
rChi21702	*Sanguibacter abtarcticus* endochitinase	P_{AOX1}	Genscript	30 U/L	(Lee et al., 2010)
EnInu	*A.niger* endoinulinase	P_{AOX1}	DNAWorks, Graphical codon	1349 U/mL	(He et al., 2014)

			usage analyzer		
CRL1	*C. rugosa* lipase	P_{AOX1}	OptimumGene	5.04 g/L	(Li et al., 2016)
AtDIR6	*Arabidopsis thaliana* dirigent protein	P_{AOX1}	GeneArt	47 mg/L	(Kazenwadel et al., 2013)
xynB	*A. usamii xylanase*	P_{AOX1}	Graphical codon usage analyzer	45225 U/mL	(Wang et al., 2016a)
Man26A	*A. niger* mannosidase	P_{GAP}	Genscript	5069 U/mL	(Zhao et al., 2011)

NA indicates not available

Optimized genes may be completely synthesized by commercial companies or created via assembly PCR and overlap extension PCR (Liu et al., 2013). Optimization of the *Trichoderma reesei* endoglucanase gene led to 24% improvement in activity relative to the native gene (Akcapinar et al., 2011). The expression of a codon-optimized endoinulinase gene was enhanced 4.8-fold compared to the native counterpart (He et al., 2014). Yang and Liu performed an optimization of the *Y. lipolytica* lipase gene by *de novo* gene design and synthesis (Yang and Liu, 2010). The optimized gene outperformed the native version in terms of biomass accumulation and enzyme secretion under the control of P_{AOX1}. Mellitzler *et al*. examined the effects of codon optimization, as well as the choice of promoters and gene copy numbers on the yield of a *T. reesei* cellulase (Mellitzer et al., 2014). A total of 48 synthetic codon-optimized variants were obtained using the Monte Carlo algorithm according to the *P. pastoris* codon usage. The highest protein yield in *P. pastoris* ever reported was 18 g/L and was achieved by combining the highest-performing synthetic gene with a synthetic derepressed promoter. Codon optimization of the *Aspergillus niger* lip2 gene resulted in 11.6- and 5.3-fold increases in enzyme activity and protein titer, respectively (Yang and Liu, 2010). Zhao *et al*. optimized the 5'-terminal of a pancreatic lipase and improved the protein yield as well as the enzymatic activity by 4- and 5-fold, respectively. A higher abundance of the pancreatic lipase mRNA was also observed in the

strain transformed with the optimized gene, indicating an enhanced efficiency of transcription bestowed by codon optimization (Zhao et al., 2014). Yang *et al*. modified the codon usages of the genes for CAL and α-MF to achieve an expression level of 3 g/L and an enzymatic activity of 6100 U/mL after optimization of fermentation conditions (Yang et al., 2013a). Li *et al*. reported the optimization of xylanase gene xynB from *A. niger* using synonymous codons (Li et al., 2012). The optimized gene had a GC content of 43.6% compared to 57.7% in the wild-type gene. This resulted in a xylanase activity of 20424.2 U/mL under fed-batch fermentation. The efficient production of a pH-stable fungal xylanase was recently described using codon-optimized sequences (Yang and Zhang, 2017). The enhanced expression of synthetic codon-optimized genes was also observed for α-Amylase (Wang et al., 2015a) and irisin (Duan et al., 2015).

2.3.4 Gene dosage

Increasing gene dosage can enhance the protein yield within a certain range of copy numbers. However, expression levels tend to plateau when heterologous protein overexpression subjects the host to metabolic burden. Furthermore, secretion capacity also limits the maximum protein titer. The generation of multi-copy strains can be achieved using *in vivo* and *in vitro* methods (Zhu et al., 2009). Screening for multi-copy clones is normally done by selection with increasing concentrations of antibiotics or by *in situ* measurements of enzymatic activity. It was shown that copy numbers may be quantified by real-time PCR. Using G418 resistance as a selection marker, a yeast strain harboring 52 copies of the porcine insulin precursor (PIP) gene was constructed. However, this high-copy strain suffered from impaired growth. An optimal expression level was achieved by a strain with 12 copies of the PIP gene. It was determined that excessively high numbers of copies of foreign genes led to protein-folding oxidative stress and created a carbon-deprived environment, as was confirmed by transcriptional analysis of key genes (Zhu et al.,

2011a). Shu and coworkers reported that the use of two copies of the *Trichoderma koningii* serine protease gene outperformed the use of one, three and four copies in terms of expression level (Shu et al., 2016). Hu *et al*. developed a visual method to rapidly select for high-production clones using a mannanase reporter (Hu et al., 2011). The target protein was fused to mannanase and high-production clones were selected based on the size of the hydrolysis zones seen on the plates which contained the substrate for mannanase. Synergistic effects can be obtained by using the combined engineering strategies mentioned above. An example of this would be that the expression level of *Citrobacter amalonaticus* phytase was increased four-fold by modifying P_{AOX1} and the α-signal, selecting a clone containing 6 copies of the phytase gene as well as co-expressing chaperone HAC1p (Li et al., 2015).

2.3.5 Systems biology methods

2.3.5.1 Genome-scale metabolic models (GEMs)

The strategies discussed above are primarily focused on the optimization of the target gene expression circuit itself. GEMs are invaluable tools for strain improvement of *P. pastoris* as they provide comprehensive knowledge of the metabolism of this yeast to predict phenotypes under certain environments, thereby allowing rational design of metabolic pathways. The construction and validation of a number of GEMs have been described since the publication of the whole genome sequences of several *P. pastoris* strains. Three fully compartmentalized GEMs including PpaMBEL1254 (Sohn et al., 2010), iPP668 (Chung et al., 2010) and iLC915 (Caspeta et al., 2012) were among the earliest models at genome-scale level and widely employed. Evaluation of carbon source utilization, constraint-based flux analysis, prediction of maximum specific growth rate and *in silico* simulation of heterologous protein production may be performed with those GEMs. Process engineering relevant factors such as oxygen limitation and substrate co-feeding

(discussed in later sections) may also be predicted by GEMs. For instance, the optimal point of oxygen uptake rate (OUR) at which maximum protein production rate could be achieved without compromising cell growth was identified using PpaMBEL1254. Chung and coworkers examined the effect of carbon sources on cell growth and found that glycerol and sorbitol are suitable candidates for protein production by supporting high growth rate and amino acid biosynthesis capacity using the model iPP668. The high energy requirement of methanol metabolism was elucidated as well in this work. The simulation of the production of an antibody using GEM iLC915 suggested that mixed feeding of glycerol with methanol may be beneficial to the production of antibody.

Existing GEMs could be extended for the production of particular proteins such as glycoprotein. Irani et al. developed such a GEM, *ihGlycopastoris*, by incorporating the native and humanized N-glycosylation pathways into iLC915 (Irani et al., 2016). This model was validated with experimental data sets and predictions showed good agreement with measured growth rates despite failing to predict the protein production under some conditions. The model suggests that N-glycosylation led to reduced yields compared with non-glycosylated counterparts. Potential gene modification targets for enhanced protein yields were predicted using Flux Scanning based on Enforced objective Function (FSEOF) by *ihGlycopastoris*, which could be verified by future experimental evidence.

Lately, a comprehensive upgrading of the three existing GEMs (PpaMBEL1254, iPP668 and iLC915) was performed to result in a merged model iMT1026 (Tomas-Gamisans et al., 2016). This model represents a more complete, versatile and non-redundant version of GEM. The GEM iMT1026 has been extensively validated and showed improved performance over the existing GEMs, indicating that it could be a powerful tool for investigating *P. pastoris*. More recently

reported was GEM iRY1243 (Ye et al., 2017), incorporating PpaMBEL1254, iPP668, iLC915 and iMT1026 to cover more metabolites and reactions. This model was verified by RNA-Seq and ^{13}C-fluxes data and showed improved predictability of growth capabilities on various carbon and nitrogen sources.

To overcome the limitation of stationary GEMs discussed above, a GEM able to predict the behaviour of *P. pastoris* under dynamic conditions such as batch and fed-batch fermentation was recently described (Saitua et al., 2017). This GEM showed robustness in analysis of flux distribution during different stages of cultivation and identification of potential gene deletion targets. The effect of feeding strategy was also evaluated using this model, indicating the versatility of this GEM for optimization of *P. pastoris* to achieve enhanced protein production.

2.3.5.2 Metabolic flux analysis (MFA)

MFA using systems biology approach, although not necessarily an engineering strategy, can offer a deeper understanding of the carbon flux redistribution during heterologous protein expression. Protocols for ^{13}C-based MFA of *P. pastoris* have been well described in literatures (Ferrer and Albiol, 2014a, b). Extensive studies particularly focusing on MFA of *Pichia* cultures using mixed feeding have been reported in recent years. MFA of chemostat cultures grown on glucose or glucose/methanol mixtures was performed to understand the effect of glucose co-feeding on carbon flux shift (Jordà et al., 2012). It was found that the elementary and macromolecular composition was largely influenced by culture conditions and genetic background of strains. Increased flux towards glycolytic pathway, TCA (tricarboxylic acid) cycle and NADH (nicotinamide adenine dinucleotide) regeneration pathways were observed in recombinant strains secreting *R. oryzae* lipase (ROL) than the control strain. The impact of methanol co-feeding on the redistribution of carbon flux was revealed by observing the shift of

carbon flux of glycolytic pathway towards pentose phosphate pathway (PPP). In mixed feeding cultivation, the majority of methanol (80%) was dissimilated to CO_2 due to the energy requirement of methanol utilization pathway. Moreover, cell stress caused by secretion of ROL instead of high level expression was also elucidated. This was also confirmed in later study using metabolomics and instationary ^{13}C-based MFA (Jorda et al., 2013). It was determined that the pool of trehalose associated with cell stress response was two-fold higher in recombinant strain than that in the control strain. Similar trend with respect to higher fluxes directed to TCA cycle and NADH regeneration was also observed. The results revealed increased fraction of methanol being directly oxidized to CO_2 due to methanol assimilation and secretion of ROL. In another study, instationary ^{13}C-based MFA of *P. pastoris* grown on glucose/methanol mixtures was conducted (Jorda et al., 2014b). The mass spectrometry-based measurements enabled more accurate profiling of several important pathways than the nuclear magnetic resonance-based counterparts. Interestingly, methanol dissimilated to CO_2 was found to be 54%, lower than that obtained by NMR-based method. The instationary MFA was able to provide insights into bidirectional reactions and metabolic fluxes which could not be obtained by NMR-based MFA. Moreover, the metabolic network was expanded by incorporating more pathways. To gain insights into the effect of glycerol/methanol mixed feeding on the carbon flux shift, MFA was performed by (Jorda et al., 2014a). The authors investigated chemostat cultures with various glycerol/methanol ratios and dilution rates. It was found that carbon fluxes shift is correlated to dilution rate while glycerol/methanol ratio played a less important role. Production of arabitol at high growth rate, which was neglected in previous studies, was identified in this work. Methanol directed to central carbon metabolism or dissimilation to CO_2 was directly related to the glycerol/methanol ratio and dilution rate. Biomass yield was found smaller at lower dilution rate.

A. Isidro and coworkers implemented a hybrid MFA, combining traditional MFA and statistical modelling, to investigate the effect of medium composition on the production of a single chain antibody fragment (Isidro et al., 2016). The hybrid MFA was able to predict the protein yield of 5 independent experiments. It was found that cell growth showed little sensitivity to medium composition in contrast with the high sensitivity of protein production. Recently, grey modelling was implemented to help understand the phenotypes of *P. pastoris* grown on different carbon sources resulted from the internal states of cells (González-Martínez et al., 2014). On-line monitoring technique such as near infrared spectroscopy (NIS) was employed as an input for MFA to achieve a real-time metabolic flux analysis (Fazenda et al., 2013). Consistent results have been obtained using this system, which paved the way for further optimization of NIR modelling to achieve the ultimate goal of on-line MFA. Celik *et al*. performed a dynamic flux balance analysis in a *P. pastoris* strain secreting human erythropoietin (rHuEPO) under three different specific growth rates. They investigated the carbon flux towards biomass production and rHuEPO at different growth phases in relation to the methanol feed rate (Celik et al., 2010). These results identified an optimal methanol feed rate for each stage to achieve optimal biomass growth and product formation.

MFA of the P_{GAP}-based systems has also been reported. Two *P. pastoris* strains secreting high and low level of a β-galactosidase were grown on glucose and investigated using MFA (Nie et al., 2014). It was found that high level expression was associated with significant up-regulation of oxidative branch of PPP and glycolytic pathway. Addition of Krebs cycle intermediate glutamate to enhance energy supply led to increased production of β-galactosidase.

MFA provides not only a framework of the carbon flux distribution but the valuable datasets for validation of existing GEMs. Metabolic engineering of *P. pastoris* for enhanced protein

production guided by GEM and MFA has been described lately. Nocon *et al*. examined the effects of the overexpression of human superoxide dismutase (hSOD) on carbon flux shift using both *in silico* genome-scale modelling as well as intracellular flux changes obtained by ^{13}C labelling (Nocon et al., 2014). Results based on both methods revealed the same trends in tricarboxylic acid (TCA) flux increase. Nine and six target genes for overexpression and deletion were identified by FSEOF and Minimization of Metabolic Adjustment (MOMA), respectively. Five out of 9 predicted genes were overexpressed and proved to be beneficial to the production of hSOD, highlighting the effectiveness of this method. This strategy is consistent with results obtained in another study where the overexpression of individual genes in the PPP improved hSOD production (Nocon et al., 2016).

Other systems biology approach such as the study of lipodome and trancriptome under hypoxia condition has been described (Adelantado et al., 2017). The pattern of membrane lipid composition differed under normal oxygen or hypoxia. The correlation of reduced ergosterols and increased protein secretion was elucidated in this study.

In short, GEMs and MFA are indispensable systems biology tools for enhancing protein production as they may provide a system-level view of the metabolism in heterologous protein-producing *P. pastoris* under different cultivation conditions. Metabolic burden could be alleviated through modification of targets identified by GEMs and MFA. Efficient fermentation strategies could be established based on the simulation of GEM and MFA results of mixed feeding, oxygen limitation and medium compositions.

2.4 Recent advances in process monitoring

2.4.1 Substrate monitoring

Methanol monitoring is crucial for the success of the P_{AOX1} systems. Excessive methanol accumulation, particularly for the MUT^- strain, is cytotoxic while insufficient methanol may reduce protein yield. Monitoring techniques are generally classified as on-line and off-line methods. Compared with conventional time-consuming off-line methods such as gas chromatography (GC) or high performance liquid chromatography (HPLC), on-line methods eliminates manual sampling and offers rapid and efficient analysis, and thus is desirable in *P. pastoris* fermentation.

In situ methanol monitoring is usually achieved by a probe immersed into the fermentation broth or off-line methanol monitor which monitors methanol through exhaust gas (Cos et al., 2006). The in-line probes must be autoclavable. Both types of monitors are based on liquid-gas equilibrium. Methanol in the culture diffuses through a membrane and vapour is detected by sensors in the probe. Such probes are commercially available and have been employed in quite a number of studies (Ding et al., 2014, Hang et al., 2009, He et al., 2014, Jin et al., 2010). The detection range of those probes is 0-20 g/L. Feed-back control system may be established for reliable methanol control using the on-line methanol probes and process control software such as LabVIEW (National Instruments, USA). The diagram of such a system has been illustrated by Panchiga and coworkers (Panchiga et al., 2016). The latter type of methanol monitor typically detects methanol vapour in exhaust gas using semiconductor sensors such as Figaro TGS822 alcohol sensor and has been reported in literatures (Curvers et al., 2001, Khatri and Hoffmann, 2006). The major drawbacks of the sensor-based monitoring are non-linear calibration and possible interference of ethanol which is formed during fermentation. Other types of on-line

methanol monitoring systems where a sampling probe was needed to withdraw aliquots such as sequential injection analysis (SIA) were also described in literatures.

Infrared spectroscopic methods have been developed for bioprocess monitoring in recent years. Near infrared (NIR), mid-infrared (MIR) and Fourier transform mid-Infrared spectroscopy (FTIR) are the most widely employed techniques for on-line monitoring of *Pichia* bioprocess. Fiber optic probes immersed into culture are required to perform *in situ* analysis. Ex-situ substrate monitoring is generally achieved by a flow-through cell (Schenk et al., 2007) or loop circulating the broth between bioreactor and monitor (Landgrebe et al., 2010). Crowley and coworkers developed a method to monitor methanol and glycerol based on NIR (Crowley et al., 2005). Extensive mathematical modelling and validation for spectra collection was performed. The models showed good fit to the transmission gathered spectra and reflectance gathered spectra, which could be potentially used for external validation. Similar models for methanol and glycerol based on FTIR was also established and validated by (Crowley et al., 2000). To address the complicated and time-consuming calibration of MIR-based monitoring, Schenk and coworkers developed a simple linear two-point calibration method which could be easily implemented *in situ*. On-line measurements showed good agreements with off-line determined data (Schenk et al., 2007).

Kim and coworkers used an on-line monitoring system to measure concentrations of glycerol and methanol as well as the relative cell density in real time based on NIR (Kim et al., 2015). This system was reported to provide accurate measurements year-round after installation.

Dahlbacka *et al*. used a FTIR-ATR system using an attenuated total reflection (ATR) probe to measure the concentrations of glycerol and methanol on-line, with an precision of 0.68 g/L and

0.13 g/L, respectively (Dahlbacka et al., 2012). Nosie signal was minimized by including training data of medium components. A mathematical model was developed to replace time-consuming calibration sampling. Goldfeld and coworkers reported the development of an online *Pichia* monitor based on NIR (Goldfeld et al. , 2014). The authors demonstrated the feasibility of a methanol feedback control system using a proportional-integral algorithm with real-time data acquisition. Methanol can be maintained as low as 1 g/L. This system showed robustness during a period of 274 days, indicating its promising potential for industrial use. Despite the obvious advantages of spectroscopy-based monitoring, its application may be limited by the high cost of equipment. A detailed review discussing bioreactor monitoring using infrared spectroscopy, may be found in literature (Landgrebe et al., 2010).

2.4.2 Biomass monitoring

The measurement of biomass is key to the process monitoring of *P. pastoris* fermentation. Commonly used on-line biomass monitoring techniques including dielectric spectroscopy, optical probing, infrared spectroscopy and fluorescence spectroscopy have been reviewed by (Kiviharju et al., 2008). FT-IR and NIR spectroscopic methods described above have been demonstrated to monitor biomass concentrations using mathematical model. However, those methods become limited when the dry cell weight reached a threshold (64.1 g/L) due to the switch from transmission to reflectance, restricting their use in high cell density cultivation process which is common for *P. pastoris*.

Biomass monitoring using permittivity measurement has been employed for on-line monitoring of *P. pastoris* fermentation (Dabros et al., 2010). This technique employs a capacitance probe plugged into broth to measure the permittivity (Kiviharju et al., 2008). Horta *et al*. evaluated this technique for the monitoring of several industrial microorganisms including *P. pastoris* which

obtained good correlation between permittivity signal and cell concentration (Horta et al., 2015). However, the tested cell concentrations were low (below 7 g/L). In a recent study, biomass based on permittivity measurement showed good agreement with off-line measurements (Goldfeld et al., 2014). However, accuracy of this method may be affected by cell morphology and physiology.

Fluorescence method exploits the cellular fluorophores such as tryptophan, NADH and riboflavin to determine biomass via fluorescence measurement. Single or multiple wavelengths could be used to obtain 1D or 2D fluorescence spectroscopy, respectively. 1D fluorescence spectroscopy is easy to implement and is suitable for only viable cells. However, this method could suffer interference of the culture conditions and background fluorescence. Chemometric tools including partial least square (PLS), principal component analysis (PCA) and neural networks are generally needed to calculate biomass for 2D fluorescence.

Estimation of biomass using on-line fluorescence of *P. pastoris* has already been described by Surribas and coworkers (Surribas et al. , 2006b). Multiple wavelengths fluorescence (MWF) coupled with PLS-1 model could provide prediction of biomass with error less than 7% (Surribas et al. , 2006a). The application of this method was limited by proteolytic degradation, which caused prediction error of 20%. Brunner *et al*. developed a method by combined use of fluorescence of four single-wavelength for biomass estimation (Brunner et al. , 2016). Various chemometric tools including multiple linear regression (MLR), PLS and principal component regression (PCR) were implemented to identify tryptophan as the most relevant variable and establish the correlation between tryptophan and biomass. Further validation of this method on bioreactor is needed to make it practical for bioprocess monitoring of *P. pastoris*.

Software sensors are another important category of indirect biomass estimation methods. They are gaining increasing interest to serve as alternatives to the direct monitoring methods. The robustness of software sensors is directly associated with the accuracy of the mathematical models used. Software sensors are generally established using data-driven or model-driven methods (Kadlec et al., 2009). The data-driven methods such as PCA, PLS, PCR and artificial neural networks (ANNs) require historical datasets for model training. In contrast, the model-driven methods are developed based on first principles such as liquid-gas equilibrium, growth kinetics and energy balance which is not always readily available. Wechselberger et al. reported the development of a model based on Monod kinetics to give real-time prediction of growth stoichiometry and specific growth rate. On-line estimation was validated with off-line measurements and showed good fit (Wechselberger et al., 2010). Wechselberger and coworkers developed a real-time soft sensor to estimate biomass using elemental balancing (Wechselberger et al., 2013). This sensor was able to estimate biomass concentrations under various yields even for induced cultures. However, it cannot differentiate soluble proteins from biomass.

Driven by the process analysis technology (PAT) initiative, a biocalorimetry-based system was developed by using heat-flow measurements which is an indicator of cellular metabolic activity (Sivaprakasam et al., 2011). In-line probes including dielectric microscopy, pH and dissolved oxygen (DO) as well as off-line gas analyser was used as input to characterize cell growth. Good correlation was found between estimated and experimentally-determined biomass for the three Crabtree effect negative yeast species tested. Process control strategy based on soft sensors was established and validated (Sagmeister et al., 2013, Schuler et al., 2012).

Recently, *in situ* microscopy (ISM) was employed to measure the optical density of *P. pastoris* cultures (Marquard et al., 2016). This method measures the optical density using an *in situ*

microscope and image analysis software to count the number of cells. A linear relationship between OD_{600} and the cell count was obtained up to an OD_{600} of 209. The accuracy of such a method is affected by cell cluster formation and by undissolved medium components.

2.5 Fed-batch cultivation

Fed-batch is an efficient operational strategy for high cell density fermentation. It is initiated with a batch phase, followed by a carbon source feeding phase to achieve high biomass accumulation for methanol induction and product formation. Carbon source depletion at the end of the batch phase is indicated by a DO spike. In *P. pastoris* fermentation, a carbon source such as glucose or glycerol is commonly used in the batch phase to develop a base of cell growth prior to feeding the experimental carbon source. The Invitrogen fed-batch fermentation protocol gives general guidelines for *P. pastoris* fermentation. Strain-specific parameters such as the maximum specific growth rate (μ_{max}), the methanol uptake rate and the specific productivity need to be determined for process development (Looser et al., 2015). A rapid approach to obtain a minimum set of process parameters has been proposed (Dietzsch et al., 2011). For the P_{AOX1} system, the rate of methanol feeding is critical to the success of protein production because methanol serves as both a carbon source as well as an inducer during the induction phase. However, accumulation of methanol is cytotoxic to cells and should therefore be avoided. For the P_{GAP} system, product formation is found to be growth-associated and is easier to control.

2.5.1 Fed-batch cultivation using P_{GAP}

Factors affecting P_{GAP} fed-batch fermentation performance include carbon source, medium composition, pH, temperature, and feeding strategy (Çalik et al., 2015). Typical fed-batch cultivation under the P_{GAP} promoter consists of a batch phase followed by a substrate feeding phase. Glycerol and glucose are routinely used for P_{GAP} cultivation. Alternative carbon sources

including fructose and ethanol have been screened for their potential use in fed-batch fermentations (Potvin et al., 2016). Among the feeding strategies for cultivation of P_{GAP} systems, exponential feeding is considered as an efficient method to achieve maximum cell growth and protein production. Substrate is fed using a pre-programmed profile to maintain the specific growth rate at a constant value. It was reported that high specific growth rate leads to enhanced production of proteins (Çalik et al., 2015). The effect of specific growth rate on the production of human antigen-binding fragment (Fab) was evaluated by implementing exponential feeding strategy recently (Garcia-Ortega et al., 2013). The authors evaluated the effect of carbon sources including glycerol and glucose and identified glycerol as a better carbon source for the production of Fab during batch cultivation. Three specific growth rates on glucose were explored: 0.05 h^{-1}, 0.1 h^{-1}, and 0.15 h^{-1}. Higher volumetric productivity was higher at 0.15 h^{-1} than that at 0.1h^{-1}, despite similar product yields were obtained. In another study performed by Garcia-Ortega *et al*., effect of specific growth rate ranging 0.025 h^{-1} to 0.15 h^{-1} was investigated using chemostat cultivations (Garcia-Ortega et al., 2016). A steady increase of the Fab production rate was observed as the specific growth rate increases. Interestingly, it was found that the implementation of short carbon-starving period during the exponential feeding led to enhanced Fab production. Notably, a staving period of 30 min in every 1.5 h resulted in 44.8% increase of Fab production compared with standard operation. The mechanism of this strategy could be due to the upregulation of the glycolytic pathways during glucose-depleted period which is beneficial to the secretion of Fab.

The general procedure to implement the exponential feeding strategy could also be extracted from this study and contains the following steps: Batch cultivations should be performed to achieve the following goals: Selection of suitable carbon source; Determination of average and

maximum specific growth rate and biomass yield on substrate. Evaluation of effect of specific growth rate on protein production; Implement the exponential feeding strategy using appropriate specific growth rate. Substrate feed rate could be determined using eq. 1.

$$Q(t)=\frac{\mu V0Cx}{CsYx/s}\exp(\mu t) \quad (1)$$

Where Q is the substrate feed rate; μ is the pre-determined specific growth rate; V_0 is the initial volume of fermentation medium; Cx is the cell concentration obtained at the end of batch phase; Cs is the substrate concentration in feeding solution; $Y_{X/S}$ is the growth yield on substrate (Çalık et al., 2015).

2.5.2 Fed-batch cultivation using P_{AOX1}

The biomass growth phase of the P_{AOX1}-system is similar to that of P_{GAP}. Glycerol is commonly used to produce a sufficient biomass prior to methanol induction. Generally, a short transitional period is required to deplete the residual glycerol and eliminate possible repression in the induction phase. Otherwise, a mixed feed of glycerol and methanol may be fed to adapt the cells to methanol cultures. Panula-Perala *et al*. proposed an interesting glucose feeding strategy using a glucose-based polymer to slowly release glucose into the medium (Panula-Perala et al., 2014). This feeding strategy maintains a low glucose concentration while avoiding cellular starvation and inactivation of P_{AOX1}, leading to a six-fold improvement in measured enzymatic activity. Maintaining methanol concentrations at an appropriate level is critical to obtaining high protein yields. Various methanol feeding strategies, either direct or indirect, have been extensively studied, the most widely used and effective of which are discussed here.

2.5.2.1 DO-stat control

Methanol utilization processes generally have a high oxygen demand. DO-stat represents an effective indirect control strategy when the concentration of methanol is not monitored. The DO is usually maintained within a desired range from the set point due to the difficulty in controlling DO at its extremes. The DO is generally maintained at above 20% air saturation by cascading the agitation speed and air flow rate. Ponte and coworkers investigated ROL production under DO levels of 5%, 10%, 25% and 50% (Ponte et al., 2016). The highest productivity, μ, methanol consumption rate, specific substrate uptake rate and specific oxygen uptake rate were each obtained at a DO of 25%, though a higher product yield was observed at a DO of 10%. The oxygen transfer rate (OTR) is an important parameter to characterize the oxygen transfer efficiency in a bioprocess. Maintaining the DO above 50% may shorten the adaption period by 4-6 hours. Furthermore, it was shown that porcine interferon-α production was enhanced due to active adenosine triphosphate (ATP) regeneration without oxygen limitations (Gao et al., 2011). In pilot-scale productions, the use of pure oxygen may be reduced by increasing the pressure of the air supply or jar air pressure (Liu et al., 2016c). This strategy is especially useful to reduce the cost of using pure oxygen under pilot-scale fermentations.

2.5.2.2 μ-stat control

This strategy controls the methanol feed rate according to a predetermined μ. Under this strategy, methanol is added at an exponential rate to the bioreactor. Normally, μ is set below μ_{max} to avoid methanol accumulation. Anasontzis *et al*. performed experiments with exponential methanol feeding under high and low values of μ (Anasontzis et al., 2014). Higher productivity and protein titer favor lower exponential feed rates, showing a volumetric productivity 1.5-fold greater than that of using a high μ. Further optimization has identified that the optimal μ is 0.05 h^{-1}. Min *et al*. studied the production of saxatilin under different values of μ by gradually increasing the rate of

methanol feeding (Min et al., 2010). The highest saxatilin yield and specific production rate was obtained under a moderate μ as opposed to under the highest μ, which may be attributed to the fact that a higher μ leads to a larger accumulation of biomass and less methanol is used for protein synthesis. They also made a comparison between constant methanol feeding and two-stage methanol feeding strategies. The latter strategies consisting of a high μ followed by a low μ resulted in optimal production with shorter fermentation times. Jacobs *et al*. employed the μ-stat control strategy for granulocyte-macrophage colony-stimulating factor production by setting μ to 25%, 50% and 75% of μ_{max} (Jacobs et al., 2010). From these experiments, maximum biomass values for accumulation and protein yield were obtained at 50% and 25% of μ_{max}, respectively. Barrigon *et al*. compared the production of ROL using three different values of μ: 0.015 h^{-1}, 0.02 h^{-1} and 0.045 h^{-1} (Barrigón et al., 2013). The highest enzymatic activity, volumetric productivity and specific productivity were achieved at a value of μ of 0.015 h^{-1}. A strategy combining the μ-stat and DO-stat controls was implemented for the production of β-galactosidase. This strategy uses a lower μ to avoid the accumulation of methanol, followed by the use of a higher μ to obtain better growth, and then finishing by using DO-stat control when oxygen becomes limited. The specific growth rate is especially critical for proteins with disulfide bonds. Aggregates of human consensus interferon mutant (cIFN) were observed under a high growth rate due to the incorrect formation of disulfide bonds (Wu et al., 2011a). To address this issue, a two-stage methanol control strategy was introduced where methanol was kept at 5 g/L in the first stage and 2.5 g/L in the second stage to maintain a value of μ of 0.02 h^{-1}.

2.5.2.3 Constant methanol feed rate

Maintaining the concentration of methanol constant during the induction phase represents an unlimited feeding strategy. This strategy is easy to implement given that the concentration of

methanol can be measured with ease. Methanol is generally monitored on-line using sensors as described previously. Gurramkonda *et al*. implemented this strategy by maintaining methanol concentrations at 2 g/L using feedback control based on on-line methanol measurements during the insulin precursor production phase (Gurramkonda et al., 2010). This strategy proved to be robust and led to a final insulin precursor titer of 3 g/L. A much lower value of μ was observed at 10 g/L despite a slightly higher ROL activity which was achieved, possibly indicating an inhibitory methanol concentration. Wu and coworkers investigated the effect of methanol concentrations ranging from 0.5 to 3.5 g/L on *Rhizopus chinensis* prolipase production (Wu et al., 2011b). The optimal concentration was found to be 1 g/L. Lipase degradation was observed to be greater at 3.5 g/L in comparison with no degradation at 0.5-1.0 g/L.

2.5.2.4 Substrate co-feeding

Methanol is essential to the induction of P_{AOX1}, but its use leads to a lower μ compared to glycerol or glucose. Moreover, methanol utilization generates a lot of heat. Maintaining a stable methanol concentration is critical for the Mut^s strain which requires longer fermentation times and is more sensitive to fluctuating methanol concentrations. To address these problems, mixed substrate feeding strategies have been frequently used. The addition of glycerol, glucose, sorbitol and mannitol as co-substrates has been reported to benefit the biomass growth and to enhance enzyme titer (Sun et al., 2016).

2.5.2.5 Methanol co-feeding with glycerol

Glycerol has been used as co-substrate despite its potential repression of protein expression. The effect of glycerol co-feeding on the production of avidin was evaluated. It was found that methanol fraction of 0.62 Cmol/Cmol in the mixed feed resulted in 1.1-fold higher volumetric production rate than that of methanol only attributed to improved biomass yield on mixed

substrate (Jungo et al., 2007). Glycerol outperforms sorbitol as a co-substrate with respect to supporting a higher growth rate. Arnau et al. tested the viability of using a glycerol/methanol combination under three values of μ for ROL production (Arnau et al., 2011). Optimal results were obtained with a value of μ of 0.02 h^{-1} in terms of lipolytic activity, product yield, as well as volumetric and specific productivities. Increasing μ to higher values of 0.05 and 0.1 h^{-1} eliminated the lag phase but led to higher proteolytic activities. Moreover, the effectiveness of a higher μ on glycerol was compromised in the Mut^s strain. The ratio of μ_{Gly} to μ_{MeOH} should be carefully selected to avoid sharp decreases in product yield and productivity. Luley-Goedl *et al*. used three glycerol/methanol ratios for co-feeding to the fermentation of *P. pastoris* KM71H producing a human sialyltransferase (Luley-Goedl et al., 2016). Biomass accumulation was 1.7-fold higher at a ratio of 2:1 (glycerol/methanol) than with methanol only. Transferase activity was nearly undetectable using a pure methanol feed while an average activity of 17.2 U/L was obtained using a 2:1 glycerol/methanol feed.

2.5.2.6 Methanol co-feeding with sorbitol

Sorbitol co-feeding has been increasingly used in *Pichia* fermentations in recent years. Niu *et al*. performed metabolic flux analysis (MFA) on a methanol/sorbitol mixed substrate culture (Niu et al., 2013). They found that specific oxygen consumption was reduced by 30% when using a mixed feeding strategy. Maximal β-galactosidase activity was achieved by maintaining a methanol fraction of 0.45-0.75 C-mol/C-mol. Zhu *et al*. used a combinatorial strategy of low induction temperature and sorbitol co-feeding to produce an alkaline β-mannanase (Zhu et al., 2011b). The highest enzymatic activities and biomass concentrations were achieved at 26°C and a methanol/sorbitol ratio of 10:1. When pure methanol is used as both the sole carbon and energy source, energy metabolism heavily relies on methanol assimilation. Celik *et al*. employed an

established stoichiometric model to perform MFA analysis under methanol/sorbitol mixed feeding (Celik et al., 2010). It was found that when methanol feed exceeds the cell demand under a high μ, a shift in energy metabolism occurs. Gao *et al*. also revealed that co-feeding with sorbitol shifted the energy metabolism from formaldehyde dissimilation to the TCA cycle (Gao et al., 2012). This shift was confirmed by comparing the activity of FLD (the first enzyme in the formaldehyde dissimilatory pathway) to those of several key enzymes including pyruvate dehydrogenase (PDH), α-ketoglutarate dehydrogenase complex and isocitrate dehydrogenase (IDH) of the TCA cycle.

Arnau *et al*. optimized the production of ROL using sorbitol as a co-substrate (Arnau et al., 2010). They tested the effects of operating with different values of μ under different methanol concentrations. The best strategy was found to be 2 g/L of methanol and a value of μ of 0.01 h^{-1}. Wang *et al*. implemented a sorbitol co-feeding strategy for the production of alkaline polygalacturonate lyase (PGL) (Wang et al., 2010). Their results suggest that the activity of AOX1 as well as the rate of methanol consumption were essentially not affected by co-feeding with sorbitol. Increased cell viability and reduced proteolytic degradation of PGL were also observed. The activity of PGL was enhanced 1.85-fold compared to that of the control group. Calik and coworkers established a sorbitol co-feeding strategy for rhGH production (Calik et al., 2011). The specific growth rate on sorbitol was determined based on the rate of sorbitol consumption. In these experiments, sorbitol was pulse-fed to the bioreactor. The highest titer of rhGH was obtained with a methanol feed rate of 0.03 h^{-1}, while the concentration of sorbitol was maintained constant at 50 g/L over the 15 h of the production phase. In another study, Calik *et al*. examined the effect of the methanol feed rate on the production of rhGH in terms of sorbitol consumption, oxygen transfer and protease formation (Çalık et al., 2010). Sorbitol consumption

was found to be independent of the methanol feed rate when μ was above 0.03 h^{-1}. The highest K_La and concentration of rhGH observed occurred at 15 h and 24 h in the experiments with a value of μ of 0.03 h^{-1}, respectively.

2.5.2.7 Methanol co-feeding with mannitol

Mannitol is an isomer of sorbitol and a non-repressive substrate for *Pichia* fermentations. Thus, its feasibility as a co-substrate for the production of rHuEPO was evaluated (Eskitoros and Çalık, 2014). Five feeding strategies using combinations of methanol with mannitol, sorbitol or glycerol as co-substrates were investigated. A strategy in which mannitol was pulse-fed at 0 h, 6 h and 12 h to maintain a concentration of 50 g/L in the reactor, led to the highest rHuEPO titer of 0.65 g/L after 9 h. Mannitol was found to be consumed simultaneously with methanol in all tested strategies. Mannitol also showed a higher rate of consumption compared to that of sorbitol, which may be due to the use of different metabolic pathways. To shorten fermentation time, mannitol therefore has the potential to become an alternative co-substrate to sorbitol. Mannitol co-feeding was tested in the production of recombinant human growth hormone (Güneş et al., 2016). Compared to sorbitol, mannitol mixed-feeding resulted in a higher biomass concentration but led to a lower protein yield.

2.5.2.8 Temperature-limited methanol feeding

Lower temperature induction offers many benefits in terms of reducing proteolytic degradation and alleviating oxygen stress. Jin *et al*. carried out experiments to compare the effect of low temperature on the production of porcine interferon-α (pIFN-α) (Jin et al., 2011). They found that the activity of pIFN-α at 20 °C improved 100-fold compared to the standard induction temperature of 30°C. This may be attributed to an increased rate of oxygen uptake at lower temperatures. Additionally, maintaining a DO above 50% was helpful to shorten the adaption

time by 4-6 h. Gao *et al*. optimized the induction temperature for pIFN-α production. The best performance in terms of each of enzymatic activity, AOX1 activity and the rate of methanol consumption was achieved at 20°C, and had the shortest adaption period of 1 h (Gao et al., 2011). Carbon flux was also found to have shifted towards central metabolism and pIFN-α synthesis. Li *et al*. conducted methanol inductions at 22°C, 26°C and 30°C for the production of ROL (Li et al., 2013b). Induction at 22°C and 26°C resulted in 1.9-fold and 1.6-fold improvements in ROL activity, respectively, compared to that at 30°C. Reduced proteolytic degradation at lower temperatures was shown by SDS-PAGE to analyze the integrity of ROL.

2.6 Production of other bio-products in *P. pastoris*

P. pastoris has been widely used as a factory for protein production. In recent years, *P. pastoris* has gained considerable interest as a host for metabolic engineering to produce value-added products. Despite the lack of autonomous plasmids and a limited choice of promoters, *P. pastoris* has been engineered into many different cellular factories, producing various products. Carotenoids such as lycopene have applications in animal feed supplements, cosmetics and pharmaceutical compounds. Bhataya et al. developed a lycopene-producing *P. pastoris* strain by introducing a synthetic pathway of three heterologous genes (Bhataya et al., 2009). Each gene was expressed under the GAP promoter or a mutant of the promoter. The synthetic pathway genes leading to lycopene production were sequentially assembled into one plasmid. Site-directed mutagenesis was employed to introduce mutations within the GAP promoter due to integration of the plasmid into the GAP locus. High-level lycopene titers were achieved under fed-batch cultivation. This work demonstrated that it is possible to achieve multiple gene expression in *P. pastoris* with a single selection marker. A similar strategy was used by Araya-Garay *et al* to construct strains of *P. pastoris* that produce lycopene and β-carotene (Araya-Garay

et al., 2012b). Further extension of the β-carotene pathway resulted in the production of astaxanthin, another valuable carotenoid compound (Araya-Garay et al., 2012a). Recent progress has been made in the production of biodiesel using engineered *P. pastoris* harboring three pathway genes, each expressed using the GAP promoter (Tao et al., 2015). The recombinant plasmid was targeted to the genome at the *HIS4* locus. In a recent report, *P. pastoris* was developed to produce polyketide, a group of secondary metabolites with various biological functions (Xue et al., 2017). The entire polyketide synthetic pathway contains a cluster of seven genes which were introduced into the genome by integrating two plasmids carrying five and two genes, respectively. Both AOX1 and GAP promoters were used for gene expression. Marx et al. reported the overexpression of the riboflavin biosynthetic pathway by replacing native promoters with a strong constitutive GAP promoter (Marx et al., 2008). Similarly to what is done with gene knockouts, an antibiotic resistance marker was repeatedly used and recycled to achieve multiple promoter replacements. Other chemicals produced by engineered *P. pastoris* include (+)-nootkatone (Wriessnegger et al., 2014), dammarenediol-II (Liu et al., 2015), glucaric acid (Liu et al., 2016d) and lactic acid (de Lima et al., 2016). These results suggest that *P. pastoris* has the potential to produce complex compounds through metabolic engineering. Expansion of the molecular toolbox for synthetic biology in *P. pastoris,* which includes synthetic promoters, signal peptides and novel genome-editing tools, has contributed to the establishment of *P. pastoris* as a metabolic engineering platform (Kang et al., 2017).

2.7 Conclusions and prospects

P. pastoris has received great attention as a powerful system for protein expression. Efficient production of recombinant proteins relies on multi-level optimization strategies incorporating promoters, codon bias, signal peptides, gene dosage and cultivation strategies. Systems biology

methods such as GEMs and MFA are particularly effective tools for enhanced protein production. Although the engineering strategies (Fig. 2-1) discussed in this review can be applied in a combinatorial fashion to achieve optimal results (Hemmerich et al., 2014, Li et al., 2015, Prabhu et al., 2016), bottlenecks are generally product-specific and need to be identified in each case due to economic considerations. The methanol-regulated P_{AOX1} as the most important promoter for recombinant protein expression is faced with both challenges and opportunities. The insights gained from recent advances regarding the regulation of P_{AOX1} and alternative promoters will contribute to the development of a methanol-free, regulated system that is capable of high-level protein production. Methanol feeding strategies in fed-batch cultivations were thoroughly discussed due to the sensitivity of *P. pastoris* to methanol. The versatility of *P. pastoris* was made evident by the production of other value-added products in addition to proteins such as biodiesel and carotenoids. The efficient expression of a single gene has been expanded to the expression of synthetic pathways to produce value-added compounds. The increasing interest in using *P. pastoris* as a promising platform for metabolic engineering was fuelled by advances in the synthetic biology toolbox of this yeast. *P. pastoris* will therefore continue to serve as a powerful workhorse for both research and industrial applications.

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2.9 References

Adelantado N, Tarazona P, Grillitsch K, Garcia-Ortega X, Monforte S, Valero F, et al. The effect of hypoxia on the lipidome of recombinant *Pichia pastoris*. Microbial cell factories. 2017;16:86.

Ahn J, Hong J, Park M, Lee H, Lee E, Kim C, et al. Phosphate-responsive promoter of a *Pichia pastoris* sodium phosphate symporter. Applied and environmental microbiology. 2009;75:3528-34.

Akcapinar GB, Gul O, Sezerman U. Effect of codon optimization on the expression of Trichoderma reesei endoglucanase 1 in *Pichia pastoris*. Biotechnology progress. 2011;27:1257-63.

Anasontzis GE, Salazar Pena M, Spadiut O, Brumer H, Olsson L. Effects of temperature and glycerol and methanol-feeding profiles on the production of recombinant galactose oxidase in *Pichia pastoris*. Biotechnology progress. 2014;30:728-35.

Araya-Garay JM, Ageitos JM, Vallejo JA, Veiga-Crespo P, Sánchez-Pérez A, Villa TG. Construction of a novel *Pichia pastoris* strain for production of xanthophylls. AMB Express. 2012a;2:24.

Araya-Garay JM, Feijoo-Siota L, Rosa-dos-Santos F, Veiga-Crespo P, Villa TG. Construction of new *Pichia pastoris* X-33 strains for production of lycopene and beta-carotene. Applied microbiology and biotechnology. 2012b;93:2483-92.

Arbulu S, Jiménez JJ, Gútiez L, Cintas LM, Herranz C, Hernández PE. Cloning and expression of synthetic genes encoding the broad antimicrobial spectrum bacteriocins SRCAM 602, OR-7, E-760, and L-1077, by recombinant *Pichia pastoris*. BioMed research international. 2015;2015.

Arnau C, Casas C, Valero F. The effect of glycerol mixed substrate on the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system. Biochemical Engineering Journal. 2011;57:30-7.

Arnau C, Ramon R, Casas C, Valero F. Optimization of the heterologous production of a *Rhizopus oryzae* lipase in *Pichia pastoris* system using mixed substrates on controlled fed-batch bioprocess. Enzyme Microb Technol. 2010;46:494-500.

Arruda A, Reis VC, Batista VD, Daher BS, Piva LC, De Marco JL, et al. A constitutive expression system for *Pichia pastoris* based on the PGK1 promoter. Biotechnology letters. 2016;38:509-17.

Ata Ö, Boy E, Güneş H, Çalık P. Codon optimization of xylA gene for recombinant glucose isomerase production in *Pichia pastoris* and fed-batch feeding strategies to fine-tune bioreactor performance. Bioprocess and biosystems engineering. 2015;38:889-903.

Ata O, Prielhofer R, Gasser B, Mattanovich D, Calik P. Transcriptional engineering of the glyceraldehyde-3-phosphate dehydrogenase promoter for improved heterologous protein production in *Pichia pastoris*. Biotechnology and bioengineering. 2017;114:2319-27.

Bai J, Swartz DJ, Protasevich II, Brouillette CG, Harrell PM, Hildebrandt E, et al. A gene optimization strategy that enhances production of fully functional P-glycoprotein in *Pichia pastoris*. PloS one. 2011;6:e22577.

Barrigón JM, Montesinos JL, Valero F. Searching the best operational strategies for *Rhizopus oryzae* lipase production in *Pichia pastoris* Mut+ phenotype: Methanol limited or methanol non-limited fed-batch cultures? Biochemical Engineering Journal. 2013;75:47-54.

Ben Azoun S, Belhaj AE, Gongrich R, Gasser B, Kallel H. Molecular optimization of rabies virus glycoprotein expression in *Pichia pastoris*. Microbial biotechnology. 2016a;9:355-68.

Ben Azoun S, Belhaj AE, Kallel H. Rabies virus glycoprotein enhanced expression in *Pichia pastoris* using the constitutive GAP promoter. Biochemical Engineering Journal. 2016b;113:77-85.

Berg L, Strand TA, Valla S, Brautaset T. Combinatorial mutagenesis and selection to understand and improve yeast promoters. BioMed research international. 2013;2013:926985.

Bhataya A, Schmidt-Dannert C, Lee PC. Metabolic engineering of *Pichia pastoris* X-33 for lycopene production. Process Biochemistry. 2009;44:1095-102.

Brunner V, Hussein M, Becker T. Biomass estimation in *Pichia pastoris* cultures by combined single-wavelength fluorescence measurements. Biotechnology and bioengineering. 2016;113:2394-402.

Byrne B. *Pichia pastoris* as an expression host for membrane protein structural biology. Current opinion in structural biology. 2015;32:9-17.

Çalık P, Ata Ö, Güneş H, Massahi A, Boy E, Keskin A, et al. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochemical Engineering Journal. 2015;95:20-36.

Çalık P, İnankur B, Soyaslan EŞ, Şahin M, Taşpınar H, Açık E, et al. Fermentation and oxygen transfer characteristics in recombinant human growth hormone production by *Pichia pastoris* in sorbitol batch and methanol fed-batch operation. Journal of Chemical Technology & Biotechnology. 2010;85:226-33.

Calik P, Sahin M, Taspinar H, Soyaslan ES, Inankur B. Dynamic flux balance analysis for pharmaceutical protein production by *Pichia pastoris*: human growth hormone. Enzyme Microb Technol. 2011;48:209-16.

Caspeta L, Shoaie S, Agren R, Nookaew I, Nielsen J. Genome-scale metabolic reconstructions of *Pichia stipitis* and *Pichia pastoris* and in silico evaluation of their potentials. BMC systems biology. 2012;6:24.

Celik E, Calik P, Oliver SG. Metabolic flux analysis for recombinant protein production by *Pichia pastoris* using dual carbon sources: Effects of methanol feeding rate. Biotechnology and bioengineering. 2010;105:317-29.

Chang S-W, Li C-F, Lee G-C, Yeh T, Shaw J-F. Engineering the expression and biochemical characteristics of recombinant *Candida rugosa* LIP2 lipase by removing the additional N-terminal peptide and regional codon optimization. Journal of agricultural and food chemistry. 2011;59:6710-9.

Chung BK, Selvarasu S, Andrea C, Ryu J, Lee H, Ahn J, et al. Genome-scale metabolic reconstruction and in silico analysis of methylotrophic yeast *Pichia pastoris* for strain improvement. Microbial cell factories. 2010;9:50.

Ciplys E, Zitkus E, Gold LI, Daubriac J, Pavlides SC, Hojrup P, et al. High-level secretion of native recombinant human calreticulin in yeast. Microbial cell factories. 2015;14:165.

Cos O, Ramon R, Montesinos JL, Valero F. Operational strategies, monitoring and control of heterologous protein production in the methylotrophic yeast *Pichia pastoris* under different promoters: a review. Microbial cell factories. 2006;5:17.

Crowley J, Arnold SA, Wood N, Harvey LM, McNeil B. Monitoring a high cell density recombinant *Pichia pastoris* fed-batch bioprocess using transmission and reflectance near infrared spectroscopy. Enzyme and Microbial Technology. 2005;36:621-8.

Crowley J, McCarthy B, Nunn NS, Harvey LM, McNeil B. Monitoring a recombinant *Pichia pastoris* fed batch process using Fourier transform mid-infrared spectroscopy (FT-MIRS). Biotechnology letters. 2000;22:1907-12.

Curvers S, Brixius P, Klauser T, Thommes J, Weuster-Botz D, Takors R, et al. Human chymotrypsinogen B production with *Pichia pastoris* by integrated development of fermentation and downstream processing. Part 1. Fermentation. Biotechnology progress. 2001;17:495-502.

Dabros M, Schuler MM, Marison IW. Simple control of specific growth rate in biotechnological fed-batch processes based on enhanced online measurements of biomass. Bioprocess and biosystems engineering. 2010;33:1109-18.

Dahlbacka J, Weegar J, von Weymarn N, Fagervik K. On-line measurement of the substrate concentrations in *Pichia pastoris* fermentations using FT-IR/ATR. Biotechnology letters. 2012;34:1009-17.

de Lima PB, Mulder KC, Melo NT, Carvalho LS, Menino GS, Mulinari E, et al. Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*. Microbial cell factories. 2016;15:158.

Dietzsch C, Spadiut O, Herwig C. A fast approach to determine a fed batch feeding profile for recombinant *Pichia pastoris* strains. Microbial cell factories. 2011;10:85.

Ding J, Gao M, Hou G, Liang K, Yu R, Li Z, et al. Stabilizing porcine interferon-α production by *Pichia pastoris* with an ethanol on-line measurement based DO-Stat glycerol feeding strategy. Journal of Chemical Technology & Biotechnology. 2014;89:1948-53.

Duan H, Umar S, Hu Y, Chen J. Both the AOX1 promoter and the FLD1 promoter work together in a *Pichia pastoris* expression vector. World Journal of Microbiology and Biotechnology. 2009;25:1779-83.

Duan H, Wang H, Ma B, Jiang P, Tu P, Ni Z, et al. Codon optimization and expression of irisin in *Pichia pastoris* GS115. International journal of biological macromolecules. 2015;79:21-6.

Eskitoros MŞ, Çalık P. Co-substrate mannitol feeding strategy design in semi-batch production of recombinant human erythropoietin production by *Pichia pastoris*. Journal of Chemical Technology & Biotechnology. 2014;89:644-51.

Fang Z, Xu L, Pan D, Jiao L, Liu Z, Yan Y. Enhanced production of *Thermomyces lanuginosus* lipase in *Pichia pastoris* via genetic and fermentation strategies. Journal of industrial microbiology & biotechnology. 2014;41:1541-51.

Fazenda ML, Dias JM, Harvey LM, Nordon A, Edrada-Ebel R, Littlejohn D, et al. Towards better understanding of an industrial cell factory: investigating the feasibility of real-time metabolic flux analysis in *Pichia pastoris*. Microbial cell factories. 2013;12:51.

Ferrer P, Albiol J. 13 C-Based Metabolic Flux Analysis in Yeast: The *Pichia pastoris* Case. Yeast Metabolic Engineering: Methods and Protocols. 2014a:209-32.

Ferrer P, Albiol J. 13 C-Based Metabolic Flux Analysis of Recombinant *Pichia pastoris*. Metabolic Flux Analysis: Methods and Protocols. 2014b:291-313.

Fickers P. *Pichia pastoris*: a workhorse for recombinant protein production. Current research in Microbiology and Biotechnology. 2014:354-63.

Gao M, Dong S, Yu R, Wu J, Zheng Z, Shi Z, et al. Improvement of ATP regeneration efficiency and operation stability in porcine interferon-α production by *Pichia pastoris* under lower induction temperature. Korean Journal of Chemical Engineering. 2011;28:1412-9.

Gao MJ, Li Z, Yu RS, Wu JR, Zheng ZY, Shi ZP, et al. Methanol/sorbitol co-feeding induction enhanced porcine interferon-alpha production by *P. pastoris* associated with energy metabolism shift. Bioprocess and biosystems engineering. 2012;35:1125-36.

Garcia-Ortega X, Adelantado N, Ferrer P, Montesinos JL, Valero F. A step forward to improve recombinant protein production in *Pichia pastoris*: From specific growth rate effect on protein secretion to carbon-starving conditions as advanced strategy. Process Biochemistry. 2016;51:681-91.

Garcia-Ortega X, Ferrer P, Montesinos JL, Valero F. Fed-batch operational strategies for recombinant Fab production with *Pichia pastoris* using the constitutive GAP promoter. Biochemical engineering journal. 2013;79:172-81.

Goldfeld M, Christensen J, Pollard D, Gibson ER, Olesberg JT, Koerperick EJ, et al. Advanced near-infrared monitor for stable real-time measurement and control ofPichia pastorisbioprocesses. Biotechnology progress. 2014;30:749-59.

Goncalves AM. *Pichia pastoris*: A Recombinant Microfactory for Antibodies and Human Membrane Proteins. Journal of microbiology and biotechnology. 2013;23:587-601.

González-Martínez JM, Folch-Fortuny A, Llaneras F, Tortajada M, Picó J, Ferrer A. Metabolic flux understanding of *Pichia pastoris* grown on heterogenous culture media. Chemometrics and Intelligent Laboratory Systems. 2014;134:89-99.

Govindappa N, Hanumanthappa M, Venkatarangaiah K, Periyasamy S, Sreenivas S, Soni R, et al. A new signal sequence for recombinant protein secretion in *Pichia pastoris*. Journal of microbiology and biotechnology. 2014;24:337-45.

Güneş H, Boy E, Ata Ö, Zerze GH, Çalık P, Özdamar TH. Methanol feeding strategy design enhances recombinant human growth hormone production by *Pichia pastoris*. Journal of Chemical Technology & Biotechnology. 2016;91:664-71.

Gurramkonda C, Polez S, Skoko N, Adnan A, Gäbel T, Chugh D, et al. Application of simple fed-batch technique to high-level secretory production of insulin precursor using *Pichia pastoris* with subsequent purification and conversion to human insulin. Microbial cell factories. 2010;9:31.

Hang H, Ye X, Guo M, Chu J, Zhuang Y, Zhang M, et al. A simple fermentation strategy for high-level production of recombinant phytase by *Pichia pastoris* using glucose as the growth substrate. Enzyme and Microbial Technology. 2009;44:185-8.

Hartner FS, Ruth C, Langenegger D, Johnson SN, Hyka P, Lin-Cereghino GP, et al. Promoter library designed for fine-tuned gene expression in *Pichia pastoris*. Nucleic acids research. 2008;36:e76.

He D, Luo W, Wang Z, Lv P, Yuan Z. Combined use of GAP and AOX1 promoters and optimization of culture conditions to enhance expression of *Rhizomucor miehei* lipase. Journal of industrial microbiology & biotechnology. 2015;42:1175-82.

He M, Wu D, Wu J, Chen J. Enhanced expression of endoinulinase from *Aspergillus niger* by codon optimization in *Pichia pastoris* and its application in inulooligosaccharide production. Journal of industrial microbiology & biotechnology. 2014;41:105-14.

Hemmerich J, Adelantado N, Barrigón JM, Ponte X, Hörmann A, Ferrer P, et al. Comprehensive clone screening and evaluation of fed-batch strategies in a microbioreactor and lab scale stirred tank bioreactor system: application on *Pichia pastoris* producing *Rhizopus oryzae* lipase. Microbial cell factories. 2014;13:36.

Horta A, da Silva A, Sargo C, Cavalcanti-Montaño I, Galeano-Suarez I, Velez A, et al. On-line monitoring of biomass concentration based on a capacitance sensor: assessing the methodology for different bacteria and yeast high cell density fed-batch cultures. Brazilian Journal of Chemical Engineering. 2015;32:821-9.

Hu F, Li X, Lü J, Mao PH, Jin X, Rao B, et al. A visual method for direct selection of high-producing *Pichia pastoris* clones. BMC biotechnology. 2011;11:23.

Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, et al. Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. PloS one. 2013;8:e58393.

Idiris A, Tohda H, Kumagai H, Takegawa K. Engineering of protein secretion in yeast: strategies and impact on protein production. Applied microbiology and biotechnology. 2010;86:403-17.

Irani ZA, Kerkhoven EJ, Shojaosadati SA, Nielsen J. Genome-scale metabolic model of *Pichia pastoris* with native and humanized glycosylation of recombinant proteins. Biotechnology and bioengineering. 2016;113:961-9.

Isidro IA, Portela RM, Clemente JJ, Cunha AE, Oliveira R. Hybrid metabolic flux analysis and recombinant protein prediction in *Pichia pastoris* X-33 cultures expressing a single-chain antibody fragment. Bioprocess and biosystems engineering. 2016;39:1351-63.

Jacobs PP, Inan M, Festjens N, Haustraete J, Van Hecke A, Contreras R, et al. Fed-batch fermentation of GM-CSF-producing glycoengineered *Pichia pastoris* under controlled specific growth rate. Microbial cell factories. 2010;9:93.

Jin H, Liu G, Dai K, Wang H, Li Z, Shi Z. Improvement of porcine interferon-alpha production by recombinant *Pichia pastoris* via induction at low methanol concentration and low temperature. Applied biochemistry and biotechnology. 2011;165:559-71.

Jin H, Liu G, Ye X, Duan Z, Li Z, Shi Z. Enhanced porcine interferon-α production by recombinant *Pichia pastoris* with a combinational control strategy of low induction temperature and high dissolved oxygen concentration. Biochemical Engineering Journal. 2010;52:91-8.

Jorda J, de Jesus SS, Peltier S, Ferrer P, Albiol J. Metabolic flux analysis of recombinant *Pichia pastoris* growing on different glycerol/methanol mixtures by iterative fitting of NMR-derived ^{13}C-labelling data from proteinogenic amino acids. New biotechnology. 2014a;31:120-32.

Jordà J, Jouhten P, Cámara E, Maaheimo H, Albiol J, Ferrer P. Metabolic flux profiling of recombinant protein secreting *Pichia pastoris* growing on glucose: methanol mixtures. Microbial cell factories. 2012;11:57.

Jorda J, Rojas HC, Carnicer M, Wahl A, Ferrer P, Albiol J. Quantitative Metabolomics and Instationary ^{13}C-Metabolic Flux Analysis Reveals Impact of Recombinant Protein Production on Trehalose and Energy Metabolism in *Pichia pastoris*. Metabolites. 2014b;4:281-99.

Jorda J, Suarez C, Carnicer M, ten Pierick A, Heijnen JJ, van Gulik W, et al. Glucose-methanol co-utilization in *Pichia pastoris* studied by metabolomics and instationary ^{13}C flux analysis. BMC systems biology. 2013;7:17.

Jungo C, Marison I, von Stockar U. Mixed feeds of glycerol and methanol can improve the performance of *Pichia pastoris* cultures: A quantitative study based on concentration gradients in transient continuous cultures. Journal of biotechnology. 2007;128:824-37.

Kadlec P, Gabrys B, Strandt S. Data-driven soft sensors in the process industry. Computers & Chemical Engineering. 2009;33:795-814.

Kang Z, Huang H, Zhang Y, Du G, Chen J. Recent advances of molecular toolbox construction expand *Pichia pastoris* in synthetic biology applications. World journal of microbiology & biotechnology. 2017;33:19.

Karaoglan M, Karaoglan FE, Inan M. Functional analysis of alcohol dehydrogenase (ADH) genes in *Pichia pastoris*. Biotechnology letters. 2016;38:463-9.

Kaushik N, Rohila D, Arora U, Raut R, Lamminmäki U, Khanna N, et al. Casamino acids facilitate the secretion of recombinant dengue virus serotype-3 envelope domain III in *Pichia pastoris*. BMC biotechnology. 2016;16:12.

Kazenwadel C, Klebensberger J, Richter S, Pfannstiel J, Gerken U, Pickel B, et al. Optimized expression of the dirigent protein AtDIR6 in *Pichia pastoris* and impact of glycosylation on protein structure and function. Applied microbiology and biotechnology. 2013;97:7215-27.

Khatri NK, Hoffmann F. Impact of methanol concentration on secreted protein production in oxygen-limited cultures of recombinant *Pichia pastoris*. Biotechnology and bioengineering. 2006;93:871-9.

Kim S, d′Anjou M, Lanz KJ, Evans CE, Gibson ER, Olesberg JT, et al. Real-time monitoring of glycerol and methanol to enhance antibody production in industrial *Pichia pastoris* bioprocesses. Biochemical Engineering Journal. 2015;94:115-24.

Kim S, Warburton S, Boldogh I, Svensson C, Pon L, d'Anjou M, et al. Regulation of alcohol oxidase 1 (AOX1) promoter and peroxisome biogenesis in different fermentation processes in *Pichia pastoris*. Journal of biotechnology. 2013;166:174-81.

Kittl R, Kracher D, Burgstaller D, Haltrich D, Ludwig R. Production of four Neurospora crassa lytic polysaccharide monooxygenases in *Pichia pastoris* monitored by a fluorimetric assay. Biotechnology for biofuels. 2012;5:79.

Kiviharju K, Salonen K, Moilanen U, Eerikainen T. Biomass measurement online: the performance of in situ measurements and software sensors. Journal of industrial microbiology & biotechnology. 2008;35:657-65.

Landes N, Gasser B, Vorauer-Uhl K, Lhota G, Mattanovich D, Maurer M. The vitamin-sensitive promoter PTHI11 enables pre-defined autonomous induction of recombinant protein production in *Pichia pastoris*. Biotechnology and bioengineering. 2016;113:2633-43.

Landgrebe D, Haake C, Hopfner T, Beutel S, Hitzmann B, Scheper T, et al. On-line infrared spectroscopy for bioprocess monitoring. Applied microbiology and biotechnology. 2010;88:11-22.

Lee SG, Koh HY, Han SJ, Park H, Na DC, Kim I-C, et al. Expression of recombinant endochitinase from the *Antarctic bacterium*, *Sanguibacter antarcticus* KOPRI 21702 in *Pichia pastoris* by codon optimization. Protein expression and purification. 2010;71:108-14.

Li C, Lin Y, Zheng X, Pang N, Liao X, Liu X, et al. Combined strategies for improving expression of *Citrobacter amalonaticus* phytase in *Pichia pastoris*. BMC biotechnology. 2015;15:88.

Li F, Yang S, Zhao L, Li Q, Pei J. Synonymous condon usage bias and overexpression of a synthetic xynB gene from *Aspergillus Niger* NL-1 in *Pichia pastoris*. BioResources. 2012;7:2330-43.

Li X, He X, Li Z, Wang F. Combined strategies for improving the production of recombinant *Rhizopus oryzae* lipase in *Pichia pastoris*. BioResources. 2013;8:2867-80.

Li X, Liu Z, Wang G, Pan D, Jiao L, Yan Y. Overexpression of *Candida rugosa* lipase Lip1 via combined strategies in Pichia pastoris. Enzyme Microb Technol. 2016;82:115-24.

Liang S, Zou C, Lin Y, Zhang X, Ye Y. Identification and characterization of P_{GCW14} : a novel, strong constitutive promoter of *Pichia pastoris*. Biotechnology letters. 2013;35:1865-71.

Lin-Cereghino GP, Stark CM, Kim D, Chang J, Shaheen N, Poerwanto H, et al. The effect of alpha-mating factor secretion signal mutations on recombinant protein expression in *Pichia pastoris*. Gene. 2013;519:311-7.

Liu B, Zhang Y, Zhang X, Yan C, Zhang Y, Xu X, et al. Discovery of a rhamnose utilization pathway and rhamnose-inducible promoters in *Pichia pastoris*. Scientific reports. 2016a;6:27352.

Liu WC, Gong T, Wang QH, Liang X, Chen JJ, Zhu P. Scaling-up Fermentation of *Pichia pastoris* to demonstration-scale using new methanol-feeding strategy and increased air pressure instead of pure oxygen supplement. Scientific reports. 2016b;6:18439.

Liu XB, Liu M, Tao XY, Zhang ZX, Wang FQ, Wei DZ. Metabolic engineering of *Pichia pastoris* for the production of dammarenediol-II. Journal of biotechnology. 2015;216:47-55.

Liu Y, Gong X, Wang C, Du G, Chen J, Kang Z. Production of glucaric acid from myo-inositol in engineered *Pichia pastoris*. Enzyme Microb Technol. 2016c;91:8-16.

Liu Y, Wu C, Wang J, Mo W, Yu M. Codon optimization, expression, purification, and functional characterization of recombinant human IL-25 in *Pichia pastoris*. Applied microbiology and biotechnology. 2013;97:10349-58.

Looser V, Bruhlmann B, Bumbak F, Stenger C, Costa M, Camattari A, et al. Cultivation strategies to enhance productivity of *Pichia pastoris*: A review. Biotechnology advances. 2015;33:1177-93.

Luley-Goedl C, Czabany T, Longus K, Schmolzer K, Zitzenbacher S, Ribitsch D, et al. Combining expression and process engineering for high-quality production of human sialyltransferase in *Pichia pastoris*. Journal of biotechnology. 2016;235:54-60.

Maity N, Thawani A, Sharma A, Gautam A, Mishra S, Sahai V. Expression and Control of Codon-Optimized Granulocyte Colony-Stimulating Factor in *Pichia pastoris*. Applied biochemistry and biotechnology. 2016;178:159-72.

Marquard D, Enders A, Roth G, Rinas U, Scheper T, Lindner P. In situ microscopy for online monitoring of cell concentration in *Pichia pastoris* cultivations. Journal of biotechnology. 2016;234:90-8.

Marsalek L, Gruber C, Altmann F, Aleschko M, Mattanovich D, Gasser B, et al. Disruption of genes involved in CORVET complex leads to enhanced secretion of heterologous carboxylesterase only in protease deficient *Pichia pastoris*. Biotechnology journal. 2017;12.

Marx H, Mattanovich D, Sauer M. Overexpression of the riboflavin biosynthetic pathway in *Pichia pastoris*. Microbial cell factories. 2008;7:23.

Mellitzer A, Ruth C, Gustafsson C, Welch M, Birner-Grunberger R, Weis R, et al. Synergistic modular promoter and gene optimization to push cellulase secretion by *Pichia pastoris* beyond existing benchmarks. Journal of biotechnology. 2014;191:187-95.

Min CK, Lee JW, Chung KH, Park HW. Control of specific growth rate to enhance the production of a novel disintegrin, saxatilin, in recombinant *Pichia pastoris*. Journal of bioscience and bioengineering. 2010;110:314-9.

Nie Y, Huang M, Lu J, Qian J, Lin W, Chu J, et al. Impacts of high beta-galactosidase expression on central metabolism of recombinant *Pichia pastoris* GS115 using glucose as sole carbon source via ^{13}C metabolic flux analysis. Journal of biotechnology. 2014;187:124-34.

Niu H, Jost L, Pirlot N, Sassi H, Daukandt M, Rodriguez C, et al. A quantitative study of methanol/sorbitol co-feeding process of a *Pichia pastoris* Mut+/pAOX1-lacZ strain. Microbial cell factories. 2013;12:33.

Nocon J, Steiger M, Mairinger T, Hohlweg J, Russmayer H, Hann S, et al. Increasing pentose phosphate pathway flux enhances recombinant protein production in *Pichia pastoris*. Applied microbiology and biotechnology. 2016;100:5955-63.

Nocon J, Steiger MG, Pfeffer M, Sohn SB, Kim TY, Maurer M, et al. Model based engineering of *Pichia pastoris* central metabolism enhances recombinant protein production. Metabolic engineering. 2014;24:129-38.

Panchiga C, Jorgen B, Yaowapha W, Jindarat P, Anan T. On-line methanol sensor system development for recombinant human serum albumin production by *Pichia pastoris*. African Journal of Biotechnology. 2016;15:2374-83.

Panula-Perala J, Vasala A, Karhunen J, Ojamo H, Neubauer P, Mursula A. Small-scale slow glucose feed cultivation of *Pichia pastoris* without repression of AOX1 promoter: towards high throughput cultivations. Bioprocess and biosystems engineering. 2014;37:1261-9.

Parashar D, Satyanarayana T. Enhancing the production of recombinant acidic α-amylase and phytase in *Pichia pastoris* under dual promoters [constitutive (GAP) and inducible (AOX)] in mixed fed batch high cell density cultivation. Process Biochemistry. 2016;51:1315-22.

Parua PK, Ryan PM, Trang K, Young ET. Pichia pastoris 14-3-3 regulates transcriptional activity of the methanol inducible transcription factor Mxr1 by direct interaction. Molecular microbiology. 2012;85:282-98.

Periyasamy S, Govindappa N, Sreenivas S, Sastry K. Isolation, characterization and evaluation of the *Pichia pastoris* sorbitol dehydrogenase promoter for expression of heterologous proteins. Protein expression and purification. 2013;92:128-33.

Ponte X, Montesinos-Seguí JL, Valero F. Bioprocess efficiency in *Rhizopus oryzae* lipase production by *Pichia pastoris* under the control of PAOX1 is oxygen tension dependent. Process Biochemistry. 2016;51:1954-63.

Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal. 2012;64:91-105.

Potvin G, Zhang Z, Defela A, Lam H. Screening of alternative carbon sources for recombinant protein production in *Pichia pastoris*. International Journal of Chemical Reactor Engineering. 2016;14:251-7.

Prabhu AA, Veeranki VD, Dsilva SJ. Improving the production of human interferon gamma (hIFN-γ) in *Pichia pastoris* cell factory: An approach of cell level. Process Biochemistry. 2016;51:709-18.

Prielhofer R, Maurer M, Klein J, Wenger J, Kiziak C, Gasser B, et al. Induction without methanol: novel regulated promoters enable high-level expression in *Pichia pastoris*. Microbial cell factories. 2013;12:5.

Qin X, Qian J, Yao G, Zhuang Y, Zhang S, Chu J. GAP promoter library for fine-tuning of gene expression in *Pichia pastoris*. Applied and environmental microbiology. 2011;77:3600-8.

Rafique N, Tabassum R, Awan MS, Orts W, Wong DW. Cloning and Expression of Pectobacterium carotovorum Endo-polygalacturonase Gene in *Pichia pastoris* for Production of Oligogalacturonates. BioResources. 2016;11:5204-14.

Razaghi A, Tan E, Lua LH, Owens L, Karthikeyan O, Heimann K. Is *Pichia pastoris* a realistic platform for industrial production of recombinant human interferon gamma? Biologicals. 2017;45:52-60.

Robert JM, Lattari FS, Machado AC, de Castro AM, Almeida RV, Torres FAG, et al. Production of recombinant lipase B from *Candida antarctica* in *Pichia pastoris* under control of the promoter PGK using crude glycerol from biodiesel production as carbon source. Biochemical Engineering Journal. 2017;118:123-31.

Ruth C, Buchetics M, Vidimce V, Kotz D, Naschberger S, Mattanovich D, et al. *Pichia pastoris* Aft1-a novel transcription factor, enhancing recombinant protein secretion. Microbial cell factories. 2014;13:120.

Sagmeister P, Wechselberger P, Jazini M, Meitz A, Langemann T, Herwig C. Soft sensor assisted dynamic bioprocess control: Efficient tools for bioprocess development. Chemical Engineering Science. 2013;96:190-8.

Saitua F, Torres P, Perez-Correa JR, Agosin E. Dynamic genome-scale metabolic modeling of the yeast *Pichia pastoris*. BMC systems biology. 2017;11:27.

Schenk J, Marison IW, von Stockar U. A simple method to monitor and control methanol feeding of *Pichia pastoris* fermentations using mid-IR spectroscopy. Journal of biotechnology. 2007;128:344-53.

Schuler MM, Sivaprakasam S, Freeland B, Hama A, Hughes KM, Marison IW. Investigation of the potential of biocalorimetry as a process analytical technology (PAT) tool for monitoring and control of Crabtree-negative yeast cultures. Applied microbiology and biotechnology. 2012;93:575-84.

Sha C, Yu XW, Lin NX, Zhang M, Xu Y. Enhancement of lipase r27RCL production in *Pichia pastoris* by regulating gene dosage and co-expression with chaperone protein disulfide isomerase. Enzyme Microb Technol. 2013;53:438-43.

Shen W, Xue Y, Liu Y, Kong C, Wang X, Huang M, et al. A novel methanol-free *Pichia pastoris* system for recombinant protein expression. Microbial cell factories. 2016;15:178.

Shu M, Shen W, Yang S, Wang X, Wang F, Wang Y, et al. High-level expression and characterization of a novel serine protease in *Pichia pastoris* by multi-copy integration. Enzyme Microb Technol. 2016;92:56-66.

Sivaprakasam S, Schuler MM, Hama A, Hughes K-M, Marison IW. Biocalorimetry as a process analytical technology process analyser; robust in-line monitoring and control of aerobic fed-batch cultures of crabtree-negative yeast cells. Journal of Thermal Analysis and Calorimetry. 2011;104:75-85.

Sohn SB, Graf AB, Kim TY, Gasser B, Maurer M, Ferrer P, et al. Genome-scale metabolic model of methylotrophic yeast *Pichia pastoris* and its use for in silico analysis of heterologous protein production. Biotechnology journal. 2010;5:705-15.

Spohner SC, Müller H, Quitmann H, Czermak P. Expression of enzymes for the usage in food and feed industry with *Pichia pastoris*. Journal of biotechnology. 2015;202:118-34.

Staley CA, Huang A, Nattestad M, Oshiro KT, Ray LE, Mulye T, et al. Analysis of the 5' untranslated region (5'UTR) of the alcohol oxidase 1 (AOX1) gene in recombinant protein expression in *Pichia pastoris*. Gene. 2012;496:118-27.

Sun FF, Bai R, Yang H, Wang F, He J, Wang C, et al. Heterologous expression of codon optimized Trichoderma reesei Cel6A in *Pichia pastoris*. Enzyme Microb Technol. 2016;92:107-16.

Surribas A, Geissler D, Gierse A, Scheper T, Hitzmann B, Montesinos JL, et al. State variables monitoring by in situ multi-wavelength fluorescence spectroscopy in heterologous protein production by *Pichia pastoris*. Journal of biotechnology. 2006a;124:412-9.

Surribas A, Montesinos JL, Valero FF. Biomass estimation using fluorescence measurements in *Pichia pastoris* bioprocess. Journal of Chemical Technology & Biotechnology. 2006b;81:23-8.

Tanaka T, Yamada R, Ogino C, Kondo A. Recent developments in yeast cell surface display toward extended applications in biotechnology. Applied microbiology and biotechnology. 2012;95:577-91.

Tanghe M, Danneels B, Camattari A, Glieder A, Vandenberghe I, Devreese B, et al. Recombinant expression of Trichoderma reesei Cel61A in *Pichia pastoris*: optimizing yield and N-terminal processing. Molecular biotechnology. 2015;57:1010-7.

Tao H, Guo D, Zhang Y, Deng Z, Liu T. Metabolic engineering of microbes for branched-chain biodiesel production with low-temperature property. Biotechnology for biofuels. 2015;8:92.

Tomas-Gamisans M, Ferrer P, Albiol J. Integration and Validation of the Genome-Scale Metabolic Models of *Pichia pastoris*: A Comprehensive Update of Protein Glycosylation Pathways, Lipid and Energy Metabolism. PloS one. 2016;11:e0148031.

Tu Y, Wang Y, Wang G, Wu J, Liu Y, Wang S, et al. High-level expression and immunogenicity of a porcine circovirus type 2 capsid protein through codon optimization in *Pichia pastoris*. Applied microbiology and biotechnology. 2013;97:2867-75.

Vadhana AK, Samuel P, Berin RM, Krishna J, Kamatchi K, Meenakshisundaram S. Improved secretion of *Candida antarctica* lipase B with its native signal peptide in *Pichia pastoris*. Enzyme Microb Technol. 2013;52:177-83.

Vogl T, Glieder A. Regulation of *Pichia pastoris* promoters and its consequences for protein production. New biotechnology. 2013;30:385-404.

Vogl T, Ruth C, Pitzer J, Kickenweiz T, Glieder A. Synthetic core promoters for *Pichia pastoris*. ACS synthetic biology. 2014;3:188-91.

Vogl T, Sturmberger L, Kickenweiz T, Wasmayer R, Schmid C, Hatzl AM, et al. A Toolbox of Diverse Promoters Related to Methanol Utilization: Functionally Verified Parts for Heterologous Pathway Expression in *Pichia pastoris*. ACS synthetic biology. 2016;5:172-86.

Wang J, Li Y, Liu D. Improved Production of Aspergillus usamii endo-beta-1,4-Xylanase in *Pichia pastoris* via Combined Strategies. BioMed research international. 2016a;2016:3265895.

Wang J, Wang X, Shi L, Qi F, Zhang P, Zhang Y, et al. Methanol-Independent Protein Expression by AOX1 Promoter with trans-Acting Elements Engineering and Glucose-Glycerol-Shift Induction in *Pichia pastoris*. Scientific reports. 2017;7:41850.

Wang JR, Li YY, Liu DN, Liu JS, Li P, Chen LZ, et al. Codon Optimization Significantly Improves the Expression Level of alpha -Amylase Gene from *Bacillus licheniformis* in *Pichia pastoris*. BioMed research international. 2015a;2015:248680.

Wang P, Huang L, Jiang H, Tian J, Chu X, Wu N. Improving the secretion of a methyl parathion hydrolase in *Pichia pastoris* by modifying its N-terminal sequence. PloS one. 2014;9:e96974.

Wang P, Huang L, Jiang H, Tian J, Chu X, Wu N. Enhanced secretion of a methyl parathion hydrolase in *Pichia pastoris* using a combinational strategy. Microbial cell factories. 2015b;14:123.

Wang X-F, Shen X-G, Sun Y-C, Zhao H-Y, Xu L, Liu Y, et al. Production of *Yarrowia lipolytica lipase* LIP2 in *Pichia pastoris* using the nitrogen source-regulated FLD1 promoter. Journal of Chemical Technology & Biotechnology. 2012a;87:553-8.

Wang X, Cai M, Shi L, Wang Q, Zhu J, Wang J, et al. PpNrg1 is a transcriptional repressor for glucose and glycerol repression of AOX1 promoter in methylotrophic yeast *Pichia pastoris*. Biotechnology letters. 2016b;38:291-8.

Wang X, Sun Y, Shen X, Ke F, Zhao H, Liu Y, et al. Intracellular expression of *Vitreoscilla* hemoglobin improves production of *Yarrowia lipolytica* lipase LIP2 in a recombinant *Pichia pastoris*. Enzyme Microb Technol. 2012b;50:22-8.

Wang X, Wang Q, Wang J, Bai P, Shi L, Shen W, et al. Mit1 Transcription Factor Mediates Methanol Signaling and Regulates the Alcohol Oxidase 1 (AOX1) Promoter in *Pichia pastoris*. The Journal of biological chemistry. 2016c;291:6245-61.

Wang Z, Wang Y, Zhang D, Li J, Hua Z, Du G, et al. Enhancement of cell viability and alkaline polygalacturonate lyase production by sorbitol co-feeding with methanol in *Pichia pastoris* fermentation. Bioresource technology. 2010;101:1318-23.

Wechselberger P, Sagmeister P, Herwig C. Real-time estimation of biomass and specific growth rate in physiologically variable recombinant fed-batch processes. Bioprocess and biosystems engineering. 2013;36:1205-18.

Wechselberger P, Seifert A, Herwig C. PAT method to gather bioprocess parameters in real-time using simple input variables and first principle relationships. Chemical Engineering Science. 2010;65:5734-46.

Wriessnegger T, Augustin P, Engleder M, Leitner E, Muller M, Kaluzna I, et al. Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metabolic engineering. 2014;24:18-29.

Wu D, Chu J, Hao Y-Y, Wang Y-H, Zhuang Y-P, Zhang S-L. High efficient production of recombinant human consensus interferon mutant in high cell density culture of *Pichia pastoris* using two phases methanol control. Process Biochemistry. 2011a;46:1663-9.

Wu D, Yu XW, Wang TC, Wang R, Xu Y. High yield *Rhizopus chinenisis* prolipase production in *Pichia pastoris*: Impact of methanol concentration. Biotechnology and Bioprocess Engineering. 2011b;16:305-11.

Wu M, Shen Q, Yang Y, Zhang S, Qu W, Chen J, et al. Disruption of YPS1 and PEP4 genes reduces proteolytic degradation of secreted HSA/PTH in *Pichia pastoris* GS115. Journal of industrial microbiology & biotechnology. 2013;40:589-99.

Xuan Y, Zhou X, Zhang W, Zhang X, Song Z, Zhang Y. An upstream activation sequence controls the expression of AOX1 gene in *Pichia pastoris*. FEMS yeast research. 2009;9:1271-82.

Xue Y, Kong C, Shen W, Bai C, Ren Y, Zhou X, et al. Methylotrophic yeast *Pichia pastoris* as a chassis organism for polyketide synthesis via the full citrinin biosynthetic pathway. Journal of biotechnology. 2017;242:64-72.

Yang H, Zhu Q, Zhou N, Tian Y. Optimized expression of prolyl aminopeptidase in *Pichia pastoris* and its characteristics after glycosylation. World Journal of Microbiology and Biotechnology. 2016;32:176.

Yang J-K, Chen F-Y, Yan X-X, Miao L-H, Dai J-H. A simple and accurate two-step long DNA sequences synthesis strategy to improve heterologous gene expression in *Pichia*. PloS one. 2012;7:e36607.

Yang J, Liu L. Codon optimization through a two-step gene synthesis leads to a high-level expression of *Aspergillus niger* lip2 gene in *Pichia pastoris*. Journal of Molecular Catalysis B: Enzymatic. 2010;63:164-9.

Yang JK, Liu LY, Dai JH, Li Q. de novo design and synthesis of *Candida antarctica* lipase B gene and alpha-factor leads to high-level expression in *Pichia pastoris*. PloS one. 2013a;8:e53939.

Yang M, Teymorian S, Olivares P, Murthy PPN. Extracellular expression of alkaline phytase in *Pichia pastoris*: Influence of signal peptides, promoters and growth medium. Biotechnology Reports. 2015;6:112-8.

Yang S, Kuang Y, Li H, Liu Y, Hui X, Li P, et al. Enhanced production of recombinant secretory proteins in *Pichia pastoris* by optimizing Kex2 P1' site. PloS one. 2013b;8:e75347.

Yang Z, Zhang Z. Codon-optimized expression and characterization of a pH stable fungal xylanase in *Pichia pastoris*. Process Biochemistry. 2017;53:80-7.

Ye R, Huang M, Lu H, Qian J, Lin W, Chu J, et al. Comprehensive reconstruction and evaluation of *Pichia pastoris* genome-scale metabolic model that accounts for 1243 ORFs. Bioresour Bioprocess. 2017;4:22.

Zhang AL, Luo JX, Zhang TY, Pan YW, Tan YH, Fu CY, et al. Recent advances on the GAP promoter derived expression system of *Pichia pastoris*. Molecular biology reports. 2009;36:1611-9.

Zhang P, Zhang W, Zhou X, Bai P, Cregg JM, Zhang Y. Catabolite repression of Aox in *Pichia pastoris* is dependent on hexose transporter PpHxt1 and pexophagy. Applied and environmental microbiology. 2010;76:6108-18.

Zhang X, Zhang X, Liang S, Ye Y, Lin Y. Key regulatory elements of a strong constitutive promoter, P_{GCW14}, from *Pichia pastoris*. Biotechnology letters. 2013;35:2113-9.

Zhao H, Chen D, Tang J, Jia G, Long D, Liu G, et al. Partial optimization of the 5-terminal codon increased a recombination porcine pancreatic lipase (opPPL) expression in *Pichia pastoris*. PloS one. 2014;9:e114385.

Zhao W, Zheng J, Zhou HB. A thermotolerant and cold-active mannan endo-1,4-beta-mannosidase from *Aspergillus niger* CBS 513.88: Constitutive overexpression and high-density fermentation in *Pichia pastoris*. Bioresource technology. 2011;102:7538-47.

Zhou W-J, Yang J-K, Mao L, Miao L-H. Codon optimization, promoter and expression system selection that achieved high-level production of *Yarrowia lipolytica* lipase in *Pichia pastoris*. Enzyme and microbial technology. 2015;71:66-72.

Zhu T, Guo M, Tang Z, Zhang M, Zhuang Y, Chu J, et al. Efficient generation of multi-copy strains for optimizing secretory expression of porcine insulin precursor in yeast *Pichia pastoris*. Journal of applied microbiology. 2009;107:954-63.

Zhu T, Guo M, Zhuang Y, Chu J, Zhang S. Understanding the effect of foreign gene dosage on the physiology of *Pichia pastoris* by transcriptional analysis of key genes. Applied microbiology and biotechnology. 2011a;89:1127-35.

Zhu T, Sun H, Li P, Xue Y, Li Y, Ma Y. Constitutive expression of alkaline β-mannanase in recombinant *Pichia pastoris*. Process Biochemistry. 2014;49:2025-9.

Zhu T, You L, Gong F, Xie M, Xue Y, Li Y, et al. Combinatorial strategy of sorbitol feeding and low-temperature induction leads to high-level production of alkaline beta-mannanase in *Pichia pastoris*. Enzyme Microb Technol. 2011b;49:407-12.

Chapter 3: Recent advances on production of 2, 3-butanediol using engineered microbes

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3.1 Abstract

Driven by increasing demand for environmental protection and combat for carbon emission, production of commodity chemicals via microbial fermentation has become an attractive alternative to petroleum-derived processes. 2, 3-butanediol (2, 3-BD) is a significant platform chemical which has found wide applications in industry. The success of microbial 2, 3-BD production was limited by the use of pathogenic microorganisms and low titer in engineered hosts. The utilization of cheaply available feedstock such as lignocellulose was another major challenge to achieve economic production of 2, 3-BD. To address those issues, engineering strategies including both genetic modifications and process optimization have been employed. In this review, we summarized the state-of-the-art progress in the biotechnological production of 2, 3-BD. Metabolic engineering and process engineering strategies were discussed.

Keywords: 2, 3-butanediol, metabolic engineering, process engineering, renewable feedstock.

3.2 Introduction

Biological production of bulk chemicals was fuelled by the demand for reduced use of fossil fuels due to limited reserve and growing environmental concern. 2, 3-butanediol (2, 3-BD) is classified as a platform chemical with enormous potential applications in industry by the US energy department. 2, 3-BD could be converted to value-added chemicals such as 2, 3-butadeine, methyl ethyl ketone, acetoin and diacetyl (Bialkowska, 2016, Ji et al., 2011b). 2, 3-BD could be used as a drop-in fuel additive owing to its high heating value comparable to other liquid fuels (Celinska and Grajek, 2009) and low vapour pressure. The optical isomers of 2, 3-BD, namely (2R, 3R)- and (2S, 3S)-2, 3-BD have seen applications in chiral synthesis and anti-freeze agent. Production of 2, 3-BD has been reported in various microorganisms including pathogenic bacteria. The biosynthesis pathways of 2, 3-BD in several typical bacteria were shown in Fig. 3-1. The highest 2, 3-BD titer achieved in bacteria was observed in *Klebsiella pneumoniae*, reaching 150 g/L (Ma et al., 2009). *K. oxytoca* was another efficient 2, 3-BD producer identified as class 2 bacterium. Up to 130 g/L of 2, 3-BD was obtained using a *K. oxytoca* strain. Despite the high titer achieved in class 2 bacteria, concern over the use of pathogenic microorganisms has led to the research in other class 1 bacteria. *Bacillus subtilis*, *B. licheniformis*, and *B. amyloliquefaciens* are potent 2, 3-BD producers with GRAS status. Native hosts generally produce 2, 3-BD in mixture of optical isomers. Extensive efforts have been committed to the identification of novel strains and improvement of optical purity. Compared with native hosts, industrially relevant hosts such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Lactococcus lactis* are more suitable for large-scale production because of their well-characterized genetics and well-established cultivation strategies. Moreover, they are GRAS microorganisms for which safety issue could be eliminated. However, heterologous hosts usually do not possess a pathway leading

to the synthesis of 2, 3-BD. Synthetic pathway needs to be introduced to achieve such goal. Titers of 2, 3-BD comparable to that of native hosts have been achieved in the engineered microbes.

In this review, we summarized the recent progress in the production of 2, 3-BD using engineered microorganisms. Strategies for strain improvement and cultivation were discussed. Feasibility of using renewable feedstocks as substrates was highlighted. Prospects in the bio-production of 2, 3-BD were also briefly discussed.

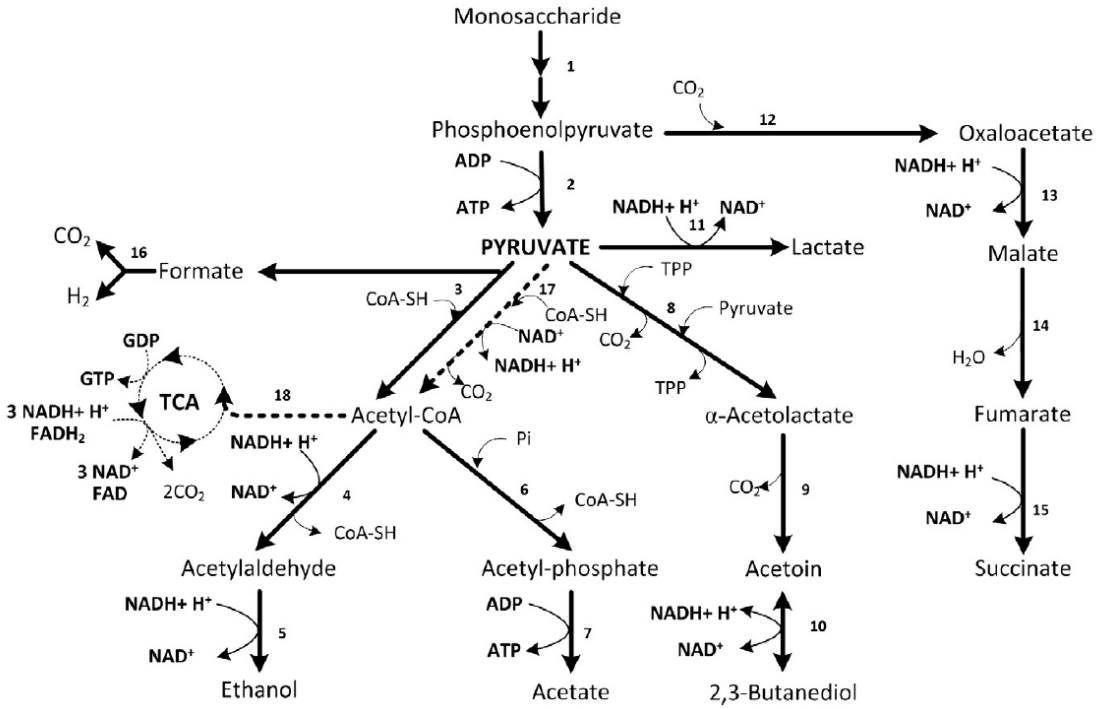

Fig. 3-1 Biosynthesis pathway of 2, 3-BD (Ji et al., 2011). TCA (tricarboxylic acid cycle); Pyruvate is formed via glycolysis or pentose phosphate pathway. 5, ethanol dehydrogenase; 7, acetate kinase; 8, α-acetolactate synthase (ALS); 9, α-acetolactate decarboxylase (ALDC); 10, 2,3-butanediol dehydrogenase (2,3-BDH); 11, lactate dehydrogenase. For a complete list of the pathway enzymes, please refer to (Ji et al., 2011).

3.3 Genetic engineering for strain improvement

3.3.1 Engineering of Bacillus sp.

B. subtilis has been reported to produce mixture of (2R, 3R)- and meso-2, 3-BD due to the presence of two butanediol dehydrogenases. Efforts have been made to produce optically pure 2, 3-BD. NADH pool has been a bottleneck for 2, 3-BD production. 2, 3-BD titer reached 49. 2 g/L in *B. subtilis* when a transhydrogenase derived from *E. coli* was overexpressed to increase the NADH availability (Fu et al., 2014). Fu *et al*. constructed a *B. subtilis* strain producing pure meso-2, 3-BD by deleting the (2R, 3R)-butanediol dehydrogenase, introducing a meso-butanediol dehydrogenase gene (*K. pneumonia budC*) and overexpressing the 2, 3-BD pathway genes (*B. subtilis alsS* and *alsD*) (Fu et al., 2016). The best strain produced 103.7 g/L meso-2, 3-BD in a fed-batch cultivation under optimized agitation and aeration codditions. Carbon flux towards formation of by-product was redistributed through the manipulation of NADH level in *B. subtilis (Yang et al., 2015b)*. The inactivation of endogenous NADH oxidase YodC by the insertion of a formate dehydrogenase led to 19.9% increase of 2, 3-BD production and 71.9% reduction of acetoin formation. *B. subtilis* was engineered to produce 2, 3-BD using cellobiose as feedstock through the overexpression of a β-glucosidase (Tanimura et al., 2016). The resulting strain was found to produce 21.2 g/L 2, 3-BD in 72h cultivation.

B. amyloliquefaciens is recognised as a safe microorganism for the production of 2, 3-BD. It was found that the co-overexpression of a glyceraldehyde-3-phosphate dehydrogenase and butanediol dehydrogenase in *B. amyloliquefaciens* B10-127 resulted in 22.7% improvement of 2, 3-BD production, reaching 132.9 g/L in fed-batch cultivation (Yang et al., 2013a). The accumulation of by-products such as acetoin, lactate and succinate was significantly reduced.

B. licheniformis produces mixture of (2R, 3R)-2, 3-BD and meso-2, 3-BD due to the presence of two BDHs. It was recently engineered to produce pure (2R, 3R)-2, 3-BD by knocking out budC (encoding meso-butanediol dehydrogenase) (Qi et al., 2014). The mutant was found to produce 30.76 g/L (2R, 3R)-2, 3-BD with a productivity of 1.28 g/L/h. To produce pure meso-2, 3-BD in *B. licheniformis*, gdh (glycerol dehydrogenase gene) was identified as the (2R, 3R)-butanediol dehydrogenase and knocked out (Qiu et al., 2016). The gdh mutant strain was found to produce only meso-2, 3-BD. Double mutant strain lacking gdh and acoR (encoding transcriptional activator for acetoin dehydrogenase system) was able to produce 98 g/L meso-2, 3-BD in fed-batch fermentation. Ge and coworkers investigated the stereo-specificity of two butanediol dehydrogenases in *B. licheniformis* (Ge et al., 2016). Two strains lacking *budC* or *gdh* were constructed. It was observed that *B. licheniformis* Δ*budC* produced 123.7 g/L (2R, 3R)-2, 3-BD while *B. licheniformis* Δ*gdh* accumulated 90.1 g/L meso-2, 3-BD.

3.3.2 Engineering of Klebsiella sp.

Native hosts such as *K. pneumoniae* and *K. oxytoca* produce 2, 3-BD with the highest reported titer. However, the optical purity was not satisfactory due to the multiple butanediol dehydrogenases. Guo and coworkers overexpressed the α-acetolactate synthase and acetoin reductase in *K. pneumoniae* (Guo et al., 2014b). The engineered strain produced 12% more 2, 3-BD compared with the wild-type strain in a batch fermentation. To eliminate the pathogenicity of *K. pneumoniae*, a mutant strain deficient in the synthesis of outer core lipopolysaccharides was constructed and used as host for 2, 3-BD production (Lee et al., 2015). The authors deleted the *budC* gene and lactate dehydrogenase gene (*ldh*A) and overexpressed the glycerol dehydrogenase genes (*gldA* and *dhaD*). The titer of (2R, 3R)-2, 3-BD reached 61 g/L using fed-batch cultivation. Redirection of carbon flux towards 2, 3-BD production through deletion of

genes responsible for by-product formation and overexpression of 2, 3-BD pathway genes has been an effective strategy. It was found that the plasmid-based overexpression of *bud*C (encoding BDH) and *bud*B (encoding α-acetolactate synthase) in *ldh*A-deficient *K. pneumoniae* KCTC2242 successfully redistributed the carbon flux towards 2, 3-BD accumulation from lactic acid formation (Kim et al., 2014a). Compared with parent strain, the titer of 2, 3-BD was improved by 40% to reach 90 g/L and lactic acid was reduced to less than 1.26 g/L in fed-batch fermentation. In another study using *K. pneumoniae* KCTC2242, the effect of overexpression of various pathway genes on 2, 3-BD production were investigated (Kim et al., 2012). Plasmids harbouring different combinations of *budA*, *budB* and *budC* were transformed to obtain various mutants. It was observed that overexpression of *budA* and *budB* led to 60% increase of 2, 3-BD compared with parental strain in shake flask culture. Fed-batch fermentation of this strain yielded 101.53 g/L 2, 3-BD with a productivity of 2.54 g/L/h. Guo and coworkers investigated the effect of deletion of *ldhA*, *adhE* (encoding alcohol dehydrogenase) and *pta* (encoding phosphate acetyltransferase) in *K. pneumoniae* KG1 (Guo et al., 2014a). Single mutant showed little effect on cell growth despite growth rate was slightly lower in the first 8 h in the *ldhA* mutant. Deletion of *adhE* and *pta* resulted in higher 2, 3-BD production and lower production rate of by-products. Double mutant lacking *adhE* and *ldhA* was cultivated in fed-batch cultivation to obtain a 2, 3-BD titer of 116 g/L with a remarkable yield on glucose of 0.49 g/g. In silico simulation based on genomic scale metabolic model and constraints-based flux analysis is particularly useful to identify gene deletion targets in *K. pneumoniae* (Park et al., 2017b). Using such strategy, a double mutant *K. pneumoniae* lacking *ldhA* and *mdh* (encoding malate dehydrogenase) was constructed to achive maximized co-production of 1, 3-PD and 2, 3-BD. This strain was able to

produce 70 g/L 1, 3-PD and 44 g/L 2, 3-BD in a pilot-scale (5000 L) fed-batch cultivation using crude glycerol as substrate.

K. oxytoca is a pathogenic bacterium producing 1, 3-propanediol and 2, 3-BD. It was found that the overexpression of *budC* in *K. oxytoca* resulted in 43% less acetoin accumulation and 1.2-fold increase of 2, 3-BD production under the agitation speed of 400 rpm (Cho et al., 2015b). Cho and coworkers deleted the *pduC* (encoding glycerol dehydratase) and *ldh*A in *K. oxytoca* M1 to direct more carbon flux towards the formation of 2, 3-BD (Cho et al., 2015a). The double mutant was able to produce 131.5 g/L 2, 3-BD using crude glycerol as carbon source. In another double mutant *K. oxytoca* strain constructed by deleting *ldhA* and *pflB* (encoding pyruvate formate lyase), production of 2, 3-BD was enhanced by 2.3-fold and 1.3-fold compared with wild-type strain and single ldhA mutant, respectively (Park et al., 2013b). In a recent study, a triple mutant *K. oxytoca* lacking *adhE*, *ackA* (acetate kinase-phosphotransacetylase) and *ldhA* was constructed (Jantama et al., 2015). This strain was found to produce 117.4 g/L 2, 3-BD with the highest reported yield of 0.49 g/g in *K. oxytoca*. By-products such as acetoin, lactate and formic acid were not detected.

3.3.3 Engineering of Enterobacter sp.

E. aerogens is an opportunistic pathogen and a potent producer of 2, 3-BD. Lactic acid is a major by-product in this species. Inactivation of *ldhA* in *E. aerogenes* KCTC 2190 led to 0. 34 g/L of lactic acid and enhanced the productivity of 2, 3-BD by 16.3% (Jung et al., 2012). In fed-batch cultivation, the *ldhA*-deficient strain produced 69.12 g/L of 2, 3-BD, which was 28.5% higher than that of the wild-type strain. The titer of 2, 3-BD was further improved to 118.05 g/L 2, 3-BD with optimized aeration and addition of 10 g/L of casamino acid.

To improve the optical purity of 2, 3-BD produced in *E. cloacae*, endogenous *bdh* (encoding BDH) was deleted and heterologous *B. pumilus bdh* was overexpressed in *E. cloacae* (Li et al., 2015). The engineered strain produces optically pure (2R, 3R)-2, 3-BD. Competing pathways leading to by-products such as lactic acid and succinic acid were blocked by eliminating *ldh* and *fr* (encoding fumarate reductase), respectively. The strain was able to efficiently co-utilize glucose and xylose and produced 152 g/L (2R, 3R)-2, 3-BD. The titer of 2, 3-BD achieved 119.4 g/L when lignocellulosic hydrolysate was used as substrate.

3.3.4 Engineering of S. marcescens

S. marcescens has become a popular host for 2, 3-BD production. The metabolic pathway for the production of 2, 3-BD was recently characterized in *S. marcescens* MG1 (Rao et al., 2012). The pathway genes for 2, 3-BD synthesis were identified to be *slaA* (encoding ALDC), *slaB* (encoding ALS) and *slaC* (encoding BDH) with two regulators encoded by *slaR* and *swrR*. It was found that *slaR* was an activator of *slaA* and *slaB* while *swrR* negatively regulated the transcription of *slaA*, *slaB* and *slaR* instead of *slaC*. Deletion of *swrR* led to earlier production of 2, 3-BD than wild type strain. Meso-2, 3-BD and (2S, 3S)-2, 3-BD were produced in this species. To produce (2R, 3R)-2, 3-BD in *S. marcescens* MG1, deletion of *slaC* (meso-BDH) and introduction of *B. subtilis bdhA* was performed, which resulted in the production of 89.81 g/L (2R, 3R)-2, 3-BD in fed-batch fermentation (Bai et al., 2015).

3.3.5 Engineering of E. coli

E. coli is a well-established bacterium for production of biofuels. Ji and coworkers constructed a synthetic pathway for the production of optically pure (2R, 3R)-2, 3-BD in *E. coli* (Ji et al., 2015). Plasmid harbouring *K. pneumoniae budB* (ALS) and *budA* (ALDC) and *B. subtilis ydj*L (BDH) was assembled by expressing the genes under the control of one IPTG-inducible

promoter. Effect of IPTG concentration was investigated. It was found that no IPTG addition resulted in the highest titer of 2, 3-BD, reaching 115 g/L in fed-batch cultivation. Constitutive production of (2R, 3R)-2, 3-BD in *E. coli* was achieved by constructing a synthetic pathway (Tong et al., 2016). Plasmid harbouring *K. pneumoniae budB, budA* and *B. subtilis ydjL* under synthesized constitutive promoter was constructed. The recombinant strain with stronger promoter P_{01} was able to produce 30.5 g/L (2R, 3R)-2, 3-BD under optimized conditions. Heterologous expression of gene clusters for 2, 3-BD synthesis from various native producers including *B. subtilis*, *B. licheniformis*, *K. pneumoniae*, *S. marcescens* and *E. cloacae* was recently investigated (Xu et al., 2014). Different promoters were tested for optimal expression. It was found that expression of *E. cloacae* gene cluster under the predicated promoter of the gene cluster (P_{abc}) resulted in the best production of 2, 3-BD. Optimization of cultivation conditions including pH, aeration and agitation speed led to 73.8 g/L of 2, 3-BD. Production of (2S, 3S)-2, 3-BD using glucose as feedstock was reported recently (Chu et al., 2015). Co-expression of *E. cloacae budB* and *budC* in *E. coli* led to the formation of (2S, 3S)-2, 3-BD using glucose as carbon source. A titer of 2.2 g/L was obtained with the addition of 10 mM iron chloride ($FeCl_3$). In a recent study, overexpression of *Enterobacter* ALS and AR using both IPTG-inducible promoter (T7) and constitutive promoter (PackA) in *E. coli* led to 2, 3-BD production (Reshamwala et al., 2017). The putative pathway for 2, 3-BD production identified in *C. autoethanogenum* was validated by overexpression of the pathway in *E. coli* (Kopke et al., 2014). This pathway was featured by a NADPH-dependent primary-secondary alcohol dehydrogenase (CaADH) which was responsible for the reduction of acetoin to 2, 3-BD. Nakashima *et al*. designed a novel method for gene expression and demonstrated its application in 2, 3-BD production in *E. coli* (Nakashima et al., 2014). Their system eliminates the use of costly inducer

such as IPTG by using the xylose-inducible promoter P_{xylF}. T7 RNA polymerase gene was expressed under the P_{xylF} to address the low activity of this weak promoter. Heterologous gene under the control of T7 promoter was integrated into the *E. coli* genome and could be expressed upon the addition of xylose as inducer. The production of 2, 3-BD using this system was tested by knocking in *B. subtilis alsS*, *alsD* and *L. lactis butA*. The resulting strain produced 54 g/L 2, 3-BD with 99% of theoretical yield, indicating the promising potential of this system for the production of 2, 3-BD.

3.3.6 Engineering of yeast

S. cerevisiae is a model eukaryote with clear genetic background. It has been gaining interest as a platform for the production of 2, 3-BD in recent years. *S. cerevisiae* is an efficient producer of ethanol. To direct more carbon flux towards 2, 3-BD production, pyruvate decarboxylase-deficient or alcohol dehydrogenase (ADH)-deficient strains were constructed. The effects of deletion of multiple ADHs, ALD6 and GPD2 were investigated in *S. cerevisiae* (Ng et al., 2012). The best strain lacking ADH1, ADH3 and ADH5 was able to produce 2.29 g/L 2, 3-BD. Kim and coworkers introduced a pathway consisting of *B. subtilis alsS*, *B. subtilis alsD* and endogenous BDH1 in *S. cerevisiae* Δpdc1Δpdc5 (Kim et al., 2013). The pdc-deficient strain was C2 compound (ethanol or acetate) dependent and was screened by evolution engineering to be C2-independent and high glucose tolerant. The engineered strain produced 96.2 g/L 2, 3-BD in fed-batch cultivation. In order to ferment xylose into 2, 3-BD, a xylose utilization pathway was integrated into the genome of *S. cerevisiae* harbouring the 2, 3-BD pathway (Kim et al., 2014b). The strain produced 43.6 g/L 2, 3-BD using xylose as carbon source in fed-batch cultivation. The pdc-deficient *S. cerevisiae* strain suffered severe growth defect. Evolution engineering could restore growth on glucose. Lian and coworkers constructed a strain by disruption of pdc1, pdc5

and pdc6 and overexpression of truncated endogenous *ilv2*, BDH1 and *B. subtilis alsD* (Lian et al., 2014). The engineered strain was capable of co-utilizing glucose and galactose by the overexpression of MTH1, a transcription factor. Enantiopure (2R, 3R)-2, 3-BD of up to 100 g/L was achieved in fed-batch fermentation. Glycerol is formed as a major by-product for the regeneration of NADH in 2, 3-BD production using *S. cerevisiae*. Disruption of *gpd*1 and *gpd*2 abolished glycerol formation but led to reduced glucose consumption rate and 2, 3-BD productivity (Kim and Hahn, 2015). The expression of a water-forming NADH oxidase *noxE* in *S. cerevisiae* resulted in a more balanced redox and improved 2, 3-BD productivity (Kim et al., 2015). The best strain lacking five ADHs and gpd1 and gpd2 produced 72.9 g/L 2, 3-BD in fed-batch shake flask cultivation. Fine-tuning the 2, 3-BD pathway could be beneficial to the titer of 2, 3-BD. The effects of overexpression of ALDCs from four bacteria were investigated in *S. cerevisiae* (Choi et al., 2016). It was found that expression of *E. aerogenes* ALDC outperformed other ALDCs and resulted in 132.4 g/L of 2, 3-BD in fed-batch cultivation. To address the C2-auxotrophy in *pdc*-deficient *S. cerevisiae*, pyruvate decarboxylase from Crabtree-negative yeasts were expressed (Kim et al., 2016a). *Candida tropicalis* PDC was selected due to its lowest V_{max} and its expression was fine-tuned with various promoters and copy numbers. The best strain expressing *C. tropicalis* PDC and *L. lactis noxE* produced the highest titer of 2, 3-BD ever reported in *S. cerevisiae*, reaching 154.3 g/L in fed-batch fermentation. *S. cerevisiae* expressing *C. tropicalis* PDC and *L. lactis noxE* was introduced with a xylose utilization pathway to convert xylose into 2, 3-BD. It was reported that 96.8 g/L 2, 3-BD was accumulated from xylose in fed-batch fermentation (Kim et al., 2017a). The engineered *S. cerevisiae* strains discussed above were based on overexpression of genes on episomal plasmids, which could lead to concern of plasmid instability. To address this issue, cocktail δ-integration of pathway genes which allows

incorporation of multi-copy genes into the *S. cerevisiae* chromosome was employed (Kato et al., 2013). The expression of four genes (*B. subtilis alsS*, *B. amyloliquefaciens alsD* and *bdhA*, and *L. lactis noxE*) was fine-tuned using a plasmid library containing 15 promoters (Yamada et al., 2017). High-producing strain was screened and evaluated. The best strain designated YPH499ΔPDC1ΔADH1ΔGPD1BD6-10 was able to produce 80 g/L 2, 3-BD in fed-batch cultivation. Cellobiose was converted to 2, 3-BD in *S. cerevisiae* strain simultaneously expressing a cellobiose utilization pathway (*Neurospora crassa cdt*-1 and *gh*1-1) and 2, 3-BD synthesis pathway (*B. subtilis alsS* and *alsD*) (Nan et al., 2014). The engineered strain could produce 5.29 g/L 2, 3-BD using 20 g/L cellobiose.

3.3.7 Engineering of other microorganisms

Corynebacterium glutamicum is an industrially-relevant microorganism with GRAS status. Production of 2, 3-BD in *C. glutamicum* was achieved by introduction of a gene cluster consisting of *als*, *alsB* and *butA* from *L. lactis* (Rados et al., 2015). The expression of the three genes was induced by isopropyl β-D-1-thiogalactopyranoside (IPTG). Four competing pathway genes encoding subunit of pyruvate dehydrogenase (*aceE*), lactate dehydrogenase (*ldhA*), pyruvate:quinone oxidoreductase (*pqo*) and malate dehydrogenase (*mdh*) were deleted. A titer of 6.3 g/L 2, 3-BD was achieved under optimized aeration conditions. In another report, overexpression of *K. pneumoniae budB* and *budA* in *C. glutamicum* was found to produce 18.9 g/L 2, 3-BD in a batch culture from 80 g/L glucose (Yang et al., 2015a).

Light-driven conversion of carbon dioxide (CO_2) to value-added chemicals such as 2, 3-BD by cyanobacteria is attracting great attention due to the sustainability of this process (Savakis and Hellingwerf, 2015). Photosynthesis of 2, 3-BD in *Synechocystis* sp. PCC6803 was recently reported (Savakis et al., 2013). The authors assembled a pathway comprising *Enterococcus*

faecalis ALS, *L. lactis* ALDC and *L. lactis* acetoin reductase (AR) in the chromosome of *Synechocystis*. Production of pure meso-2, 3-BD was observed at a titer of 0.47 g/L using CO_2 as feedstock. *Synechococcus elongatus* PCC7492, another cyanobacterium, was also engineered to convert CO_2 to 2, 3-BD (Oliver et al., 2013). The authors constructed an oxygen-insensitive pathway by using O_2-insensitive ALDCs and tested 6 ALDC candidates for acetoin accumulation. *Aeromonas hydrophila* ALDC was identified as the best enzyme by comparing the acetoin titer. *Clostridium beijerinckii adh* was identified as most suitable ADH through screening and was used for the production of 2, 3-BD. The strain overexpressing *B. subtilis alsS*, *A. hydrophila* ALDC and *C. beijerinckii adh* was found to produce 2.38 g/L of 2, 3-BD after 21 days. To achieve balanced expression of *B. subtilis alsS*, *E. aerogenes alsD* and *C. beijerinckii adh* under a single operon in *S. elongatus* PCC7492, four ribosome binding sites (RBS) of various strength were used to modulate the expression of the three pathway genes (Oliver et al., 2014). The best construct consisting of *alsS* preceded by RBS-B, *alsD* preceded by RBS-B and *adh* preceded by RBS-D was found to produce 0.496 g/L 2, 3-BD, which represents 1.8-fold increase compared with the non-optimized strain.

Zymomonas mobilis is recognized as an efficient ethanol producer which can utilize a diverse range of feedstocks such as lignocellulosic biomass (Yang et al., 2016a). Yang and coworkers recently developed *Z. mobilis* strains to convert glucose and xylose to 2, 3-BD (Yang et al., 2016c). The synthetic pathway consists of native or codon-optimized *als*, *aldC* and *bdh* from *B. licheniformis* and *E. cloacae*. Expression of the genes was optimized using inducible or constitutive promoters. The best strain harbouring codon-optimized *B. subtilis als* and *E. cloacae aldC* and *bdh* produced 15 g/L of 2, 3-BD under optimized dissolved oxygen (DO) condition in bioreactor cultivation.

L. lactis has been mainly used as a producer of lactic acid. Rational engineering of *L. lactis* based on genomic scale metabolic model for the production of diacetyl and (2S, 3S)-2, 3-BD was reported (Liu et al., 2016). The authors first constructed a respiration-dependent strain which accumulates large amounts of α-acetolactate. Diacetyl can be converted from α-acetolactate via acetoin reductase or metal ion-based catalysis. Diacetyl production was extended to the 2, 3-BD production by overexpression of *E. cloacae bdh*. A titer of 6.7 g/L of enantiomerically pure (2S, 3S)-2, 3-BD was achieved with the addition of 10 mM iron (Fe^{3+}) in shake flask cultivation.

Clostridium acetobutylicum is a major butanol producer which can produce acetoin as minor product under certain conditions. To produce 2, 3-BD in this bacterium, *C. beijerinkii* acetoin reductase (*acr*) was overexpressed. (2R, 3R)-2, 3-BD was produced in *C. acetobutylicum* with a titer of 1.8 g/L (Siemerink et al., 2011).

A comparison of 2, 3-BD production in various engineered microorganisms was provided in Table 3-1.

Table 3-1 Comparison of 2, 3-butanediol production in engineered microorganisms. (Adapted from Yang et al., 2017)

Microorganism	Substrate	Yield (g/g)	Titer (g/L)	Description	Reference
E. coli	Glucose	0.42	115	Fed-batch, highest titer in *E. coli*	(Ji et al., 2015)
E. coli	Glucose	0.41	73.8	Fed-batch	(Xu et al., 2014)
E. coli	Glucose	0.381	30.5	Fed-batch	(Tong et al., 2016)
E. coli	Glucose	0.08	2.2	Shake flask	(Chu et al.,

					2015)
E. coli	Glucose, xylose	0.49	54	Shake flask, highest yield in *E. coli*	(Nakashima et al., 2014)
E. coli	Glucose	0.29	1.12	Shake flask	(Nielsen et al., 2010)
E. coli	Glucose	0.303	14.5	Shake flask	(Li et al., 2010)
E. coli	Glycerol	0.332	9.56	Shake flask	(Shen et al., 2012)
B. subtilis	Glucose	0.46	49.29	Shake flask	(Fu et al., 2014)
B. subtilis	Glucose	0.487	103.7	Fed-batch, highest titer in *B. subtilis*.	(Fu et al., 2016)
B. subtilis	Glucose	0.2	2.4	Shake flask	(de Oliveira and Nicholson, 2016)
B. subtilis	Cellobiose	0.35	21.2	Batch	(Tanimura et al., 2016)
B. licheniformis	Glucose	NA	30.76	Shake flask	(Qi et al., 2014)
B. licheniformis	Xylose	0.365	13.8	Batch	(Wang et al., 2012)
B. licheniformis	Glucose	0.4	98	Fed-batch	(Qiu et al., 2016)
B. licheniformis	Glucose	NA	123.7	Fed-batch	(Ge et al.,

					2016)
B. amyloliquefaciens	Glucose	0.45	132.9	Fed-batch	(Yang et al., 2013a)
B. amyloliquefaciens	Crude glycerol	0.44	102.3	Fed-batch	(Yang et al., 2015d)
K. pneumoniae	Glucose	NA	116	Fed-batch	(Guo et al., 2014a)
K. pneumoniae	Glucose	0.38	90	Fed-batch	(Kim and Hahn, 2014)
K. pneumoniae	Glucose	0.2	61	Fed-batch	(Lee et al., 2015)
K. pneumoniae	Glucose	0.37	24.48	Shake flask	(Guo et al., 2014b)
K. pneumoniae	Glucose	0.461	31.1	Shake flask	(Jung et al., 2014)
K. pneumoniae	Crude glycerol	0.44	131.5	Fed-batch, highest titer in an engineered *K. pneumoniae*	(Cho et al., 2015a)
K. pneumoniae	Starch	0.269	53.8	Batch	(Tsvetanova et al., 2014)
K. pneumoniae	Glucose	NA	101.53	Fed-batch	(Kim et al., 2012)
K. pneumoniae	Glucose	NA	44	Fed-batch	(Park et al., 2017b)
K. oxytoca	Glucose	0.42	142.5	Fed-batch, highest	(Cho et al.,

				titer in engineered K. oxytoca	2015b)
K. oxytoca	Crude glycerol	0.44	131.5	Fed-batch	(Cho et al., 2015a)
K. oxytoca	Glucose	0.41	115		
K. oxytoca	Glucose	0.48	130	Fed-batch	(Ji et al., 2010)
K. oxytoca	Glucose	0.49	117.4	Fed-batch	(Jantama et al., 2015)
K. oxytoca	Glucose	0.4	106.7	Fed-batch	(Park et al., 2015)
K. oxytoca	Glucose	0.45	113	Fed-batch	(Park et al., 2013a)
K. oxytoca	Glucose, xylose	NA	23.9	Shake flask	(Ji et al., 2011a)
K. oxytoca	Glucose	0.48	130	Fed-batch	(Ji et al., 2010)
E. aerogens	Sugarcane molasses	NA	140	Fed-batch, highest titer in *E. aerogens*	(Jung et al., 2015)
E. aerogens	Glucose	NA	118.05	Fed-batch	(Jung et al., 2012)
E. aerogens	Sugarcane molasses	0.366	98.69	Fed-batch	(Jung et al., 2013)
E. aerogens	Sugarcane molasses	0.395	21	Batch	(Um et al., 2017)
E. cloacae	Glucose	0.475	119.4	Fed-batch	(Li et al., 2015)

S. marcescens	Sucrose	0.35	89.81	Fed-batch	(Bai et al., 2015)
S. marcescens	Sucrose	0.463	152	Fed-batch	(Zhang et al., 2010)
S. cerevisiae	Glucose	0.28	96.2	Fed-batch	(Kim et al., 2013)
S. cerevisiae	Xylose	0.27	43.6	Fed-batch	(Kim et al., 2014b)
S. cerevisiae	Xylose	NA	96.8	Fed-batch	(Kim et al., 2017a)
S. cerevisiae	Glucose	0.367	31	Shake flask	(Kim et al., 2015)
S. cerevisiae	Glucose	0.41	72.9	Shake flask	(Kim and Hahn, 2015)
S. cerevisiae	Glucose	0.404	154.3	Fed-batch, highest titer in *S. cerevisiae*	(Kim et al., 2016a)
S. cerevisiae	Glucose	0.278	80	Fed-batch flask	(Yamada et al., 2017)
S. cerevisiae	Glucose, galactose	NA	100	Fed-batch	(Lian et al., 2014)
S. cerevisiae	Glucose	NA	29.1	Fed-batch	(Kim and Hahn, 2014)
S. elongatus	CO2	NA	0.496	Photosynthesis	(Oliver et al., 2014)
S. elongatus	CO2	NA	0.58	Photosynthesis	(Savakis et al.,

					2013)
C. glutamicum	Glucose	0.33	6.3	Batch	(Rados et al., 2015)
C. glutamicum	Glucose	NA	18.9	Batch	(Yang et al., 2015a)
Z. mobilis	Glucose, xylose	0.16	15	Batch	(Yang et al., 2016c)

3.4 Characterization of novel 2, 3-BD dehydrogenases

BDH or AR catalyzes the reversible conversion between acetoin and 2, 3-BD. The BDHs characterized to date belong to the short chain or medium chain alcohol dehydrogenase family. They are generally NAD^+ or $NADP^+$ dependent and featured by a zinc-containing motif. The stereo isomer of 2, 3-BD is determined by the specificity of BDH. A number of BDHs of various sources have been identified and characterized. Some BDHs, although not annotated as BDH, are playing a similar role and thus will also be included here. These novel BDHs could contribute to the construction of novel engineered hosts for efficient production of optically pure 2, 3-BD. Moreover, BDH could be used for the synthesis of other entatiopure chiral diols (Calam et al., 2016). Their enzymatic properties were summarized in Table 3-2.

Table 3-2 Properties of novel 2, 3-butanediol dehydrogenases

BDH	Major substrates		Stereo-specificity	Enzymatic properties		Reference
	Reduction	Oxidation		Reduction	Oxidation	

K. pneumoniae budC	Meso-2,3-BD	acetoin	Meso-2,3-BD	T=35 °C pH 8.0	pH 9.0	(Zhang et al., 2012)
K. pneumoniae dhaD	Diacetyl, Racemic acetoin, R-acetoin	Glycerol, (2R, 3R)-2,3-BD, Meso-2,3-BD	(2R, 3R)-2,3-BD, Meso-2,3-BD	NA	NA	(Chen et al., 2014)
K. pneumoniae gldA	Acetoin	Glycerol, (2R, 3R)-2,3-BD, Meso-2,3-BD	(2R, 3R)-2,3-BD, Meso-2,3-BD	NA	NA	(Wang et al., 2014)
B. licheniformis budC	Diacetyl, acetoin, 2,3-hexanedione, 2,3-pentanedione,	racemic 2,3-BD	(2S, 3S)-2, 3-BD	T=37 °C pH 5.0	pH 10.0	(Xu et al., 2016)
S. marcescens gldA	acetoin, diacetyl	Meso-2,3-BD, glycerol	(2R, 3R)-2,3-BD, Meso-2,3-BD	T=60 °C pH 7.0	pH 8.0	(Zhang et al., 2014a)
S. marcescens budC	Diacetyl, 3R-acetoin, 3S-	Meso-2,3-BD,	Meso-2,3-BD,	T=40 °C pH 5.0	pH 8.0	(Zhang et al.,

	acetoin	(2S,3S)-2,3-BD	(2S,3S)-2,3-BD			2014b)
C. glutamicum butA	R-acetoin, S-acetoin	(2S,3S)-2,3-BD, Meso-2,3-BD	(2S,3S)-2,3-BD , Meso-2,3-BD	NA	NA	(Radoš et al., 2016)
C. cretanum BDH	Acetoin, diacetyl	2,3-BD	NA	T=35 °C pH 4.0	pH 10.0	(Zhao et al., 2015)
Serratia sp. T241 BDH1	3S-acetoin, 3R-acetoin	(2S,3S)-2,3-BD, Meso-2,3-BD	(2S,3S)-2,3-BD, Meso-2,3-BD	NA	NA	(Zhang et al., 2016)
Serratia sp. T241 BDH2	3S-acetoin, 3R-acetoin	(2S,3S)-2,3-BD, Meso-2,3-BD	(2S,3S)-2,3-BD, Meso-2,3-BD	NA	NA	(Zhang et al., 2016)
Serratia sp. T241 BDH3	3S-acetoin, 3R-acetoin	Meso-2,3-BD, (2R,3R)-2,3-BD	Meso-2,3-BD, (2R,3R)-2,3-BD	NA	NA	(Zhang et al., 2016)
Serratia sp. T241 GDH	3S-acetoin, 3R-acetoin	Meso-2,3-BD, (2R,3R)-2,3-BD	Meso-2,3-BD, (2R,3R)-2,3-BD	NA	NA	(Zhang et al., 2016)
P. polymyxa	Diacetyl,	(2R,3R)-	(2R,3R)-	T=40 °C	pH 11.0	(Yu et al.,

BDH	3R-acetoin, 3S-acetoin	2,3-BD, meso-2,3-BD, 1,2-propandio, 1,2-pentandiol	2,3-BD, meso-2,3-BD	pH 8.0		2011)
S. cerevisiae BDH1	Acetoin, 1-hydroxy-2-propanone	(2R,3R)-2,3-BD	(2R,3R)-2,3-BD	pH 7.0	pH 8.0	(Gonzalez et al., 2000)
Bacillus sp. BRC1 *bdh*	Acetoin, diacetyl	(2R,3R)-2,3-BD, meso-2,3-BD, 1,2-BD, 1,2-propanediol	(2R,3R)-2,3-BD, meso-2,3-BD	NA	NA	(Kang et al., 2015)
Clostridium ljungdahlii CLJU_c23220	Acetoin, diacetyl	Racemic 2, 3-BD, 1, 2-propanediol	NA	pH 8.0 T=45 °C	NA	(Tan et al., 2015)
Mycobacterium sp. B-009 *mbd1*		(2R, 3R)-2, 3-BD, 1, 2-butanediol, 1, 2-propanediol, meso-2, 3-BD	(2R, 3R)-2, 3-BD	T=85 °C	pH 8.5	(Takeda et al., 2011)

Rhodococcus erythropolis WZ010 ReBDH	Acetoin, diacetyl	(2R, 3R)-2, 3-BD, meso-2, 3-BD	(2R, 3R)-2, 3-BD	pH 6.5 T=55 °C	pH 10.0 T=45 °C	(Yu et al., 2015)
C. autoethanogenum ADH	Acetoin, acetone, butanone	(2R, 3R)-2, 3-BD	(2R, 3R)-2, 3-BD	NA	NA	(Kopke et al., 2014)

NA indicates not available

3.5 Process engineering strategies to enhance 2, 3-BD production

Production of 2, 3-BD can be achieved in a diverse range of microorganisms. The growth conditions vary amongst the microbes. Therefore, optimization of fermentation conditions is generally species-specific.

3.5.1 Medium optimization

Statistical optimization of medium composition is an efficient approach to boost the production of 2, 3-BD. Plackett-Burman design is constantly employed to identify key factors of the medium constituents. Response surface methodology such as Box-Behnken design and central composite design is used for further optimization of level points of key factors. It was found that inulin, potassium phosphate dibasic (K_2HPO_4) and ammonium chloride (NH_4Cl) were key factors in the production of 2, 3-BD using *P. polyxyma* ZJ-9. Titer of 2, 3-BD reached 36.92 g/L using optimized medium. Similar techniques were used to optimize the production of 2, 3-BD in *P. polymyxa* ICGEB2008, boosting the yield to 0.33 g/g sugar using cellulosic hydrolysate, which is similar to that of rich medium (Adlakha and Yazdani, 2015). The highest reported titer of 2, 3-BD achieved in *K. pneumoniae* was obtained by optimizing the medium based on two-

level Plackett-Burman design (Ma et al., 2009). Using an optimized medium based on Plackett-Burman design, central composite design and response surface analysis, cultivation of *B. amyloliquefaciens* B10-127 was found to produce 62.7 g/L 2, 3-BD in contrast with 45.7 g/L with unoptimized medium (Yang et al., 2012). Notably, the optimized medium could be scaled up to a working volume of 20 L with similar titer. Li *et al*. optimized the medium composition using *B. licheniformis* strain 10-1-A (Li et al., 2013). Inexpensive corn steep liquor powder was used to partially replace yeast extract as nitrogen source. The titer of 2, 3-BD achieved 115.7 g/L using optimized medium with a productivity of 2.4 g/L/h.

3.5.2 Effect of complex nitrogen source

Nitrogen source is essential for the cell growth of 2, 3-BD producers. Complex nitrogen sources are rich in nutrients such as amino acids, vitamins and trace metals. Yeast extract and urea were normally added to the medium. Supplementation of yeast extract in the fermentation of *P. polymyxa* significantly improved the production rate of 2, 3-BD (Hassler et al., 2012). The tier of 2, 3-BD reached 111 g/L when up to 60 g/L yeast extract was added. Fu *et al*. also found that more 2, 3-BD and biomass was obtained in an engineered *B. subtilis* strain using rich medium than simple M9 medium (Fu et al., 2016). Addition of 5 g/L yeast extract and 10 g/L casamino acid was proved beneficial to 2, 3-BD production using *Raoultella ornithinolytica* B6 (Kim et al., 2017b). The titer was improved from 60.66 g/L to 79.25 g/L and fermentation duration was also prolonged. Similar effect was also observed in *K. oxytoca* M1 when supplemented with yeast extract and casamino acid. Cell biomass and 2, 3-BD titer was enhanced by 1.4 fold and 1.6 fold, respectively (Cho et al., 2015b).

3.5.3 Effect of pH

Cell growth and metabolite production is affected by pH. The effect of pH on the production of 2, 3-BD in some native producers in which 2, 3-BD is produced through mixed-acid pathway is even more significant. It was reported that the formation of neutral 2, 3-BD could be a countermeasure against acidification. The acetoin reductase activity was affected by pH, which offers a pH control strategy to manipulate the conversion of acetoin to 2, 3-BD by keeping pH value above a certain limit. It was found that the highest activity of acetoin reductase of *B. amyloliquefaciens* was observed at pH 6.5 (Yang et al., 2015d). The pH was maintained at pH 6.5 when the pH dropped to this value. The titer of 2, 3-BD obtained with pH 6.5 was 14.8% higher than that without pH control. Similar control strategy was applied in the fermentation of *R. ornithinolytica* B6 by maintaining pH 5.5 (Kim et al., 2016b) or pH 6.0 (Kim et al., 2017b) after pH dropped from initial value of 7.0. In one study using *E. cloacae*, two-stage pH control strategy was employed to favour cell growth in the first stage and product accumulation in the second stage (Priya et al., 2016). Compared with constant pH control (pH 7.5), dual pH strategy (pH7.5 and pH6.5) boosts the titer and yield of 2, 3-BD despite lower biomass was obtained. It was found that metabolite profile was influenced by pH values. In 2, 3-BD production using *Klebsiella* sp. Zmd30, pH 6.0 was optimal for 2, 3-BD accumulation while lactate and acetate was accumulated at higher pH values and citrate was the major by-product at lower pH values (Wong et al., 2014). The essentiality of pH control varies amongst different strains used. It was reported that lowering pH below 6.0 resulted in reduced substrate uptake rate and biomass formation of *K. oxytoca*, which was likely due to the inefficient transport of maltodextrin at low pH (Chan et al., 2016). Forced pH fluctuation through the addition of sodium hydroxide (NaOH) was implemented to enhance the production of 2, 3-BD in *K. pneumoniae* (Petrov and Petrova,

2010). This strategy was established based on the finding that 2, 3-BD formation was stimulated by a pH drop. A titer of 70 g/L was achieved using forced pH fluctuation compared with 52.5 g/L obtained without pH control. In a study where 2, 3-BD and succinic acid was co-produced, pH 6 was found optimal to achieve highest titer of 2, 3-BD (Cheng et al., 2013). In fed-batch fermentation, 77.1 g/L 2, 3-BD and 28.7 g/L succinic acid was obtained.

3.5.4 Effect of oxygen level

Oxygen supply is crucial for process development of 2, 3-BD production. The $NADH/NAD^+$ ratio is regulated by oxygen level. Micro-aeration favours higher production of 2, 3-BD but leads to lower biomass accumulation and decreased substrate uptake rate. Therefore, it is imperative to optimize aeration to balance product yield and biomass formation. Oxygen control by means of k_La (Hassler et al., 2012) or respiratory quotient (RQ) (Zhang et al., 2010) has been reported. Those strategies are complicated to implement despite being effective. Dissolved oxygen (DO) is governed by aeration rate and agitation speed, thereby controlling aeration and agitation would be more straightforward. It was found that DO could affect the ratio of 1, 3-propanediol (1, 3-PD) to 2, 3-BD in *Klebsiella* sp. Ana-WS5 (Yen et al., 2014). Higher 2, 3-BD was observed when DO was above 80% saturation level while lower DO (below 20%) was in favour of the 1, 3-PD formation. The ratio between two diols was 0.2 and 9.9 under high and low DO, respectively. Xu *et al*. optimized the agitation and aeration rates and found that agitation speed of 400 rpm and aeration of 1.5 vvm was optimal for 2, 3-BD production and biomass accumulation in an engineered *E. coli* strain (Xu et al., 2014). Two stage aeration or agitation control has been implemented in recent studies. The establishment of multi-stage oxygen control is based on the kinetic parameters including specific growth rate (μ), substrate consumption rate (q_s) and product formation rate (q_p). In the production of 2, 3-BD using *K. oxytoca*, agitation speed of 300 rpm

was used in the first 15 h to allow higher specific growth rate (μ). The agitation speed was reduced to 200 rpm for 2, 3-BD accumulation. This strategy enhanced the titer of 2, 3-BD to 95.5 g/L, which is 6.23% higher than that obtained with constant agitation (200 rpm) (Ji et al., 2009). In another study using *B. amyloliquefaciens* to ferment crude glycerol, a three-stage agitation control strategy consisting of 300 rpm in the first 5h, 400 rpm until 22h and 350 rpm until the end of fermentation was implemented (Yang et al., 2015d). Titer of 2, 3-BD was enhanced by 9.85% compared with constant agitation speed. Two-stage agitation speed control was implemented in the fermentation of a double mutant *K. oxytoca* (Park et al., 2013b). The authors investigated the effect of agitation speed between 150 rpm and 450 rpm and found that the highest 2, 3-BD productivity was obtained under 450 rpm while the highest titer and yield of 2, 3-BD was achieved under 150 rpm. Agitation speed was first maintained at 450 rpm and switched to 150 rpm when the acetoin titer reached 10 g/L to avoid the toxic effect of excessive acetoin. It was found that 113 g/L 2, 3-BD was obtained with 2.1 g/L/h, representing a 95.2% and 27.3% increase of titer and productivity over the constant speed of 450 rpm, respectively.

3.6 Alternative substrates

Biotechnological production of 2, 3-BD has shown great potential due to the diverse feedstock available as substrates. Utilization of alternative substrates is limited by the carbon metabolism of specific strain used. Introduction of certain pathway genes is generally needed to achieve this purpose.

3.6.1 Crude glycerol

Crude glycerol is a by-product of biodiesel production and has been investigated as a substrate for fermentation. Crude glycerol has been used as carbon source for efficient phytase production in *Pichia pastoris* (Tang et al., 2009). Glycerol could be metabolized by a wide range of 2, 3-BD

producing microorganisms. *R. ornithinolytica* B6 was recently cultivated with crude glycerol to produce 79.25 g/L 2, 3-BD (Kim et al., 2017b). It was found that the titer obtained with crude glycerol was 73% that of refined glycerol, which can be attributed to the accumulation of inhibitory impurities in crude glycerol. Pre-treatment of crude glycerol to remove soap and free fatty acids and adjustment of pH was beneficial to prolong the fermentation time and enhance the production yield. Yang *et al*. explored the effect of molasses as co-substrate of crude glycerol on the production of 2, 3-BD (Yang et al., 2013b). It was reported that crude glycerol could support cell growth and 2, 3-BD formation in *B. amyloliquefaciens* equally as purified glycerol, which is in contrast with the results mentioned above. The supplementation of sugars such as glucose, sucrose and molasses showed a positive effect in reducing fermentation duration and enhancing yield. A titer of 83.3 g/L was achieved using molasses as a co-substrate. Ripoll *et al*. described the isolation of two species belonging to *Raoultella* genus for efficient conversion of crude glycerol into 2, 3-BD (Ripoll et al., 2016). It was found that crude glycerol did not compromise the cell growth compared with pure glycerol. *R. planticola* and *R. terrigena* were able to produce 27.5 g/L and 33.6 g/L 2, 3- BD using 90 g/L crude glycerol as substrate, respectively. Metsoviti et al. investigated the cultivation of *K. oxytoca* for the co-production of 1, 3-propanediol, 2, 3-BD and ethanol using crude glycerol of various sources (Metsoviti et al., 2012). It was found that production of 2, 3-BD was boosted through the use of glucose as co-substrate. Highest titer of 2, 3-BD was detected at 8 g/L using 30 g/L glycerol and 10 g/L glucose as substrates.

3.6.2 Lignocellulosic hydrolysate

Utilization of waste product such as sugarcane bagasse for 2, 3-BD production is attracting great attention due to economic considerations. The hydrolysate of sugarcane bagasse mainly consists of glucose and xylose. Carbon catabolite repression (CCR) is the major challenge for mixed-

sugar fermentation due to the preferential utilization of glucose. Simultaneous assimilation of the two sugars derived from sugarcane bagasse hydrolysate by *E. aerogenes* for efficient production of 2, 3-BD was described recently (Um et al., 2017). Sugarcane bagasse was pre-treated to improve the enzymatic digestibility. Gene *ptsG* was deleted to eliminate CCR for co-utilization of xylose and glucose. Combined with overexpression of *gal*P (encoding glucose transporter) and *glk* (encoding glucose kinase) and inactivation of *ldhA* and *pflB*, the resulting strain achieved a 2, 3-BD yield of 0.39 g/g. Sucrose is the major component of sugarcane molasses. The efficient use of sucrose by *E. aerogenes* was limited the regulation of sucrose regulator ScrR (Jung et al., 2015). Inactivation of the regulator led to improved sucrose consumption using sugarcane molasses as carbon source. The mutant strain was reported to produce 98.69 g/L 2, 3-BD in fed-batch cultivation. Disruption of *cra* gene of *E. aerogenes* improved the fructose utilization but led to sucrose accumulation. Plasmid-based expression of scrAB (encoding genes for sucrose catabolism) in the double mutant *E. aerogenes*Δ*cra*Δ*scrR* restored the sucrose utilization rate. The resulting strain was able to produce 140 g/L 2, 3-BD using sugarcane molasses as feedstock in fed-batch fermentation. Pre-treatment of lignocellulosic biomass to remove lignin is a key step before the enzymatic hydrolysis. Effect of chemical treatment of rice waste biomass was studied by (Saratale et al., 2016). The ratio of sodium carbonate and sodium sulfite was optimized to achieve optimal lignin removal efficiency and subsequent hydrolysis yield. The enzymatic hydrolysate of rice waste biomass was used to cultivate *K. pneumoniae* for 2, 3-BD production and showed good performance, highlighting the significance of chemical pre-treatment. A thermophilic *B. licheniformis* strain X10 which was able to co-utilize glucose and xylose without carbon catabolite repression was reported (Li et al., 2014b). Corn stover hydrolysate was used as

substrate to produce 74 g/L 2, 3-BD in fed-batch fermentation. The high tolerance of inhibitors of this strain was also beneficial for its utilization of lignocellulosic hydrolysate.

3.6.3 Other substrates

Jerusalem artichoke tuber (JAT), a rich source of inulin, has been used as a feedstock for 2, 3-BD synthesis. Inulinase activity is essential for efficient utilization of JAT. Inuline was utilized by an engineered *P. polymyxa* strain to produce 51.3 g/L 2, 3-BD. Putative inulinase gene *sacC* was identified in the genome of *Bacillus* sp. BRC1 and overexpression of this gene under a xylose-inducible promoter resulted in increased activity of inulin hydrolysis (Park et al., 2017a). High level production of 2, 3-BD at 28.6 g/L with 92.3% of theoretical yield was achieved in fed-batch cultivation. Mixed sugars derived from empty palm fruit bunches were reported to support high production of 2, 3-BD in *Bacillus* sp. BRC1 (Kang et al., 2015). Apple pomace is a waste of the juice industry. The hydrolysate of apple pomace after thermal pre-treatment and enzymatic saccharification consists of mainly reducing sugars such as glucose and fructose (Bialkowska et al., 2015). The inhibitors in the hydrolysate were below detection level. The viability of using the apple pomace hydrolysate as carbon source for the production of 2, 3-BD in *B. licheniformis* was evaluated (Bialkowska et al., 2015). The titer of 2, 3-BD reached 87.71 g/L in fed-batch fermentation, indicating that this hydrolysate could be a suitable feedstock for economic production of 2, 3-BD. Sweet sorghum is a promising energy crop and its juice contains highly fermentable sugars such as sucrose, glucose and fructose. Recently, a medium using sweet sorghum juice has been developed for the cultivation of *S. marcescens* H30 to obtain a titer of 109.44 g/L (Yuan et al., 2017). The use of waste gas from steel industry as sole carbon and energy source for the production of 2, 3-BD has been recently explored (Kopke et al., 2011). Three autotrophic species belonging to the *Clostridium* genus were found to harbour putative 2,

3-BD pathway genes homologue to those in existing 2, 3-BD producers through genome analysis and gene expression studies (Kopke et al., 2011). Carbon monoxide was assimilated through the Wood-Ljungdahl pathway to form acetyl-CoA, which was further converted to pyruvate. Around 2 mM 2, 3-BD was detected in *C. autoethanogenum* after 200 h incubation. Direct conversion of spirit-based distiller's grain (SDG), a waste produced during wine-brewing process, to 2, 3-BD by *B. amyloliquefaciens* B10-127 was recently investigated (Yang et al., 2015c). SDG was abundant in starch, amino acids and other nutrients. It could be metabolized by this strain without hydrolysis due to its endogenous amylase activity. 2, 3-BD titer of 40.7 g/L was achieved from 400 g/L SDG with a productivity of 0.81g/L/h, suggesting that SDG could be used as a promising raw material for 2, 3-BD production. Other reports using waste product and raw materials for the production of 2, 3-BD were summarized in Table 3-3.

Table 3-3 Production of 2, 3-BD using waste product and raw material

Species	Substrate	Operation mode	2,3-BD titer (g/L)	References
R. ornithinolytica B6	Crude glycerol	Fed-batch	79.25	(Kim et al., 2017b)
R. planticola	Crude glycerol	Batch	27.5	(Ripoll et al., 2016)
R. terrigena	Crude glycerol	Batch	33.6	(Ripoll et al., 2016)
E. coli	Crude glycerol	Batch	6.9	(Yang et al., 2016b)
K. pneumoniae KCTC12133BP	Crude glycerol	Fed-batch	44	(Park et al., 2017b)
K. oxytoca M1	Crude glycerol	Fed-batch	131.5	(Cho et al., 2015a)
B. amyloliquefaciens	Crude glycerol, molasses	Fed-batch	83.3	(Yang et al., 2013b)
B. amyloliquefaciens B10-127	Crude glycerol	Fed-batch	102.3	(Yang et al., 2015d)

B. amyloliquefaciens B10-127	Spirit-distiller's grain	Batch	40.7	(Yang et al., 2015c)
K. oxytoca FMCC-197	Crude glycerol, glucose	Batch	8	(Metsoviti et al., 2012)
K. oxytoca CICC 22912	*Jatropha* hull hydrolysate	Batch	31.41	(Jiang et al., 2012)
K. oxytoca CICC 22912	*Jatropha* hull hydrolysate	Shake flask	33.49	(Jiang et al., 2013)
K. pneumoniae CGMCC 1.9131	Sugarcane bagasse hydrolysate	Shake flask	9	(Zhao et al., 2011)
E. aerogenes	Sugarcane bagasse hydrolysate	Batch	21	(Um et al., 2017)
E. aerogenes	Sugarcane molasses	Fed-batch	140	(Jung et al., 2015)
E. cloacae	Sugarcane molasses	Fed-batch	90.8	(Dai et al., 2015)
E. cloacae	Oil palm front hydrolysate	Batch	7.67	(Hazeena et al., 2016)
B. subtilis	Sugarcane molasses	Batch	NA	(Deshmukh et al., 2016)
K. pneumoniae KMK-05	Rice waste hydrolysate	Shake flask	11.44	(Saratale et al., 2016)
B. licheniformis X10	Corn stover	Fed-batch	74	(Li et al., 2014b)

	hydrolysate			
B. licheniformis ATCC 14580	Inulin	Fed-batch	103	(Li et al., 2014a)
Bacillus sp. BRC1	Empty palm fruit bunches hydrolysate	Batch	18	(Kang et al., 2015)
Bacillus sp. BRC1	Jerusalem artichoke tuber	Fed-batch	28.6	(Park et al., 2017a)
S. marcescens H30	Sweet sorghum juice	Fed-batch	109.44	(Yuan et al., 2017)
P. polymyxa ICGEB2008	Lignocellulosic hydrolysate	Fed-batch	17	(Adlakha and Yazdani, 2015)

NA indicates not available

3.7 Conclusions and prospects

Economic production of commodity chemical 2, 3-BD via microbial fermentation is recognized as a promising approach to alleviate the dependency on the fossil fuel-based chemical production routes. To this end, genetic modifications to debottleneck the 2, 3-BD synthesis pathway were extensively employed. Process engineering strategies were crucial to the scale up of engineered strains to reach industrially-relevant levels. General strategies were presented in Fig. 3-1 which could be used as a simple guideline. Detailed methodology has to be established based on specific requirement due to the diversity of 2, 3-BD producers and substrates. Novel BDHs were invaluable candidates for fine-tuning the pathway for efficient synthesis of 2, 3-BD. Expansion of feedstock range for cost-effective production of 2, 3-BD relies on the engineering of carbon

metabolism in microorganisms. Advances in metabolic engineering tools would certainly facilitate the construction of more robust hosts. More raw materials and waste products will be explored to evaluate their potential as substrates for 2, 3-BD production.

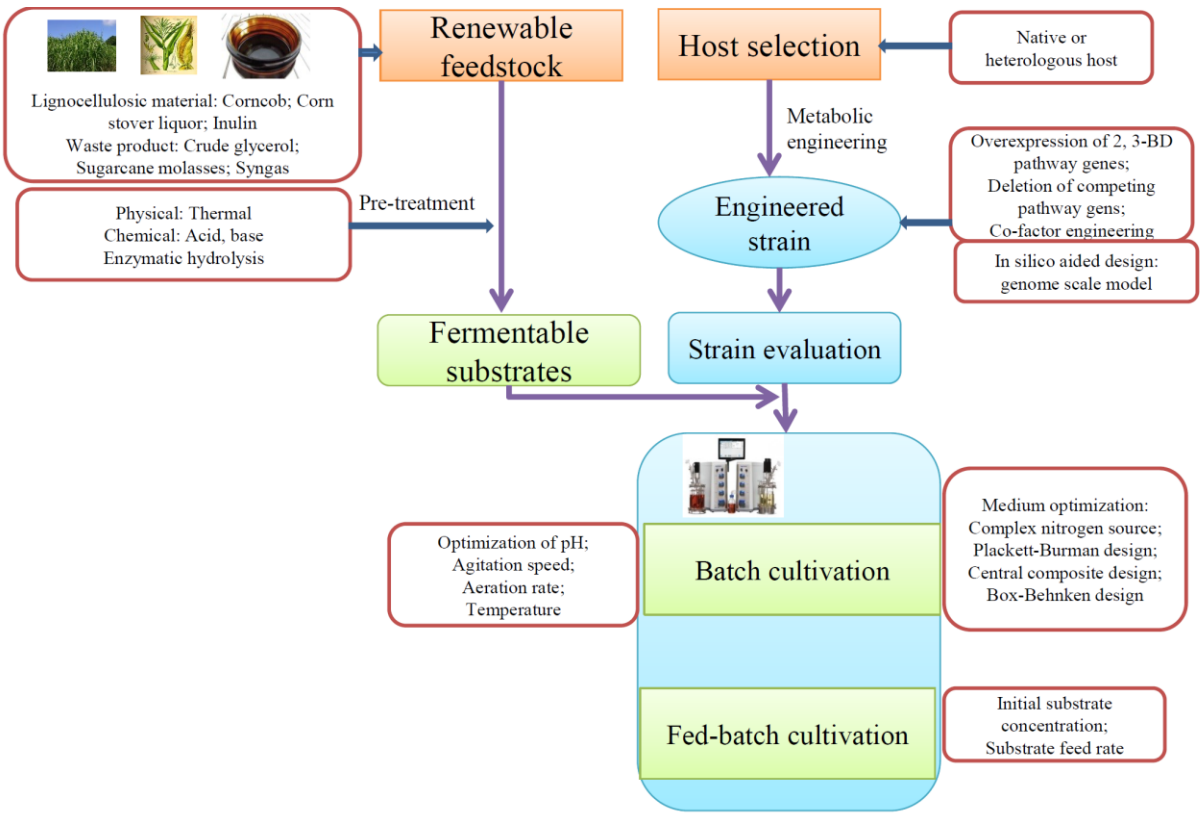

Fig. 3-2 Strategies for enhanced production of 2, 3-BD using renewable feedstocks

3.8 Acknowledgements

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3.9 References

Adlakha N, Yazdani SS. Efficient production of (R, R)-2, 3-butanediol from cellulosic hydrolysate using *Paenibacillus polymyxa* ICGEB2008. Journal of industrial microbiology & biotechnology. 2015;42:21-8.

Bai F, Dai L, Fan J, Truong N, Rao B, Zhang L, et al. Engineered *Serratia marcescens* for efficient (3R)-acetoin and (2R,3R)-2,3-butanediol production. Journal of industrial microbiology & biotechnology. 2015;42:779-86.

Bialkowska AM. Strategies for efficient and economical 2,3-butanediol production: new trends in this field. World journal of microbiology & biotechnology. 2016;32:200.

Bialkowska AM, Gromek E, Krysiak J, Sikora B, Kalinowska H, Jedrzejczak-Krzepkowska M, et al. Application of enzymatic apple pomace hydrolysate to production of 2,3-butanediol by alkaliphilic *Bacillus licheniformis* NCIMB 8059. Journal of industrial microbiology & biotechnology. 2015;42:1609-21.

Calam E, Gonzalez-Roca E, Fernandez MR, Dequin S, Pares X, Virgili A, et al. Enantioselective Synthesis of Vicinal (R,R)-Diols by *Saccharomyces cerevisiae* Butanediol Dehydrogenase. Applied and environmental microbiology. 2016;82:1706-21.

Celinska E, Grajek W. Biotechnological production of 2,3-butanediol--current state and prospects. Biotechnology advances. 2009;27:715-25.

Chan S, Jantama SS, Kanchanatawee S, Jantama K. Process Optimization on Micro-Aeration Supply for High Production Yield of 2,3-Butanediol from Maltodextrin by Metabolically-Engineered *Klebsiella oxytoca*. PloS one. 2016;11:e0161503.

Chen C, Wei D, Shi J, Wang M, Hao J. Mechanism of 2,3-butanediol stereoisomer formation in *Klebsiella pneumoniae*. Applied microbiology and biotechnology. 2014;98:4603-13.

Cheng KK, Wu J, Wang GY, Li WY, Feng J, Zhang JA. Effects of pH and dissolved CO_2 level on simultaneous production of 2,3-butanediol and succinic acid using *Klebsiella pneumoniae*. Bioresource technology. 2013;135:500-3.

Cho S, Kim T, Woo HM, Kim Y, Lee J, Um Y. High production of 2,3-butanediol from biodiesel-derived crude glycerol by metabolically engineered *Klebsiella oxytoca* M1. Biotechnology for biofuels. 2015a;8:146.

Cho S, Kim T, Woo HM, Lee J, Kim Y, Um Y. Enhanced 2,3-Butanediol Production by Optimizing Fermentation Conditions and Engineering *Klebsiella oxytoca* M1 through Overexpression of Acetoin Reductase. PloS one. 2015b;10:e0138109.

Choi M-H, Kim S-J, Kim J-W, Park Y-C, Seo J-H. Molecular cloning and expression of Enterobacter aerogenes α-acetolactate decarboxylase in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae* for efficient 2,3-butanediol production. Process Biochemistry. 2016;51:170-6.

Chu H, Xin B, Liu P, Wang Y, Li L, Liu X, et al. Metabolic engineering of *Escherichia coli* for production of (2S,3S)-butane-2,3-diol from glucose. Biotechnology for biofuels. 2015;8:143.

Dai J-Y, Zhao P, Cheng X-L, Xiu Z-L. Enhanced production of 2, 3-butanediol from sugarcane molasses. Applied biochemistry and biotechnology. 2015;175:3014-24.

de Oliveira RR, Nicholson WL. Synthetic operon for (R,R)-2,3-butanediol production in *Bacillus subtilis* and *Escherichia coli*. Applied microbiology and biotechnology. 2016;100:719-28.

Deshmukh AN, Nipanikar-Gokhale P, Jain R. Engineering of *Bacillus subtilis* for the Production of 2,3-Butanediol from Sugarcane Molasses. Applied biochemistry and biotechnology. 2016;179:321-31.

Fu J, Huo G, Feng L, Mao Y, Wang Z, Ma H, et al. Metabolic engineering of *Bacillus subtilis* for chiral pure meso-2,3-butanediol production. Biotechnology for biofuels. 2016;9:90.

Fu J, Wang Z, Chen T, Liu W, Shi T, Wang G, et al. NADH plays the vital role for chiral pure D-(-)-2,3-butanediol production in *Bacillus subtilis* under limited oxygen conditions. Biotechnology and bioengineering. 2014;111:2126-31.

Ge Y, Li K, Li L, Gao C, Zhang L, Ma C, et al. Contracted but effective: production of enantiopure 2,3-butanediol by thermophilic and GRAS *Bacillus licheniformis*. Green Chem. 2016;18:4693-703.

Gonzalez E, Fernandez MR, Larroy C, Sola L, Pericas MA, Pares X, et al. Characterization of a (2R,3R)-2,3-butanediol dehydrogenase as the *Saccharomyces cerevisiae* YAL060W gene product. Disruption and induction of the gene. The Journal of biological chemistry. 2000;275:35876-85.

Guo X, Cao C, Wang Y, Li C, Wu M, Chen Y, et al. Effect of the inactivation of lactate dehydrogenase, ethanol dehydrogenase, and phosphotransacetylase on 2, 3-butanediol production in *Klebsiella pneumoniae* strain. Biotechnology for biofuels. 2014a;7:44.

Guo XW, Zhang YH, Cao CH, Shen T, Wu MY, Chen YF, et al. Enhanced production of 2,3-butanediol by overexpressing acetolactate synthase and acetoin reductase in *Klebsiella pneumoniae*. Biotechnol Appl Biochem. 2014b;61:707-15.

Hassler T, Schieder D, Pfaller R, Faulstich M, Sieber V. Enhanced fed-batch fermentation of 2,3-butanediol by *Paenibacillus polymyxa* DSM 365. Bioresource technology. 2012;124:237-44.

Hazeena SH, Pandey A, Binod P. Evaluation of oil palm front hydrolysate as a novel substrate for 2,3-butanediol production using a novel isolate *Enterobacter cloacae* SG1. Renewable Energy. 2016;98:216-20.

Jantama K, Polyiam P, Khunnonkwao P, Chan S, Sangproo M, Khor K, et al. Efficient reduction of the formation of by-products and improvement of production yield of 2,3-butanediol by a combined deletion of alcohol dehydrogenase, acetate kinase-phosphotransacetylase, and lactate dehydrogenase genes in metabolically engineered *Klebsiella oxytoca* in mineral salts medium. Metabolic engineering. 2015;30:16-26.

Ji X-J, Huang H, Du J, Zhu J-G, Ren L-J, Hu N, et al. Enhanced 2, 3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. Bioresource technology. 2009;100:3410-4.

Ji X-J, Huang H, Zhu J-G, Ren L-J, Nie Z-K, Du J, et al. Engineering *Klebsiella oxytoca* for efficient 2, 3-butanediol production through insertional inactivation of acetaldehyde dehydrogenase gene. Applied microbiology and biotechnology. 2010;85:1751-8.

Ji X-J, Nie Z-K, Huang H, Ren L-J, Peng C, Ouyang P-K. Elimination of carbon catabolite repression in *Klebsiella oxytoca* for efficient 2, 3-butanediol production from glucose–xylose mixtures. Applied microbiology and biotechnology. 2011a;89:1119-25.

Ji XJ, Huang H, Ouyang PK. Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnology advances. 2011b;29:351-64.

Ji XJ, Liu LG, Shen MQ, Nie ZK, Tong YJ, Huang H. Constructing a synthetic metabolic pathway in *Escherichia coli* to produce the enantiomerically pure (R, R)-2,3-butanediol. Biotechnology and bioengineering. 2015;112:1056-9.

Jiang L-q, Fang Z, Li X-K, Luo J. Production of 2, 3-butanediol from cellulose and Jatropha hulls after ionic liquid pretreatment and dilute-acid hydrolysis. AMB Express. 2013;3:48.

Jiang LQ, Fang Z, Guo F, Yang LB. Production of 2,3-butanediol from acid hydrolysates of Jatropha hulls with *Klebsiella oxytoca*. Bioresource technology. 2012;107:405-10.

Jung M-Y, Mazumdar S, Shin SH, Yang K-S, Lee J, Oh M-K. Improvement of 2, 3-butanediol yield in *Klebsiella pneumoniae* by deletion of the pyruvate formate-lyase gene. Applied and environmental microbiology. 2014;80:6195-203.

Jung MY, Jung HM, Lee J, Oh MK. Alleviation of carbon catabolite repression in *Enterobacter aerogenes* for efficient utilization of sugarcane molasses for 2,3-butanediol production. Biotechnology for biofuels. 2015;8:106.

Jung MY, Ng CY, Song H, Lee J, Oh MK. Deletion of lactate dehydrogenase in *Enterobacter aerogenes* to enhance 2,3-butanediol production. Applied microbiology and biotechnology. 2012;95:461-9.

Jung S-G, Jang J-H, Kim A-Y, Lim M-C, Kim B, Lee J, et al. Removal of pathogenic factors from 2, 3-butanediol-producing *Klebsiella* species by inactivating virulence-related *wabG* gene. Applied microbiology and biotechnology. 2013;97:1997-2007.

Kang IY, Park JM, Hong W-K, Kim YS, Jung YR, Kim S-B, et al. Enhanced production of 2, 3-butanediol by a genetically engineered *Bacillus* sp. BRC1 using a hydrolysate of empty palm fruit bunches. Bioprocess and biosystems engineering. 2015;38:299-305.

Kato H, Matsuda F, Yamada R, Nagata K, Shirai T, Hasunuma T, et al. Cocktail delta-integration of xylose assimilation genes for efficient ethanol production from xylose in *Saccharomyces cerevisiae*. Journal of bioscience and bioengineering. 2013;116:333-6.

Kim B, Lee S, Jeong D, Yang J, Oh MK, Lee J. Redistribution of carbon flux toward 2,3-butanediol production in *Klebsiella pneumoniae* by metabolic engineering. PloS one. 2014a;9:e105322.

Kim B, Lee S, Park J, Lu M, Oh M, Kim Y, et al. Enhanced 2, 3-butanediol production in recombinant *Klebsiella pneumoniae* via overexpression of synthesis-related genes. Journal of microbiology and biotechnology. 2012;22:1258-63.

Kim JW, Kim J, Seo SO, Kim KH, Jin YS, Seo JH. Enhanced production of 2,3-butanediol by engineered *Saccharomyces cerevisiae* through fine-tuning of pyruvate decarboxylase and NADH oxidase activities. Biotechnology for biofuels. 2016a;9:265.

Kim JW, Seo SO, Zhang GC, Jin YS, Seo JH. Expression of *Lactococcus lactis* NADH oxidase increases 2,3-butanediol production in Pdc-deficient Saccharomyces cerevisiae. Bioresource technology. 2015;191:512-9.

Kim S-J, Sim H-J, Kim J-W, Lee Y-G, Park Y-C, Seo J-H. Enhanced production of 2, 3-butanediol from xylose by combinatorial engineering of xylose metabolic pathway and cofactor regeneration in pyruvate decarboxylase-deficient *Saccharomyces cerevisiae*. Bioresource technology. 2017a.

Kim S, Hahn J-S. Synthetic scaffold based on a cohesin–dockerin interaction for improved production of 2, 3-butanediol in *Saccharomyces cerevisiae*. Journal of biotechnology. 2014;192:192-6.

Kim S, Hahn JS. Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing. Metabolic engineering. 2015;31:94-101.

Kim SJ, Seo SO, Jin YS, Seo JH. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. Bioresource technology. 2013;146:274-81.

Kim SJ, Seo SO, Park YC, Jin YS, Seo JH. Production of 2,3-butanediol from xylose by engineered *Saccharomyces cerevisiae*. Journal of biotechnology. 2014b;192 Pt B:376-82.

Kim T, Cho S, Lee SM, Woo HM, Lee J, Um Y, et al. High Production of 2,3-Butanediol (2,3-BD) by *Raoultella ornithinolytica* B6 via Optimizing Fermentation Conditions and Overexpressing 2,3-BD Synthesis Genes. PloS one. 2016b;11:e0165076.

Kim T, Cho S, Woo HM, Lee SM, Lee J, Um Y, et al. High production of 2,3-butanediol from glycerol without 1,3-propanediol formation by *Raoultella ornithinolytica* B6. Applied microbiology and biotechnology. 2017b;101:2821-30.

Kopke M, Gerth ML, Maddock DJ, Mueller AP, Liew F, Simpson SD, et al. Reconstruction of an acetogenic 2,3-butanediol pathway involving a novel NADPH-dependent primary-secondary alcohol dehydrogenase. Applied and environmental microbiology. 2014;80:3394-403.

Kopke M, Mihalcea C, Liew F, Tizard JH, Ali MS, Conolly JJ, et al. 2,3-butanediol production by acetogenic bacteria, an alternative route to chemical synthesis, using industrial waste gas. Applied and environmental microbiology. 2011;77:5467-75.

Lee S, Kim B, Yang J, Jeong D, Park S, Lee J. A non-pathogenic and optically high concentrated (R,R)-2,3-butanediol biosynthesizing *Klebsiella* strain. Journal of biotechnology. 2015;209:7-13.

Li L, Chen C, Li K, Wang Y, Gao C, Ma C, et al. Efficient simultaneous saccharification and fermentation of inulin to 2, 3-butanediol by thermophilic *Bacillus licheniformis* ATCC 14580. Applied and environmental microbiology. 2014a;80:6458-64.

Li L, Li K, Wang K, Chen C, Gao C, Ma C, et al. Efficient production of 2,3-butanediol from corn stover hydrolysate by using a thermophilic *Bacillus licheniformis* strain. Bioresource technology. 2014b;170:256-61.

Li L, Li K, Wang Y, Chen C, Xu Y, Zhang L, et al. Metabolic engineering of *Enterobacter cloacae* for high-yield production of enantiopure (2R,3R)-2,3-butanediol from lignocellulose-derived sugars. Metabolic engineering. 2015;28:19-27.

Li L, Zhang L, Li K, Wang Y, Gao C, Han B, et al. A newly isolated *Bacillus licheniformis* strain thermophilically produces 2, 3-butanediol, a platform and fuel bio-chemical. Biotechnology for biofuels. 2013;6:123.

Li Z-J, Jian J, Wei X-X, Shen X-W, Chen G-Q. Microbial production of meso-2, 3-butanediol by metabolically engineered *Escherichia coli* under low oxygen condition. Applied microbiology and biotechnology. 2010;87:2001-9.

Lian J, Chao R, Zhao H. Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (2R,3R)-butanediol. Metabolic engineering. 2014;23:92-9.

Liu J, Chan SH, Brock-Nannestad T, Chen J, Lee SY, Solem C, et al. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. Metabolic engineering. 2016;36:57-67.

Ma C, Wang A, Qin J, Li L, Ai X, Jiang T, et al. Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Applied microbiology and biotechnology. 2009;82:49-57.

Metsoviti M, Paraskevaidi K, Koutinas A, Zeng A-P, Papanikolaou S. Production of 1,3-propanediol, 2,3-butanediol and ethanol by a newly isolated *Klebsiella oxytoca* strain growing on biodiesel-derived glycerol based media. Process Biochemistry. 2012;47:1872-82.

Nakashima N, Akita H, Hoshino T. Establishment of a novel gene expression method, BICES (biomass-inducible chromosome-based expression system), and its application to the production of 2,3-butanediol and acetoin. Metabolic engineering. 2014;25:204-14.

Nan H, Seo SO, Oh EJ, Seo JH, Cate JH, Jin YS. 2,3-butanediol production from cellobiose by engineered *Saccharomyces cerevisiae*. Applied microbiology and biotechnology. 2014;98:5757-64.

Ng C, Jung M-y, Lee J, Oh M-K. Production of 2, 3-butanediol in *Saccharomyces cerevisiae* by in silico aided metabolic engineering. Microbial cell factories. 2012;11:68.

Nielsen DR, Yoon SH, Yuan CJ, Prather KL. Metabolic engineering of acetoin and meso-2, 3-butanediol biosynthesis in *E. coli*. Biotechnology journal. 2010;5:274-84.

Oliver JW, Machado IM, Yoneda H, Atsumi S. Cyanobacterial conversion of carbon dioxide to 2, 3-butanediol. Proceedings of the National Academy of Sciences. 2013;110:1249-54.

Oliver JW, Machado IM, Yoneda H, Atsumi S. Combinatorial optimization of cyanobacterial 2,3-butanediol production. Metabolic engineering. 2014;22:76-82.

Park JM, Oh BR, Kang IY, Heo SY, Seo JW, Park SM, et al. Enhancement of 2,3-butanediol production from Jerusalem artichoke tuber extract by a recombinant *Bacillus* sp. strain BRC1 with increased inulinase activity. Journal of industrial microbiology & biotechnology. 2017a;44:1107-13.

Park JM, Rathnasingh C, Song H. Enhanced production of (R, R)-2, 3-butanediol by metabolically engineered *Klebsiella oxytoca*. Journal of industrial microbiology & biotechnology. 2015;42:1419-25.

Park JM, Rathnasingh C, Song H. Metabolic engineering of *Klebsiella pneumoniae* based on *in silico* analysis and its pilot-scale application for 1,3-propanediol and 2,3-butanediol co-production. Journal of industrial microbiology & biotechnology. 2017b;44:431-41.

Park JM, Song H, Lee HJ, Seung D. Genome-scale reconstruction and in silico analysis of *Klebsiella oxytoca* for 2, 3-butanediol production. Microbial cell factories. 2013a;12:20.

Park JM, Song H, Lee HJ, Seung D. In silico aided metabolic engineering of *Klebsiella oxytoca* and fermentation optimization for enhanced 2,3-butanediol production. Journal of industrial microbiology & biotechnology. 2013b;40:1057-66.

Petrov K, Petrova P. Enhanced production of 2,3-butanediol from glycerol by forced pH fluctuations. Applied microbiology and biotechnology. 2010;87:943-9.

Priya A, Dureja P, Talukdar P, Rathi R, Lal B, Sarma PM. Microbial production of 2,3-butanediol through a two-stage pH and agitation strategy in 150 l bioreactor. Biochemical Engineering Journal. 2016;105:159-67.

Qi G, Kang Y, Li L, Xiao A, Zhang S, Wen Z, et al. Deletion of meso-2, 3-butanediol dehydrogenase gene *budC* for enhanced D-2, 3-butanediol production in *Bacillus licheniformis*. Biotechnology for biofuels. 2014;7:16.

Qiu Y, Zhang J, Li L, Wen Z, Nomura CT, Wu S, et al. Engineering *Bacillus licheniformis* for the production of meso-2,3-butanediol. Biotechnology for biofuels. 2016;9:117.

Rados D, Carvalho AL, Wieschalka S, Neves AR, Blombach B, Eikmanns BJ, et al. Engineering *Corynebacterium glutamicum* for the production of 2,3-butanediol. Microbial cell factories. 2015;14:171.

Radoš D, Turner DL, Catarino T, Hoffart E, Neves AR, Eikmanns BJ, et al. Stereospecificity of *Corynebacterium glutamicum* 2, 3-butanediol dehydrogenase and implications for the stereochemical purity of bioproduced 2, 3-butanediol. Applied microbiology and biotechnology. 2016;100:10573-83.

Rao B, Zhang LY, Sun J, Su G, Wei D, Chu J, et al. Characterization and regulation of the 2,3-butanediol pathway in *Serratia marcescens*. Applied microbiology and biotechnology. 2012;93:2147-59.

Reshamwala SM, Deb SS, Lali AM. A shortened, two-enzyme pathway for 2, 3-butanediol production in *Escherichia coli*. Journal of industrial microbiology & biotechnology. 2017:1-5.

Ripoll V, de Vicente G, Morán B, Rojas A, Segarra S, Montesinos A, et al. Novel biocatalysts for glycerol conversion into 2,3-butanediol. Process Biochemistry. 2016;51:740-8.

Saratale GD, Jung MY, Oh MK. Reutilization of green liquor chemicals for pretreatment of whole rice waste biomass and its application to 2,3-butanediol production. Bioresource technology. 2016;205:90-6.

Savakis P, Hellingwerf KJ. Engineering cyanobacteria for direct biofuel production from CO2. Current opinion in biotechnology. 2015;33:8-14.

Savakis PE, Angermayr SA, Hellingwerf KJ. Synthesis of 2,3-butanediol by *Synechocystis* sp. PCC6803 via heterologous expression of a catabolic pathway from lactic acid- and enterobacteria. Metabolic engineering. 2013;20:121-30.

Shen X, Lin Y, Jain R, Yuan Q, Yan Y. Inhibition of acetate accumulation leads to enhanced production of (R, R)-2, 3-butanediol from glycerol in *Escherichia coli*. Journal of industrial microbiology & biotechnology. 2012;39:1725-9.

Siemerink MA, Kuit W, Lopez Contreras AM, Eggink G, van der Oost J, Kengen SW. D-2,3-butanediol production due to heterologous expression of an acetoin reductase in *Clostridium acetobutylicum*. Applied and environmental microbiology. 2011;77:2582-8.

Takeda M, Muranushi T, Inagaki S, Nakao T, Motomatsu S, Suzuki I, et al. Identification and characterization of a mycobacterial (2R, 3R)-2, 3-butanediol dehydrogenase. Bioscience, biotechnology, and biochemistry. 2011;75:2384-9.

Tan Y, Liu ZY, Liu Z, Li FL. Characterization of an acetoin reductase/2,3-butanediol dehydrogenase from *Clostridium ljungdahlii* DSM 13528. Enzyme Microb Technol. 2015;79-80:1-7.

Tang S, Boehme L, Lam H, Zhang Z. *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochemical Engineering Journal. 2009;43:157-62.

Tanimura K, Takashima S, Matsumoto T, Tanaka T, Kondo A. 2,3-Butanediol production from cellobiose using exogenous beta-glucosidase-expressing *Bacillus subtilis*. Applied microbiology and biotechnology. 2016;100:5781-9.

Tong YJ, Ji XJ, Shen MQ, Liu LG, Nie ZK, Huang H. Constructing a synthetic constitutive metabolic pathway in *Escherichia coli* for (R, R)-2,3-butanediol production. Applied microbiology and biotechnology. 2016;100:637-47.

Tsvetanova F, Petrova P, Petrov K. 2, 3-butanediol production from starch by engineered *Klebsiella pneumoniae* G31-A. Applied microbiology and biotechnology. 2014;98:2441-51.

Um J, Kim DG, Jung MY, Saratale GD, Oh MK. Metabolic engineering of *Enterobacter aerogenes* for 2,3-butanediol production from sugarcane bagasse hydrolysate. Bioresource technology. 2017.

Wang Q, Chen T, Zhao X, Chamu J. Metabolic engineering of thermophilic *Bacillus licheniformis* for chiral pure D-2,3-butanediol production. Biotechnology and bioengineering. 2012;109:1610-21.

Wang Y, Tao F, Xu P. Glycerol dehydrogenase plays a dual role in glycerol metabolism and 2, 3-butanediol formation in *Klebsiella pneumoniae*. Journal of Biological Chemistry. 2014;289:6080-90.

Wong CL, Yen HW, Lin CL, Chang JS. Effects of pH and fermentation strategies on 2,3-butanediol production with an isolated *Klebsiella* sp. Zmd30 strain. Bioresource technology. 2014;152:169-76.

Xu GC, Bian YQ, Han RZ, Dong JJ, Ni Y. Cloning, Expression, and Characterization of *budC* Gene Encoding meso-2,3-Butanediol Dehydrogenase from *Bacillus licheniformis*. Applied biochemistry and biotechnology. 2016;178:604-17.

Xu Y, Chu H, Gao C, Tao F, Zhou Z, Li K, et al. Systematic metabolic engineering of *Escherichia coli* for high-yield production of fuel bio-chemical 2,3-butanediol. Metabolic engineering. 2014;23:22-33.

Yamada R, Wakita K, Mitsui R, Nishikawa R, Ogino H. Efficient production of 2,3-butanediol by recombinant *Saccharomyces cerevisiae* through modulation of gene expression by cocktail delta-integration. Bioresource technology. 2017.

Yang J, Kim B, Kim H, Kweon Y, Lee S, Lee J. Industrial Production of 2,3-Butanediol from the Engineered *Corynebacterium glutamicum*. Applied biochemistry and biotechnology. 2015a;176:2303-13.

Yang S, Fei Q, Zhang Y, Contreras LM, Utturkar SM, Brown SD, et al. *Zymomonas mobilis* as a model system for production of biofuels and biochemicals. Microbial biotechnology. 2016a;9:699-717.

Yang S, Mohagheghi A, Franden MA, Chou Y-C, Chen X, Dowe N, et al. Metabolic engineering of *Zymomonas mobilis* for 2, 3-butanediol production from lignocellulosic biomass sugars. Biotechnology for biofuels. 2016b;9:189.

Yang S, Mohagheghi A, Franden MA, Chou YC, Chen X, Dowe N, et al. Metabolic engineering of *Zymomonas mobilis* for 2,3-butanediol production from lignocellulosic biomass sugars. Biotechnology for biofuels. 2016c;9:189.

Yang T, Rao Z, Hu G, Zhang X, Liu M, Dai Y, et al. Metabolic engineering of *Bacillus subtilis* for redistributing the carbon flux to 2,3-butanediol by manipulating NADH levels. Biotechnology for biofuels. 2015b;8:129.

Yang T, Rao Z, Zhang X, Xu M, Xu Z, Yang S-T. Economic conversion of spirit-based distillers' grain to 2,3-butanediol by *Bacillus amyloliquefaciens*. Process Biochemistry. 2015c;50:20-3.

Yang T, Rao Z, Zhang X, Xu M, Xu Z, Yang ST. Improved production of 2,3-butanediol in *Bacillus amyloliquefaciens* by over-expression of glyceraldehyde-3-phosphate dehydrogenase and 2,3-butanediol dehydrogenase. PloS one. 2013a;8:e76149.

Yang T, Rao Z, Zhang X, Xu M, Xu Z, Yang ST. Enhanced 2,3-butanediol production from biodiesel-derived glycerol by engineering of cofactor regeneration and manipulating carbon flux in *Bacillus amyloliquefaciens*. Microbial cell factories. 2015d;14:122.

Yang T, Zhang X, Rao Z, Gu S, Xia H, Xu Z. Optimization and scale-up of 2,3-butanediol production by *Bacillus amyloliquefaciens* B10-127. World journal of microbiology & biotechnology. 2012;28:1563-74.

Yang T, Rao Z, Zhang X, Xu M, Xu Z, Yang ST. Metabolic engineering strategies for acetoin and 2,3-butanediol production: advances and prospects. Critical reviews in biotechnology. 2017:1-16.

Yang TW, Rao ZM, Zhang X, Xu MJ, Xu ZH, Yang ST. Fermentation of biodiesel-derived glycerol by *Bacillus amyloliquefaciens*: effects of co-substrates on 2,3-butanediol production. Applied microbiology and biotechnology. 2013b;97:7651-8.

Yen HW, Li FT, Chang JS. The effects of dissolved oxygen level on the distribution of 1,3-propanediol and 2,3-butanediol produced from glycerol by an isolated indigenous *Klebsiella* sp. Ana-WS5. Bioresource technology. 2014;153:374-8.

Yu B, Sun J, Bommareddy RR, Song L, Zeng A-P. Novel (2R, 3R)-2, 3-butanediol dehydrogenase from potential industrial strain *Paenibacillus polymyxa* ATCC 12321. Applied and environmental microbiology. 2011;77:4230-3.

Yu M, Huang M, Song Q, Shao J, Ying X. Characterization of a (2R,3R)-2,3-Butanediol Dehydrogenase from *Rhodococcus erythropolis* WZ010. Molecules. 2015;20:7156-73.

Yuan J, He Y-Z, Guo Z-W, Gao H-F, Chen F-B, Li L-Z, et al. Utilization of Sweet Sorghum Juice for Efficient 2, 3-Butanediol Production by *Serratia marcescens* H30. BioResources. 2017;12:4926-42.

Zhang GL, Wang CW, Li C. Cloning, expression and characterization of meso-2,3-butanediol dehydrogenase from *Klebsiella pneumoniae*. Biotechnology letters. 2012;34:1519-23.

Zhang L, Guo Z, Chen J, Xu Q, Lin H, Hu K, et al. Mechanism of 2,3-butanediol stereoisomers formation in a newly isolated *Serratia* sp. T241. Scientific reports. 2016;6:19257.

Zhang L, Xu Q, Peng X, Xu B, Wu Y, Yang Y, et al. Cloning, expression and characterization of glycerol dehydrogenase involved in 2,3-butanediol formation in *Serratia marcescens* H30. Journal of industrial microbiology & biotechnology. 2014a;41:1319-27.

Zhang L, Xu Q, Zhan S, Li Y, Lin H, Sun S, et al. A new NAD(H)-dependent meso-2, 3-butanediol dehydrogenase from an industrially potential strain *Serratia marcescens* H30. Applied microbiology and biotechnology. 2014b;98:1175-84.

Zhang L, Yang Y, Sun Ja, Shen Y, Wei D, Zhu J, et al. Microbial production of 2, 3-butanediol by a mutagenized strain of *Serratia marcescens* H30. Bioresource technology. 2010;101:1961-7.

Zhao X, Song Y, Liu D. Enzymatic hydrolysis and simultaneous saccharification and fermentation of alkali/peracetic acid-pretreated sugarcane bagasse for ethanol and 2,3-butanediol production. Enzyme Microb Technol. 2011;49:413-9.

Zhao X, Zhang X, Rao Z, Bao T, Li X, Xu M, et al. Identification and characterization of a novel 2,3-butanediol dehydrogenase/acetoin reductase from *Corynebacterium crenatum* SYPA5-5. Lett Appl Microbiol. 2015;61:573-9.

Chapter 4: Codon-optimized expression and characterization of a pH stable fungal xylanase in *Pichia pastoris*

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4.1 Abstract

Novel xylanase (EC 3.2.1.8) is in great demand due to its industrial significance. In this study, we have developed and characterized a novel xylanase-producing yeast strain. This mature xylanase gene *xyn*11A consists of 870 base pairs and belongs to GH11 family. The gene sequence was optimized and synthesized, and was then cloned into yeast vector pGAPZαA under the control of the constitutive GAP promoter. SDS-PAGE analysis indicates that Xyn11A is extracellularly expressed as a glycosylated protein in *P. pastoris*. Xyn11A is optimally active at 70 °C and pH 7.4. This xylanase retained more than 90% of its activity after incubation at 50 °C and 60 °C for up to 1 hour. Xyn11A is also stable over a wide range of pH (2.0-11.0). Most metal ions tested such as copper (Cu^{2+}) and lead (Pb^{2+}) have little inhibitory effects on Xyn11A. It is also resistant to pepsin and proteinase K digestion, retaining 80% and 90% of its activity after digestion at 37 °C for 1 hour, respectively. Those superior properties make Xyn11A a robust xylanase with great potential for industrial use. To the best of our knowledge, this is the first report of xylanase from the fungus *Corynascus thermophilus*.

Keywords: Fungal xylanase; Codon optimization; *Pichia pastoris*; High cell density fermentation

4.2 Introduction

Depleting fossil fuels and growing concerns on climate change has sparked great interest in biofuels such as bioethanol. Xylan is the major component of hemicellulose and represents the second most abundant polysaccharide on the planet. The efficient enzymatic degradation of xylan requires the synergistic action of several key enzymes such as xylanase (EC 3.2.1.8) and xylosidase (EC 3.2.1.37) (Van Dyk and Pletschke, 2012). Xylanase can catalyze the hydrolysis of β-1, 4-xylosidic bond and randomly break down xylan into xylo-oligosaccharides. Xylanase is also an important industrial enzyme and has enormous potential applications in food, animal feed additive, pharmaceutical, textile, paper and pulp industry (Juturu and Wu, 2012). Xylanase has been mainly classified into families 5, 7, 8, 10, 11 and 43 based on the amino acid composition and hydrophobic cluster analysis in the catalytic domain (Collins et al., 2005). GH11 family xylanases are characterized by their relatively low molecular weight and single domain. GH11 family xylanase has been extensively investigated due to their high substrate specificity (Paes et al., 2012). Xylanases from various microorganisms including bacteria, yeast and fungus have been identified and characterized (Polizeli et al., 2005). Thermophilic fungi have been an important reservoir for the identification and characterization of novel thermostable xylanases. Thermophilic xylanase has shown robust properties at elevated temperatures and attracted great industrial interest. Genome sequencing of thermophilic fungi has rendered it possible for the discovery of novel xylanase through gene annotation. Thermal and pH stable xylanases has been reported and widely used in the pulp industry (Bajpai, 1999). However, broad pH stable xylanase has been relatively less reported. Identification of novel thermal and pH stable xylanase is gaining great interest for paper and biobleaching industry.

Pichia pastoris has become an important workhorse for heterologous expression of proteins (Macauley-Patrick et al., 2005). Recently, it has been widely employed as the expression host for numerous lignocellulosic extremozymes (Ergun and Calik, 2016). As a eukaryotic platform, it offers many advantages in terms of high yield, high secretion efficiency and post-translational modification such as glycosylation, a distinctive advantage for the expression of glycoproteins. Two types of promoters are commonly used to drive the heterologous protein expression in *P. pastoris*: the methanol-inducible alcohol oxidase 1 (AOX1) and the constitutive glyceraldehyde 3-phosphate dehydrogenase (GAP) promoters. High cell density fermentation (HCDF) strategies for *P. pastoris* have been well established. High protein yield can be achieved in the methanol-inducible system through HCDF. However, accumulation of excessive methanol is detrimental to cell growth thus complicating the process control. GAP promoter avoids the use of flammable methanol without compromising the protein yield and thus has been widely used in recent years. Despite *P. pastoris* is regarded as an important industrial host for protein expression, codon usage of the heterologous genes is often biased from the host. Codon optimization is imperative to achieve high level expression. In this study, an endo-1, 4-β-xylanase from a thermophilic fungus *C. thermophilus* was codon-optimized and expressed in *P. pastoris* for the first time to our knowledge. Biochemical characterization of this enzyme was performed. Effects of temperature, pH and metal ions on enzyme activity were also investigated. This study provided guidance for the expression of other xylanases from this thermophilic fungus.

4.3 Materials and methods

4.3.1 Strains and media

Escherichia coli XL1-Blue (Agilent technologies) was used for plasmid construction and propagation. *P. pastoris* X33 was used as a host for protein expression. Restriction enzymes

were purchased from Thermo scientific. Low salt LB broth consisting of 1% peptone, 0.5% yeast extract and 0.5% sodium chloride was used for *E. coli* cultivation with 25 µg/mL of zeocin (Invitrogen, USA). YPD medium (10 g/L yeast extract, 20 g/L bactopeptone and 20 g/L dextrose) was used for yeast cultivation and shake flask fermentation. YPDS (10 g/L yeast extract, 20 g/L bactopeptone and 20 g/L dextrose, 184 g/L sorbitol) plates with 100 µg/mL of zeocin were used for yeast transformants screening.

4.3.2 Sequence analysis

Signal peptide was predicted by SignalP 4.1 server (Petersen et al., 2011). The molecular mass of the protein was calculated by Compute pI/MW tool (http://web.expasy.org/compute_pi/). BLASTP was used to search homology in Genbank. Sequence alignment of xylanases was performed using ClustalW and phylogenetic tree was constructed by MEGA6.0 using the neighbour-joining tree method. Putative glycosylation site was predicted using a web-based tool NetNglyc1.0 (http://www.cbs.dtu.dk/services/NetNGlyc/).

4.3.3 Codon-optimization and gene synthesis of xyn11A

Sequence of xylanase gene *xyn*11A from *C. thermophilus* was retrieved from http://fungalgenomics.ca/wiki/Fungal_Genomes. Mature coding sequence without the intron was codon-optimized and the optimized sequence was synthesized (Genscript, USA) and inserted into cloning vector pUC57, resulting in plasmid pUC57-*xyn*11A. Primers were ordered from Invitrogen.

4.3.4. Construction of recombinant plasmids

Truncated xyn11A excluding the native signal peptide was amplified from pUC57-*xyn*11A using two primers xyn11F: 5'-CCGGAATTCACACCTACCCAGAATGGAG-3' and xyn11R: 5'-

TGCTCTAGATTACAAGCACTGTGAATACCAATCG-3' by polymerase chain reaction (PCR). Restriction sites *EcoR* I and *Xba* I (underlined) were added to the forward and reverse primers, respectively. PCR was performed as follows: one cycle at 95°C for 5 min, 34 cycles at 98 °C for 20 s, 61 °C for 15 s, 72°C for 1 min. PCR product of *xyn*11A and pGAPZαA (Invitrogen, USA) were digested by *EcoR* I and *Xba* I and gel-purified using Qiaquick Gel Purification Kit (QIAGEN, USA). Ligation mixture was transformed into *E. coli* XL1-Blue competent cells using heat shock method. Transformants were selected on low salt LB plates with zeocin (25 μg/mL). Plasmid construction was verified by double digestion and sequencing.

4.3.5 Transformation of yeast and screen of transformants

Recombinant plasmid pGAPZαA-*xyn*11A (5 μg) was linearized with *BspH* I and transformed into competent *P. pastoris* X33 cells using an electroporator (Eppendorf, Germany) according to the manufacturer′s instructions. Yeast transformants were plated on YPDS agar plates with zeocin (100 μg/mL) and incubated at 30 °C for 3 days. Genomic DNA of positive transformants was extracted using a Wizard DNA kit (Promega, USA). PCR was performed to verify the integration of expression cassette using the genomic DNA as templates.

4.3.6 Protein expression in shake flask and purification

Single yeast colony was picked up from YPD plates and grown in 10 mL YPD medium at 30 °C overnight. 0.5 mL yeast culture was inoculated into 50 mL YPD medium in a 250 mL shake flask and grown for 48 hours. Supernatant of yeast culture was collected by centrifugation at 13000 rpm for 5 minutes. The supernatant was heated at 60 °C for 1 h and centrifuged at 13000 rpm for 20 min to remove the precipitate. Supernatant was then concentrated using an Amicon ultra filter (Millipore) with 30K molecular weight cut-off. Protein concentration was measured by the protein assay (Bio-rad) using bovine serine albumin as the protein standard.

4.3.7 Deglycosylation and SDS-PAGE

Purified Xyn11A was treated with Endo H (New England Biolabs, USA) at 37 °C for 1 hour according to the manufacturer′s instructions. Reaction mixture was analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Untreated enzyme and Endo H were used as control. Protein samples were boiled with 5X loading buffer and loaded on a precast 4-12% SDS-PAGE gel (Bio-rad, USA) and run at 80 volts for 80 minutes. The protein bands were visualized by Coomassie Brilliant Blue-R250 (Bio-Rad, USA) staining. SDS-PAGE image was captured by a gel imager (Alpha Innotech, USA).

4.3.8 Zymography

Zymography was performed according to the protocol described by Royer *et al.* (Royer and Nakas, 1990) with slight modifications. Native Xyn11A was first loaded onto SDS-PAGE gel and run at 80 volts for 90 minutes. The agarose gel was made by dissolving 0.5% xylan and 1% agarose in 0.05M citrate buffer. The agarose gel containing xylan was solidified and used as substrate for zymogram. After the SDS-PAGE gel run was finished, the gel was washed with DD water and then overlaid onto the agarose gel containing xylan. The gel sandwich was incubated at 50 °C and then the gels were separated. The SDS-PAGE gel was visualized by comassie staining. The agarose gel was stained in 1 mg/mL congo red solution for 15 min and destained in 1 M sodium chloride solution. Images of both gels were taken separately using the gel imager.

4.3.9 Enzyme activity assay

Xylanase activity was assayed according to the 3, 5-dinitrosalicyclic acid (DNS) method described by Bailey *et al*. (Bailey et al., 1992). The modified DNS reagent described by Miller *et al*. (Miller, 1959) consisting of per liter: 182 g potassium sodium tartrate, 2 g phenol, 10 g

sodium hydroxide , 10 g DNS and 0.5 g sodium sulfite was used. Briefly, 0.5% of birchwood xylan dissolved in 0.05M citrate buffer was used as substrate. The standard reaction mixture containing 200 µL diluted enzyme and 1.8 mL substrate was incubated at 50 °C and pH 6.0. Reaction was stopped by adding 3 mL DNS reagent to the mixture and boiled for 15 minutes then cooled. Absorbance at 540 nm was measured. Pure xylose (Sigma, USA) was used as standard of reducing sugar. One unit of xylanase activity was defined as the amount of enzyme that produced 1 µmol of xylose in 1 minute. All activity assays were performed in triplicate.

4.3.10 Biochemical characterization of the recombinant xylanase

4.3.10.1. Effect of pH and pH stability

To determine optimal pH, enzyme assay was carried out at 70 °C in buffer with pH ranging from 3.0 to 10.0. Various pH buffers were used: 0.05M citrate-sodium citrate buffer for pH 3.0-6.0, 0.05M Tris-HCl buffer for pH 7.0-10.0 and 0.05M Na_2HPO_4-NaOH buffer for pH 11.0-12.0. For pH stability determination, 20 µL of enzyme solution was incubated in buffer with pH ranging 2.0-12.0 at 4 °C for 24 hours. Residual activity was measured under standard assay conditions.

4.3.10.2. Effect of temperature and thermal stability

To investigate optimal temperature, enzyme activity was determined with temperature ranging 30 °C to 90 °C at pH 7.4. Thermal stability was measured at pH7.4 by incubating enzyme at 50 °C, 60 °C and 70°C in citrate buffer for 60 minutes. Aliquots were taken at interval of 10 minutes. Residual activity was measured under standard condition.

4.3.10.3. Effect of metal ions and other chemicals and proteinase digestion

Various metal ions (KCl, $CaCl_2$, LiCl, $CoCl_2$, $Cr(NO_3)_3$, $Ni(NO_3)_2$,$CuSO_4$, $MgSO_4$, $FeCl_3$, $MnSO_4$, $ZnSO_4$, $Pb(CH_3COO)_2$) or chemical reagents (SDS, EDTA, β-mercaptoethanol) were

added to the assay system in 5 mM final concentration. Residual activity was determined under standard condition. To test the resistance of enzyme to proteinase digestion, 20 μL enzyme was incubated with 10 μg/mL pepsin (pH 2.0) or proteinase K (pH 6.9) at 37 °C for 1 hour. Residual activity was measured using the standard assay.

4.3.10.4. Substrate specificity and kinetic parameters of Xyn11A

Birchwood xylan, beechwood xylan and carboxyl methyl cellulose sodium (CMC-Na) were purchased from sigma and used as substrate to test the specificity of Xyn11A. Enzyme activity was assayed using standard assay. The K_m and V_{max} of Xyn11A was determined using 1-10 mg/mL birchwood xylan as substrate. The kinetic parameters were estimated by Lineweaver-Burk plots. Experiments were done in triplicate.

4.3.11. High cell density fermentation

Fed-batch fermentation was performed in a 5 L Bioflo 320 bioreactor (Eppendorf, Canada). Basal salts media (BSM) consisting of per liter: 42.9 g KH_2PO_4, 5.17 g $(NH_4)_2SO_4$, 14.33 g K_2SO_4, 0.6 g $CaSO_4$ $2H_2O$, 5.71 g $MgSO_4$ $7H_2O$, 0.5 mL H_2SO_4, 4mL PTM1 trace salts were used for cultivation. Reactors contained 3 L of BSM media and 10 g/L glycerol at the beginning of each run. Inoculum was prepared by growing a single yeast colony in 300 mL YPD medium overnight. Feed solution contained 400 g/L glycerol and 4 ml/L PTM1 trace salts solution. Step-wise feeding strategy was used to add the feeding solution to the reactor when the initial glycerol was depleted which was indicated by a sharp increase of dissolved oxygen. Reactors were kept at 30 °C and sparged with 4-6 L/min air to maintain the dissolved oxygen level of 20%. Agitation and air flow rate were cascaded to dissolved oxygen. pH was regulated to 5.0 by adding 30% ammonium hydroxide. Aliquots were taken at different time intervals for optical density measurements and enzyme activity assay.

4.4 Results

4.4.1 Sequence analysis and codon-optimization

The whole genome of *Corynascus thermophilus* CBS 405.69 has been recently sequenced (http://fungalgenomics.ca/wiki/Fungal_Genomes). A xylanase gene xyn11A was obtained from the annotated genome sequence of Corynasucus thermophilus. The mature xyn11A gene sequence consists of 870 bp, encoding 289 amino acids. A putative signal peptide was located in the first 19 amino acids. The calculated molecular weight of Xyn11A was 30.5 KD. The isoelectric point (pI) was predicted to be pH 5.75. Sequence similarity search was performed using BLASTp. A neighbour-joining tree was constructed using clustalW and MEGA 6.0 (Fig. 4-1). Xyn11A shares 81% and 71% of amino acid similarity with a putative xylanase from *Myceliophthora thermophila* ATCC 42464 which has been whole-genome sequenced recently (Berka et al., 2011) and XynC81 from *Achaetomium* sp. Xz-8 (Zhao et al., 2013).

Codon optimization can improve the expression level of recombinant proteins in *P. pastoris* (Hu et al., 2013). It has proved an effective strategy for the expression of lignocellulolytic enzymes in *P. pastoris* (Mellitzer et al., 2012). High level expression of xylanase from *Streptomyces* sp. S38 and hyperthermostable xylanase from *Thermotoga maritima* was achieved through codon-optimization (Fu et al., 2011, Jia et al., 2012). In this study, the xyn11A sequence was optimized to the codon usage of *P. pastoris*. Codon adaption index (CAI) was increased from 0.53 to 0.85 and GC content was significantly lowered to 45.74%, which is closer to that of the *P. pastoris* genome (41%).

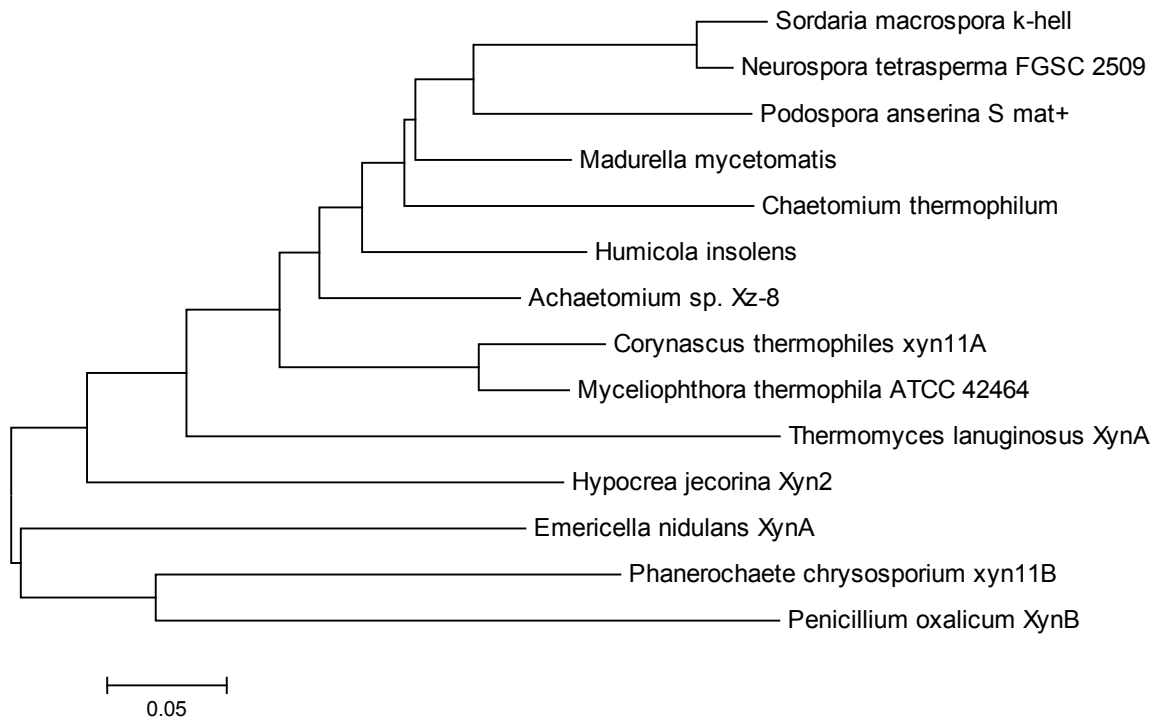
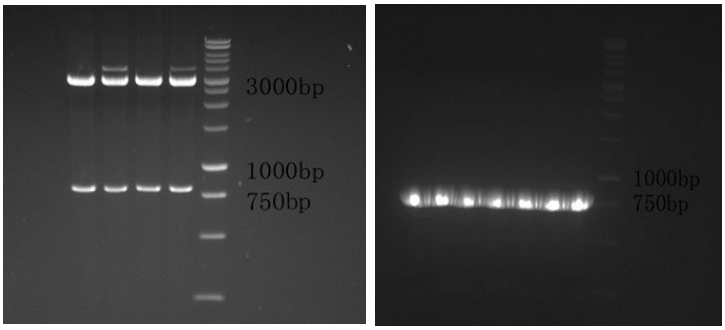


Fig. 4-1 Phylogenetic tree of Xyn11A with its homologs. Amino acids of xylanases were obtained from NCBI. Accession numbers of the xylanases were: B7SIW1.1, BAA08650.1, E7EF85.1, CAD48749.1, XP_661217.1, CAA49293.1, O43097.1, XP_003662402.1, AHE13929.1, XP_001903201.1, KXX79513.1, AJF98581.1, XP_003344557.1 and EGZ75942.1. Scale bar indicates number of amino acid substitutions per site.

4.4.2 Construction of expression plasmids and screening of yeast transformants

The codon-optimized gene of *xyn11A* was amplified using pUC57-*xyn11A* as template. Four colonies were picked up and plasmids were analyzed by double digestion with *Eco*R I and *Xba* I. Insertion of *xyn11A* into pGAPZαA was confirmed through the double digestion by *Eco*R I and *Xba* I (Figure 4-2a). Seven transformants were picked up and analyzed for the integration of *xyn11A* in the genome. PCR amplification indicated the presence of *xyn11A* in *P. pastoris* X33 genome (Fig. 4-2b)



(a) (b)

Fig. 4-2 (a) *Eco*R I and *Xba* I digestion of pGAPZαA-*xyn11A*. Four plasmids were tested; (b) PCR confirmation of *xyn11A* in *P. pastoris* transformants. Seven colonies were tested.

4.4.3 SDS-PAGE and deglycosylation

Xyn11A was purified to electrophoretic homogeneity after heat treatment and ultrafiltration (Fig. 4-3). Analysis of Xyn11A on SDS-PAGE revealed a molecular weight of approximately 37 KD, which is larger than the theoretical molecular weight. This could be attributed to glycosylation. Glycosylation is the most common post-translational modification in *P. pastoris* (Macauley-Patrick et al., 2005). Two types of glycosylation: O- and N-linked glycosylation have been frequently reported. Putative N-linked glycosylation sites prediction was identified using a web-based tool NetNglyc1.0. Prediction results suggest possible N-linked glycosylation might occur at amino acid Asn89. Treatment with Endo H resulted in a band of 30 KD, which is consistent with the deduced molecular weight of Xyn11A (Fig. 4-4). Therefore, Xyn11A was expressed as a glycoprotein in *P. pastoris*. Glycosylation has been reported to play a role in the thermal stability of xylanase (Cheng et al., 2015).

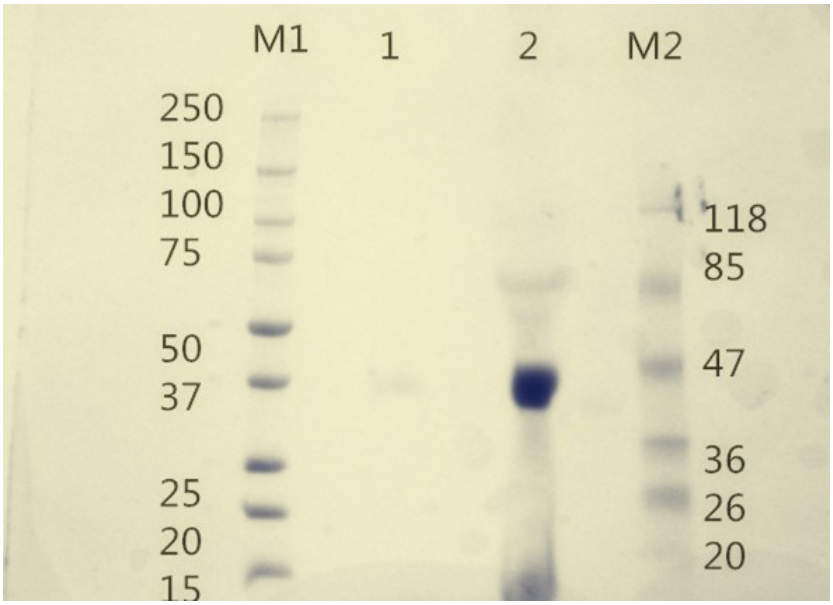


Fig. 4-3 SDS-PAGE analysis of Xyn11A. Lane M1, protein molecular marker 1 (Bio-rad); Lane 1, crude extract of Xyn11A; Lane 2, purified Xyn11A; Lane M2, protein molecular marker 2 (Fisher scinentific).

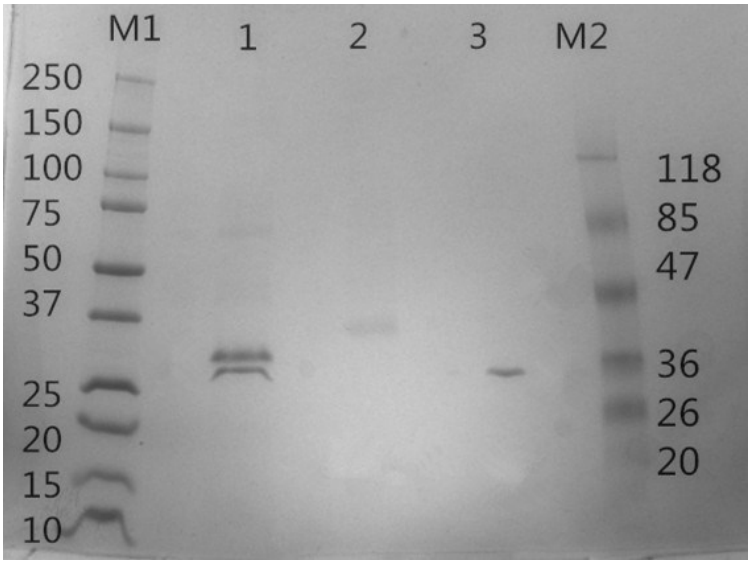


Fig. 4-4 Deglycosyalation of Xyn11A. Lane M1, protein standard marker 1 (Bio-rad); Lane 1, deglycosylated Xyn11A; Lane 2, purified Xyn11A; Lane 3, Endo H; Lane M2, protein molecular marker 2 (Fisher scientific).

4.4.4 Zymography

Zymogrphy was performed to confirm the xylanase activity of Xyn11A on gel. As shown in Fig. 4-5, two clear zones corresponding to the positions on SDS-PAGE were observed on the agarose gel, which clearly indicated the xylanase activity of this enzyme.

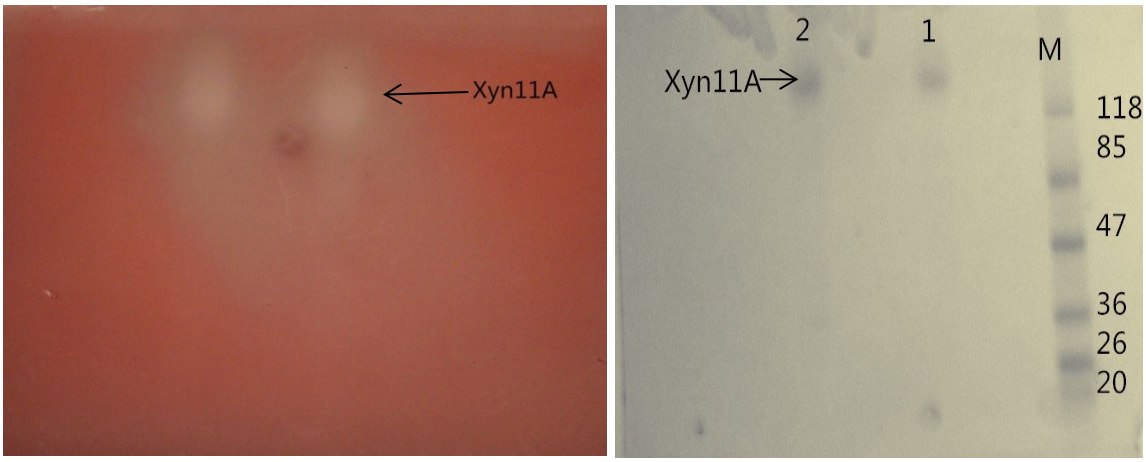


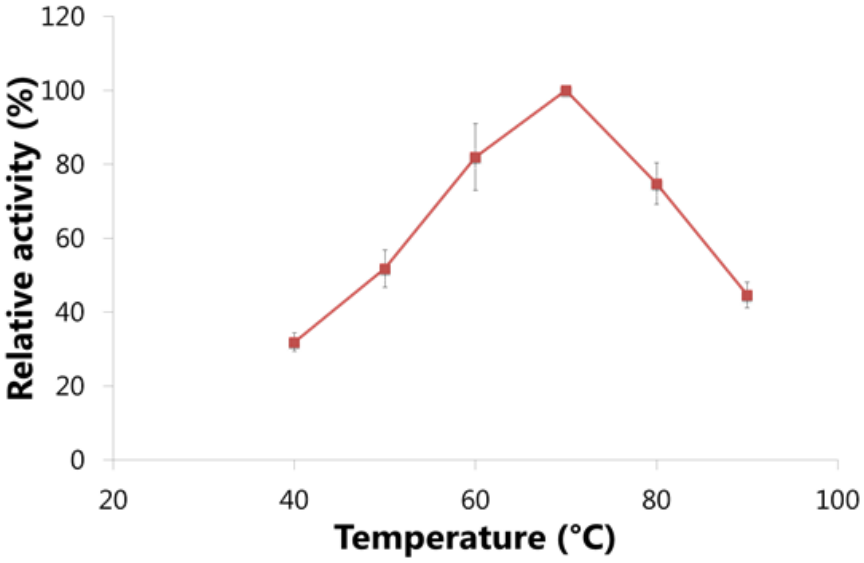
Fig. 4-5 Zymography of Xyn11A. (a) Xylanase activity on agarose gel containing 0.5% birchwood xylan; (b) Native Xyn11A on a SDS-PAGE gel. Lane M, protein standard marker (Fisher scientific); Lane 1 and 2, native Xyn11A on SDS-PAGE gel.

4.4.5 Biochemical characterization of Xyn11A

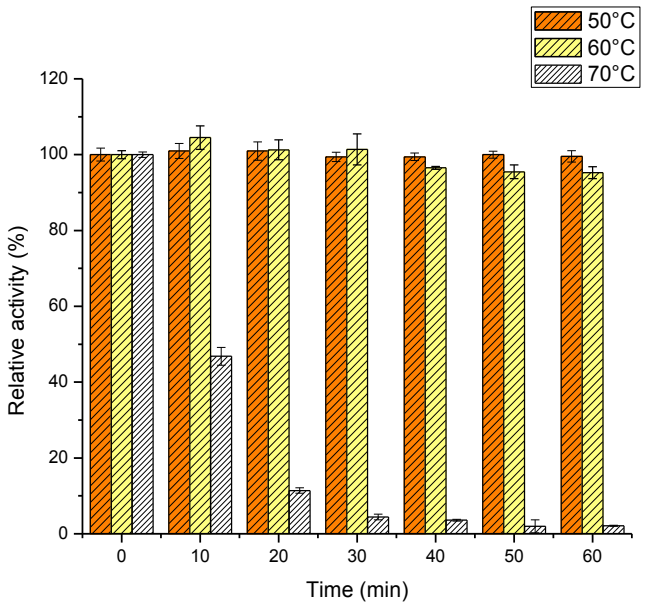
4.4.5.1 Effects of temperature and thermal stability

Thermophilic and thermostable xylanase has great applications in industry due to the benefits of operating at elevated temperature. Xyn11A was active at between 40 and 90 °C with the optimal temperature at 70 °C (Fig. 4-6a). Xyn11A was stable at 50 °C and 60 °C for up to 1 hour, retaining more than 90% of its activity (Fig. 4-6b). To test the potential of Xyn11A for industrial use, we incubated Xyn11A at 50 °C, pH 5.0 for up to 72 h (data not shown). Xyn11A retained about 80%, 64% and 60% of its activity after 6 h, 48h and 72 h, respectively. However, Xyn11A

was not stable at its optimal temperature, losing half of its activity after incubation for 10 min. This can be addressed using rational protein engineering.



(a)

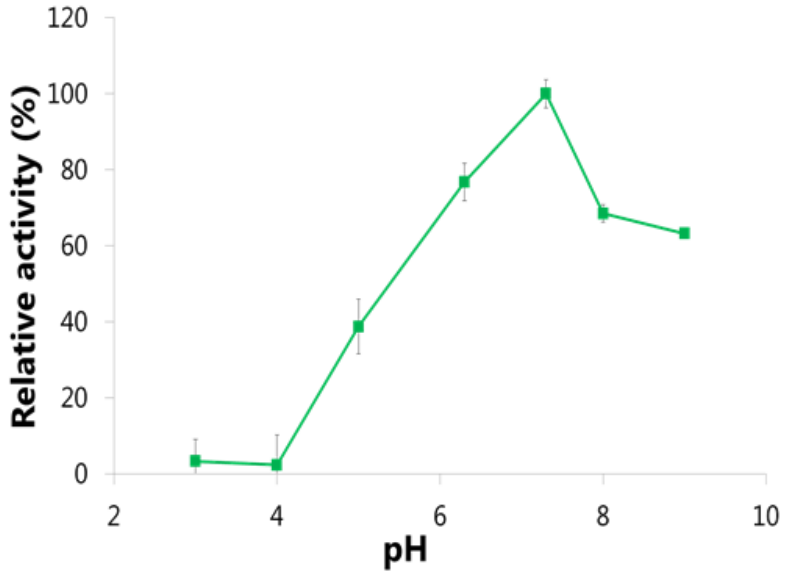


(b)

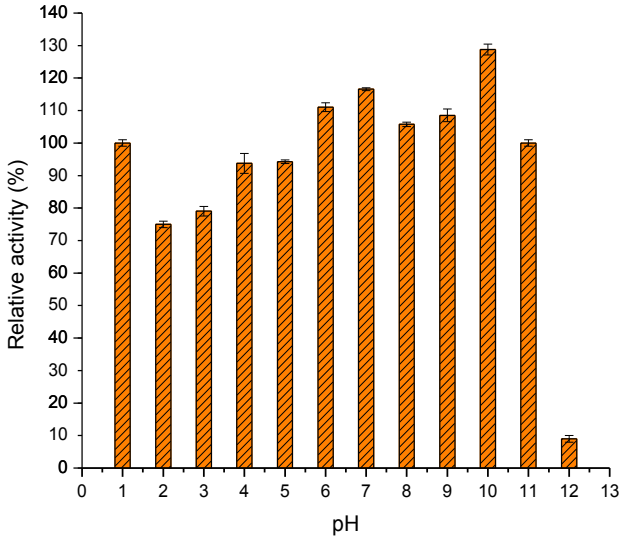
Fig. 4-6 (a) Effect of temperature on the activity of Xyn11A. Enzyme activity was determined in citrate buffer (pH 7.4) at 40-90 °C. (b) Thermal stability of Xyn11A. Xyn11A was incubated at 50, 60 and 70 °C in citrate buffer (pH 7.4). Aliquots were taken at different time points. Residual activity was measured in triplicate under standard assay condition.

4.4.5.2 Effects of pH and pH stability

Enzyme assay was carried out in various buffers to determine the optimal pH of Xyn11A. Interestingly, Xyn11A was optimally active at pH 7.4 (Fig. 4-7a), which is consistent with the GH11 family xylanase properties. Xyn11A retained more than 70% of maximum activity between pH 6.0-9.0. pH stability is a pivotal property for the industrial application of xylanase such as biobleaching where harsh environment occurs. Xyn11A was able to maintain more than 80% of its maximum activity after incubation at a wide range of pH values (Fig. 4-7b). The broad range pH stability and a maximal pH at 7.4 makes Xyn11A a potential candidate for feed additive.



(a)



(b)

Fig. 4-7 (a) Effect of pH on the activity of Xyn11A. The enzyme activity was measured at 70 °C. (b) pH stability of Xyn11A. pH=1 indicates the control group without pre-incubation in buffers. The enzyme activity was determined in triplicates under standard assay condition.

4.4.5.3 Effects of metal ions and chemical reagents

Various metal ions and chemicals were tested for their effects on xylanase activity (Fig. 4-8). Most ions tested in this study had little effect on the activity of Xyn11A. Copper can strongly inhibit some xylanase, which limits their industrial application. Cobalt, copper and lead ions (Zhang et al., 2012) have been reported to inhibit some GH11 family xylanase but showed little effect on Xyn11A. Ag^{+} is a strong inhibitor to enzymes and reduced the enzyme activity by 50%. SDS can inhibit many xylanase at a low concentration but didn't inhibit Xyn11A at 5 mM concentration, which was also confirmed in the zymography experiment where a SDS-PAGE containing 5mM SDS was used. Xyn11A displayed strong resistance to proteinase digestion, retaining more than 80% and 90% of the activity after digestion by proteinase K and pepsin for 1 h, respectively. Proteinase-resistant xylanase has been reported by Ning et al. (Li et al., 2008) The strong resistance to proteinase digestion can make Xyn11A a potential candidate for the application in food industry and animal feeding additive.

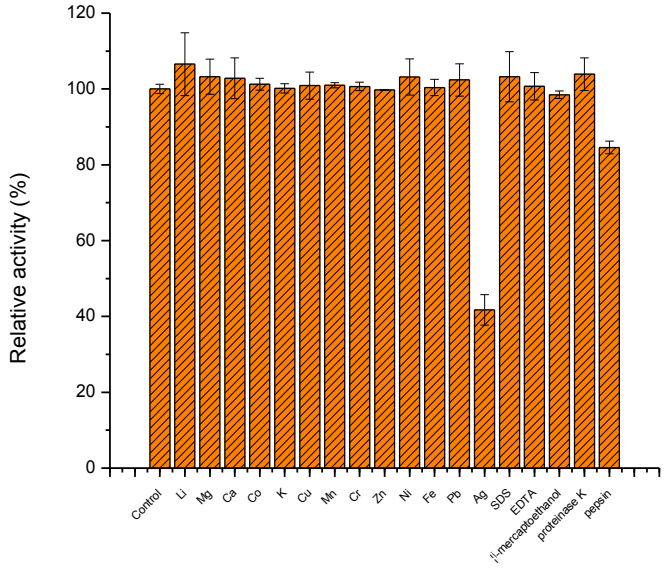


Fig. 4-8 Effects of metal ions and proteinase digestion on the activity of Xyn11A. Metal ions, SDS, EDTA and β-mercaptoethanol were added to the standard assay in 5mM final concentration. Xyn11A was incubated with pepsin (pH 2.0) and proteinase K (pH 6.9) for 1 h at 37 °C. Residual activity was determined under standard assay condition.

4.4.5.4 Substrate specificity and kinetic parameters

Xyn11A were active on birchwood xylan (100%), beechwood xylan (170%). No cellulase activity of Xyn11A was observed using CMC-Na as the substrate. Kinetic parameters were determined using the Lineweaver-Burk plot (Fig. 4-9). The K_m and V_{max} for Xyn11A under standard assay condition were 12.09 mg/mL and 10,245 U/mg, respectively.

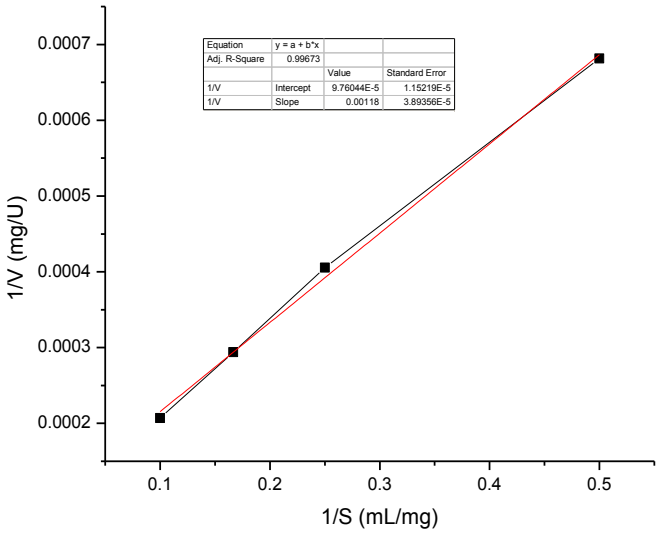


Fig. 4-9 Lineweaver-Burk plot of Xyn11A. Xylanase activity was measured using different concentrations of birchwood xylan as substrate under standard assay condition. Xylanase activity assay was done in triplicates.

4.4.6 Production of Xyn1 using high cell density fermentation

Production of Xyn11A was first carried out in shake flask, with xylanase activity reaching 100 U/mL after 72 h of cultivation (data not shown). To test the production of Xyn11A in a larger scale, fed-batch fermentation was performed in a 5 L bioreactor. In this work, *P. pastoris* was grown to an OD_{600} of 300 without optimizing fermentation conditions. The highest xylanase activity obtained was more than 2000 U/mL after 72 h of fermentation (Fig. 4-10).

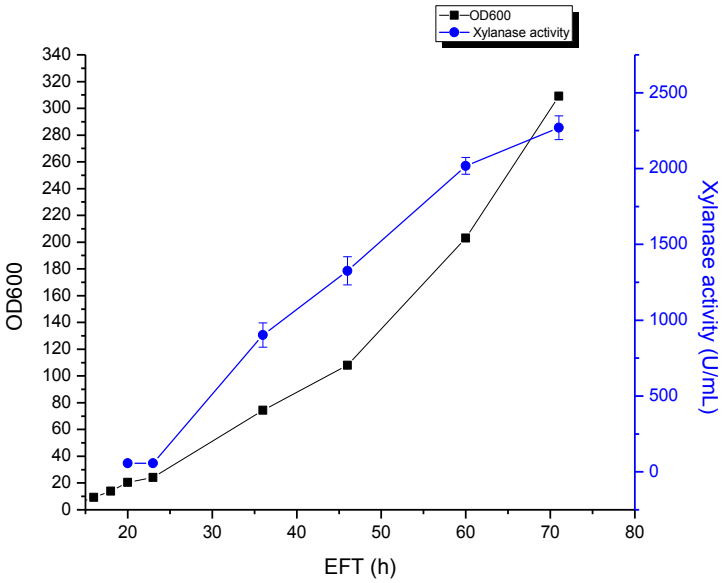


Fig. 4-10 Time course of Xyn11A production in a 5 L bioreactor. EFT indicates the elapsed fermentation time since the inoculation. Xylanase activity assay was done in triplicates. Xylanase activity was indicated by blue circles. OD_{600} was indicated by black squares.

4.5 Discussion

GH11 family consists of only one category of hydrolase: xylanases. Members of GH11 family are characterized by their high substrate selectivity, smaller molecular weight and high catalytic efficiency (Collins et al., 2005). GH11 family xylanases have potential applications in food and feed industry, paper and pulp industry and biofuel production (Paes et al., 2012). GH11 xylanases with various temperature and pH optima have been reported in recent years. In this work, a GH11 family xylanase from *C. thermophilus* was described for the first time. Two putative glutamic acid residues in Xyn11A responsible for GH11 xylanase activity: Glu116 and Glu207 were identified as the catalytic residues. Those two catalytic residues were the same as XynC81 from *Achaetomium* sp. Xz-8 (Zhao et al., 2013). Robust properties of Xyn11A were revealed by biochemical characterization. Higher optimal temperature and thermal stability is

desirable for application in biobleaching industry. Thxyn11A was retained over 90% activity after incubation of 30 min at 70 °C (Zhang et al., 2012). Xyn10B from *Thermotoga thermarum* was stable at 75 °C for 2 h (Shi et al., 2014). Xyn10A isolated also from the extremely thermophilic bacterium *T. thermarum* was optimally active at 95 °C and is stable between 55-90 °C (Shi et al., 2013). Xyn11A has an optimal temperature of 70 ˚C, which falls into the typical temperature range between 62 ˚C and 85 ˚C of thermophilic fungal xylanases (Paes et al., 2012). Good thermal stability was observed for Xyn11A. Xyn11A retained more than 90% of its activity after 1 h incubation at 50°C and 60 °C. To test its potential for industrial use, we incubated Xyn11A for up to 72 h at 50 °C. More than 80%, 60% and 40% of residual activity was detected after incubation of 24h, 48h and 72 h at 50 °C, respectively. These results suggest Xyn11A is quite stable at 50 °C. Despite cellulose binding module (CBM) of family 1 was present at the C-terminal of Xyn11A, no cellulase activity was detected, which is beneficial to its potential application in paper industry. Xylanases with broad pH stability can be used for textile industry. Several such GH11 xylanases have been characterized. XynB119 from *Streptomyces* sp. TN119 is stable over pH1.0-11.0 (Zhou et al., 2011). PoXyn2 isolated from *Penicillium occitanis* Pol6 retained 100% of its activity over pH2.0-9.0 (Driss et al., 2012). Ren *et al*. reported a xylanase from *Streptomonospora* sp. YIM90494 with pH stability between pH4.0-10.0 (Ren et al., 2013). XynSW3 from *Streptomyces* sp. SWU10 displayed stability within pH0.6-10.3 (Sukhumsirichart et al., 2014). Most fungal GH11 xylanases displayed optimal activity at pH 5.5 or below (Polizeli et al., 2005). Xyn11A has a neutral pH optimum and is alkaline-tolerant. Xyn11A showed optimal activity at pH7.4, similar to that of XynB119. The excellent thermal stability and cellulase-free property make Xyn11A a suitable candidate for paper industry. Xyn11A demonstrated strong resistance to some chemicals such as SDS, EDTA, Hg^{2+} and Cu^{2+}

which are reported to be strong inhibitors of several xylanases. For example, XynZG of *Plectosphaerella cucumerina*, XynSW3 and XynB119 were nearly 100% inhibited by Hg^{2+}. SDS even at 1mM, completely inhibited the activity of XYL11B from *Bispora* sp. MEY-1. Xyn11B activity was significantly reduced by 5mM SDS (Shi et al., 2015). Cu^{2+} showed a strong inhibitory effect on Xyn10B (Shi et al., 2014). Xylanase with strong resistance to protease could be used as feed additive for improved nutrition utilization. XYL11B was resistant to trypsin and pepsin digestion (Luo et al., 2009). XynB119 was reported be unaffected by a number of protease including pepsin, trypsin, collagenase, α-chymotrypsin and proteinase K. Xyn11A showed strong resistance to pepsin and proteinase K digestion. Combined with its neutral pH optimum, Xyn11A could be a potential candidate for animal feed additive. In summary, Xyn11A exhibited some robust properties in terms of high thermal stability, broad pH stability and strong resistance to metal ions and proteinase digestion, indicating potential applications for this xylanase in the sector of feed additive, food and pulp industry.

Heterologous expression of fungal xylanase has been achieved mostly in *E. coli*, *P. pastoris* and other fungal hosts (Ahmed et al., 2009). Among them, *P. pastoris* combined the merits of simple cultivation strategies, fast growth rate, high protein yield and easy downstream processing requirements, making it an attractive host for mass production of recombinant proteins. The methylotrophic *P. pastoris* has been a successful workhorse for recombinant protein expression. Methanol-inducible promoter AOX1 has been widely used for heterologous expression of proteins due to its high protein yield and tight regulation by methanol. Many xylanases have been expressed under the AOX1 promoter (Fan et al., 2012, Gaffney et al., 2009, Luo et al., 2009). AOX1-driven expression is suitable for the production of toxic proteins. However, the shift from accumulation of biomass to methanol-induced protein production complicates the

process control. Moreover, methanol is highly flammable and hazardous, which is undesirable for large scale production (Ahmad et al., 2014). GAP promoter possesses the advantages over the methanol-inducible AOX1 promoter in terms of associating the yield to cell growth and less toxic intermediates such as formaldehyde and hydrogen peroxide (Çalık et al., 2015). Feeding strategies can be refined and optimized to obtain high yield and productivity. High xylanase activity was obtained after 72 h of cultivation. Further process optimization concerning feeding strategies and carbon source screening could be investigated to enhance the production of Xyn11A.

4.6 Conclusions

In this study, we described the cloning, high level expression and characterization of a codon-optimized xylanase gene *xyn11*A from *C. thermophilus* in *P. pastoris*. Xyn11A showed optimal activity at 70 °C and pH 7.4. Xyn11A demonstrated good thermostability at 60 °C or below. Xyn11A is stable over a wide range of pH values. Xyn11A is resistant to proteinase digestion and some metal ions. Those superior properties make Xyn11A a good candidate for industrial use. High cell density cultivation was achieved by growing *P. pastoris* in bioreactor. This xylanase is feasible for mass production in *P. pastoris*. To our knowledge, this study is the first time to clone and express a codon optimized xylanase from the thermophilic fungus *Corynascus thermophilus* in *P. pastoris*. Process optimization can be investigated to further increase the production yield of Xyn11A.

4.7 Acknowledgements

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4.8 References

Ahmad M, Hirz M, Pichler H, Schwab H. Protein expression in *Pichia pastoris*: recent achievements and perspectives for heterologous protein production. Applied microbiology and biotechnology. 2014;98:5301-17.

Ahmed S, Riaz S, Jamil A. Molecular cloning of fungal xylanases: an overview. Applied Microbiology and Biotechnology. 2009;84:19-35.

Bailey MJ, Biely P, Poutanen K. Interlaboratory testing of methods for assay of xylanase activity. Journal of biotechnology. 1992;23:257-70.

Bajpai P. Application of enzymes in the pulp and paper industry. Biotechnology progress. 1999;15:147-57.

Berka RM, Grigoriev IV, Otillar R, Salamov A, Grimwood J, Reid I, et al. Comparative genomic analysis of the thermophilic biomass-degrading fungi *Myceliophthora thermophila* and *Thielavia terrestris*. Nat Biotechnol. 2011;29:922-7.

Çalık P, Ata Ö, Güneş H, Massahi A, Boy E, Keskin A, et al. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochemical Engineering Journal. 2015;95:20-36.

Cheng Y-S, Chen C-C, Huang J-W, Ko T-P, Huang Z, Guo R-T. Improving the catalytic performance of a GH11 xylanase by rational protein engineering. Applied microbiology and biotechnology. 2015;99:9503-10.

Collins T, Gerday C, Feller G. Xylanases, xylanase families and extremophilic xylanases. FEMS Microbiol Rev. 2005;29:3-23.

Driss D, Bhiri F, Chaabouni SE. Cloning and constitutive expression of His-tagged xylanase GH 11 from *Penicillium occitanis* Pol6 in *Pichia pastoris* X33: purification and characterization. Protein expression and purification. 2012;83:8-14.

Ergun BG, Calik P. Lignocellulose degrading extremozymes produced by *Pichia pastoris*: current status and future prospects. Bioprocess and biosystems engineering. 2016;39:1-36.

Fan G, Katrolia P, Jia H, Yang S, Yan Q, Jiang Z. High-level expression of a xylanase gene from the thermophilic fungus *Paecilomyces thermophila* in *Pichia pastoris*. Biotechnology letters. 2012;34:2043-8.

Fu XY, Zhao W, Xiong AS, Tian YS, Peng RH. High expression of recombinant *Streptomyces* sp. S38 xylanase in *Pichia pastoris* by codon optimization and analysis of its biochemical properties. Mol Biol Rep. 2011;38:4991-7.

Gaffney M, Carberry S, Doyle S, Murphy R. Purification and characterisation of a xylanase from *Thermomyces lanuginosus* and its functional expression by *Pichia pastoris*. Enzyme and Microbial Technology. 2009;45:348-54.

Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, et al. Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. PloS one. 2013;8:e58393.

Jia H, Fan G, Yan Q, Liu Y, Yan Y, Jiang Z. High-level expression of a hyperthermostable *Thermotoga maritima* xylanase in *Pichia pastoris* by codon optimization. Journal of Molecular Catalysis B: Enzymatic. 2012;78:72-7.

Juturu V, Wu JC. Microbial xylanases: engineering, production and industrial applications. Biotechnology advances. 2012;30:1219-27.

Li N, Yang P, Wang Y, Luo H, Meng K, Wu N, et al. Cloning, expression, and characterization of protease-resistant xylanase from *Streptomyces fradiae* var. k11. J Microbiol Biotechnol. 2008;18:410-6.

Luo H, Wang Y, Li J, wang H, Yang J, Yang Y, et al. Cloning, expression and characterization of a novel acidic xylanase, XYL11B, from the acidophilic fungus *Bispora* sp. MEY-1. Enzyme and Microbial Technology. 2009;45:126-33.

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM. Heterologous protein production using the *Pichia pastoris* expression system. Yeast. 2005;22:249-70.

Mellitzer A, Weis R, Glieder A, Flicker K. Expression of lignocellulolytic enzymes in *Pichia pastoris*. Microbial cell factories. 2012;11:1.

Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Analytical chemistry. 1959;31:426-8.

Paes G, Berrin JG, Beaugrand J. GH11 xylanases: Structure/function/properties relationships and applications. Biotechnology advances. 2012;30:564-92.

Petersen TN, Brunak S, von Heijne G, Nielsen H. SignalP 4.0: discriminating signal peptides from transmembrane regions. Nat Methods. 2011;8:785-6.

Polizeli M, Rizzatti A, Monti R, Terenzi H, Jorge JA, Amorim D. Xylanases from fungi: properties and industrial applications. Applied microbiology and biotechnology. 2005;67:577-91.

Ren W, Zhang F, Yang X, Tang S, Ming H, Zhou E, et al. Purification and properties of a SDS-resistant xylanase from halophilic *Streptomonospora* sp. YIM 90494. Cellulose. 2013;20:1947-55.

Royer JC, Nakas J. Simple, sensitive zymogram technique for detection of xylanase activity in polyacrylamide gels. Applied and environmental microbiology. 1990;56:1516-7.

Shi H, Zhang Y, Li X, Huang Y, Wang L, Wang Y, et al. A novel highly thermostable xylanase stimulated by Ca^{2+} from *Thermotoga thermarum*: cloning, expression and characterization. Biotechnology for biofuels. 2013;6:1.

Shi H, Zhang Y, Zhong H, Huang Y, Li X, Wang F. Cloning, over-expression and characterization of a thermo-tolerant xylanase from *Thermotoga thermarum*. Biotechnol Lett. 2014;36:587-93.

Shi P, Du Y, Yang H, Huang H, Zhang X, Wang Y, et al. Molecular characterization of a new alkaline-tolerant xylanase from *Humicola insolens* Y1. BioMed research international. 2015;2015:149504.

Sukhumsirichart W, Deesukon W, Kawakami T, Matsumoto S, Seesom W, Sakamoto T. Expression and characterization of recombinant GH11 xylanase from thermotolerant *Streptomyces* sp. SWU10. Applied biochemistry and biotechnology. 2014;172:436-46.

Van Dyk J, Pletschke B. A review of lignocellulose bioconversion using enzymatic hydrolysis and synergistic cooperation between enzymes—factors affecting enzymes, conversion and synergy. Biotechnology advances. 2012;30:1458-80.

Zhang F, Chen J-J, Ren W-Z, Lin L-B, Zhou Y, Zhi X-Y, et al. Cloning, expression, and characterization of an alkaline thermostable GH11 xylanase from *Thermobifida halotolerans* YIM 90462T. Journal of industrial microbiology & biotechnology. 2012;39:1109-16.

Zhao L, Meng K, Bai Y, Shi P, Huang H, Luo H, et al. Two family 11 xylanases from *Achaetomium* sp. Xz-8 with high catalytic efficiency and application potentials in the brewing industry. Journal of agricultural and food chemistry. 2013;61:6880-9.

Zhou J, Shi P, Zhang R, Huang H, Meng K, Yang P, et al. Symbiotic *Streptomyces* sp. TN119 GH 11 xylanase: a new pH-stable, protease-and SDS-resistant xylanase. Journal of industrial microbiology & biotechnology. 2011;38:523-30.

Chapter 5: Enhanced xylanase production by GAP promoter using high cell density cultivation in *Pichia pastoris*

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5.1 Abstract

Fuelled by growing demand of biofuel production using lignocellulose, xylanase is attracting great attention due to its capacity to break down xylan into xylose and oligosaccharides. *Pichia pastoris* has been increasingly used for heterologous expression of xylanase due to the high titer and low cost of downstream processing. The GAP promoter is becoming increasingly popular for protein expression in *P. pastoris* owing to simple cultivation strategies and high level constitutive expression. Carbon source and feeding strategies are crucial factors to achieving high level production and thus should be optimized. In this work, we investigated the effect of carbon source and feeding strategies including constant feeding, stepwise feeding and exponential feeding on the production of a pH-stable xylanase using GAP promoter. Xylanase activity was enhanced to 15000 U/mL when glucose was exponentially fed to bioreactor, which was increased by seven-fold compared with our previous report.

Keywords: *Pichia pastoris*, GAP promoter, feeding strategy, high cell density cultivation.

5.2 Introduction

Driven by the increasing demand to build a sustainable economy due to growing concern over the scarce fossil fuel reserve, research regarding efficient utilization of renewable feedstock such as lignocellulose has intensified. Xylan, a polymer of xylose and a major component of lignocellulose, has the potential to become a feedstock for bioethanol production. Enzymatic hydrolysis of xylan is achieved by the synergistic action of multiple enzymes among which xylanase (EC 3.2.1.8) is of particular industrial interest. Xylanase is responsible for the cleavage of β-1, 4-xylosidic bond to break down the backbone of xylan. Xylanase has a variety of industrial applications widely used for pulp bleaching (Bajpai, 1999, Birijlall et al., 2011), animal feed additive, wine-brewing (Polizeli et al., 2005) and bakery (Elgharbi et al., 2015, Xu et al., 2016).

Xylanase is naturally secreted by some potent biomass-degrading filamentous fungus such as *Aspergillus niger* and *Trichoderma reesei* (Juturu and Wu, 2012). Heterologous production of xylanase in bacteria or yeast has been successfully achieved in recent years (Mellitzer et al., 2012). *Pichia pastoris* is an industrially-relevant host widely used for recombinant protein production. It has gained popularity primarily due to the tightly-regulated strong alcohol oxidase 1 promoter (P_{AOX1}), its ability to achieve high biomass concentration on defined media, the post-translational modifications such as glycosylation and disulfide bond formation to yield correctly-folded product and most notably, secretion of protein into culture, a merit greatly valued in industrial enzyme production (Potvin et al., 2012).

The GAP (glyceraldehyde-3-phosphate dehydrogenase) promoter is an alternative to P_{AOX1} suitable for the production of non-toxic proteins due to its growth-associated product formation (Çalık et al., 2015). The popularity of GAP promoter is garnered mainly attributed to its high

level constitutive expression, which eliminates the handling of hazardous methanol. High level expression of industrial enzymes such as phytase (Tang et al., 2009) or xylanase using the P_{GAP}-based system has been reported recently (Yang and Zhang, 2017). Optimization of cultivation strategies was imperative to achieve high level production. Cultivation conditions such as carbon source and specific growth rate (μ) are crucial factors to yield high level production in P_{GAP}-based systems.

Cost-effective production of xylanase via large-scale fermentation is critical for the commercial use of xylanase. The objective of this paper was to establish an efficient fermentation strategy to achieve enhanced production of a xylanase, which could be potentially used for the production of biofuels. In our previous report, xylanase was produced at 2000 U/mL (Yang and Zhang, 2017). In this work, xylanase activity was enhanced to over 14,000 U/mL after optimization.

5.3 Materials and methods

5.3.1 Strain and media

Pichia strain X33-Xyn11A secreting a pH stable endo-β-1, 4-xylanase was kept on YPD agar plates (containing 10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, 15 g/L agar powder and 100 mg/L Zeocin) and streaked on fresh plates every two weeks. Other chemicals were of analytical grade and purchased from Fisher scientific (Canada) or Sigma-Aldrich (USA). BSM (basal salts medium) consisting of per liter: 42.9 g KH_2PO_4, 14.33 g K_2SO_4, 0.6 g $CaSO_4$, 5.17 g $(NH_4)_2SO_4$, 5.71 g $MgSO_4 \cdot 7H_2O$, 0.5 mL H_2SO_4 and 4 mL PTM1 trace metal solution was used for bioreactor cultivations. PTM1 solution consists of per liter: 6 g $CuSO_4 \cdot 5H_2O$, 0.08 g NaI, 3 g $MnSO_4 \cdot H_2O$, 0.2 g $Na_2MoO_4 \cdot 2H_2O$, 0.02 g HBO_3, 0.5 g $CoCl_2$, 20 g $ZnCl_2$, 65 g $FeSO_4 \cdot 7H_2O$, 0.2 g biotin and 5 mL H_2SO_4. Glucose was separately autoclaved and added to the media after.

5.3.2 Inoculum preparation

Single yeast colony was inoculated into a 500 mL shake flask containing 100 mL YPD media and incubated overnight on a rotary shaker (New Brunswick, USA) at 30 °C and 250 rpm. Yeast culture was used to inoculate bioreactors containing sterilized fermentation media.

5.3.3 Bioreactor setup

All batch and fed-batch cultivations were performed in 5 L Bioflo320 bioreactors (Eppendorf, Canada) equipped with dual Rushton impellers. Bioreactors were kept at 30 °C and sparged with filtered air at a rate of 4-8 L/min. Agitation speed was maintained between 400-1100 rpm. The dissolved oxygen (DO) was cascaded to air flow rate and agitation speed to maintain above 20% air saturation. pH was kept at pH 5.0 by adding 30% ammonium hydroxide. DO and pH were measured by probes (Mettler-Toledo, Canada). Foaming was controlled by adding 5% antifoam (Sigma-Aldrich) solution regulated by a level probe. For online glucose monitoring, a sampling

probe (Flowmics, USA) was plugged into the bioreactor. Cultures were pumped out of the bioreactor through the sitini pump (YSI 2900 biochemical analyzer, Mandel, Canada). Glucose was automatically measured and concentrations were collected and displayed on the control station of Bioflo320 bioreactors. Aliquots were periodically taken for analysis of biomass concentration and substrate concentration and enzyme activity assay.

Exponential feeding strategy

To implement the specific growth rate-based feeding strategy, carbon source (glycerol or glucose) was fed to the bioreactor according to the following equation:

$$F = \frac{\mu_{set} X_0 V_0 \exp(\mu_{set} t)}{Y_{X/S} * Cs_0} \quad (5\text{-}1)$$

Where F was feed rate (L/h), μ_{set} was the predetermined specific growth rate, X_0 (g/L) was the DCW at the end of batch phase. V_0 (L) was the initial media volume of batch phase, $Y_{X/S}$ (g/g) was the biomass yield on substrate and Cs_0 (g/L) was the substrate concentration of feeding solution. The calculated feed rate was implemented using the NBS Biocommand software.

5.3.4 Analytical methods

Biomass concentration of properly diluted aliquots was determined by measuring optical density at 600 nm using a Biochrom Ultrospec 60 spectrophotometer (UK). Dry cell weight (DCW) was determined according to the calibration curve. One OD_{600} is equivalent to 0.378 g/L DCW. Samples were centrifuged for 10 min at 13,000 rpm and supernatant was used for analysis of substrate and metabolites. Glucose and glycerol concentrations were determined using a YSI 2900 Biochemical Analyzer (Mandel, Canada) equipped with a glucose or glycerol membrane, respectively. Protein concentrations in supernatant were determined based on the Bradford

method (Bio-Rad protein assay, USA) using bovine serum albumin (BSA) as standards. Absorbance at 595 nm was measured to quantify the protein concentration by comparing to standard curve. Xylanase activity was assayed using 3, 5-dinitrosalicyclic acid (DNS) method as described previously using birchwood xylan as substrate (Yang and Zhang, 2017). Absorbance was measured at 540 nm to quantify xylanase activity based on standard curve. All absorbance was read by a micro-plate reader (Biotek, USA). One unit of xylanase activity was defined as the amount of enzyme required for the release of 1 μmol reducing sugar in 1 min.

5.4 Results and discussions

5.4.1 Batch cultivation using glycerol

Despite a number of carbon sources that could be used for the fermentation of *P. pastoris* (Potvin et al., 2016), glucose and glycerol remain the most popular ones, particularly for P_{GAP}-based systems, as they sustain high cell growth rate and are easy to monitor. We first performed batch cultivations using various concentrations of glycerol to evaluate its performance for HCDC of *P. pastoris*. The results were shown in Table 5-1. As can be seen from Table 5-1, the biomass yield was obtained between 0.5-0.6 g/g glycerol, which was slightly higher than previous report (Tang et al., 2009). Increased biomass and xylanase activity were obtained with increased glycerol concentration. Xylanase yield on glycerol was not significantly changed within the 20-40 g/L glycerol range. Glycerol of 40 g/L was selected in subsequent fed-batch fermentations to achieve a higher xylanase concentration in batch phase prior to glycerol feeding phase.

Table 5-1 Batch cultivations with various concentrations of glycerol

Glycerol (g/L)	Cultivation time (h)	DCW (g/L)	Xylanase activity (U/mL)	Biomass yield (gDCW/gGlycerol)	Xylanase yield (U/g)
20	26	11.91	401.6	0.596	20080
30	30	15.82	579.7	0.527	19323
40	40	22.26	792.8	0.556	19820

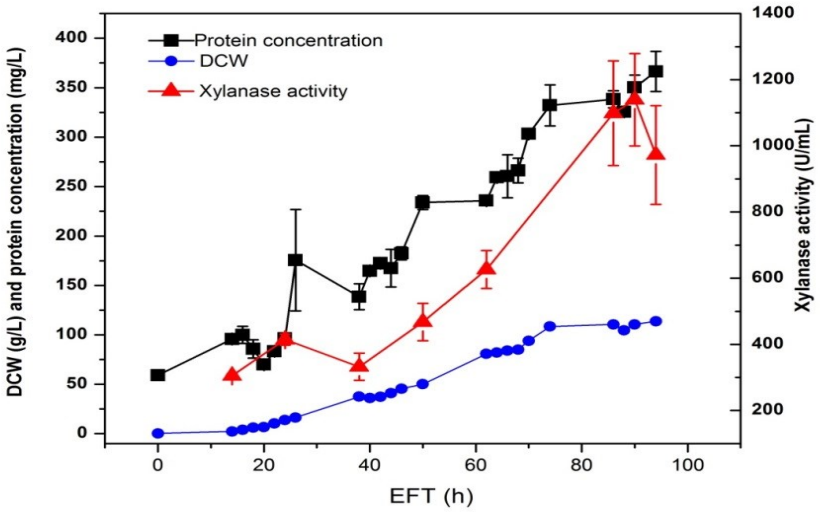
5.4.2 Fed-batch cultivation with glycerol

5.4.2.1 Constant and step-wise feeding

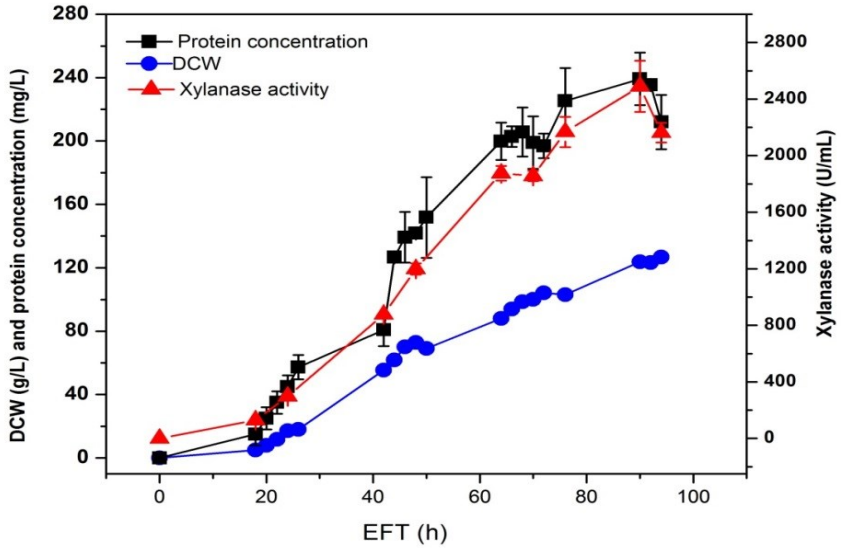
Constant feed rate is a feeding strategy simple to implement. The glycerol feeding rate was kept constant throughout the feeding phase until the end of fermentation. Step-wise feeding is another form of feeding strategy where feed rate is adjusted in different fermentation periods. DO-stat was used in those two feeding strategies. DO was kept above 20% by cascading to agitation speed and aeration rate. Several feed rates were tested in this work to evaluate the efficiency of this feeding strategy. Time course of the fermentation was shown in Fig. 5-1. Comparison of three fed-batch cultivations were summarized in Table 5-2. The highest protein concentration and xylanase activity was achieved when glycerol feeding was maintained at a higher rate. Feed rate below 0.6 mL/min led to the lowest xylanase activity despite similar DCW was obtained as the high feed rate. The DCW obtained at constant feed rate of 0.6 mL/min was slightly higher than those of the other two feeding strategies. Notably, the fermentation time was significantly shortened using a higher feed rate, which enhanced the volumetric productivity. The results obtained here suggest that a higher rate could be investigated to achieve better process performance.

Table 5-2 Comparison of fed-batch cultivations using constant and stepwise feeding

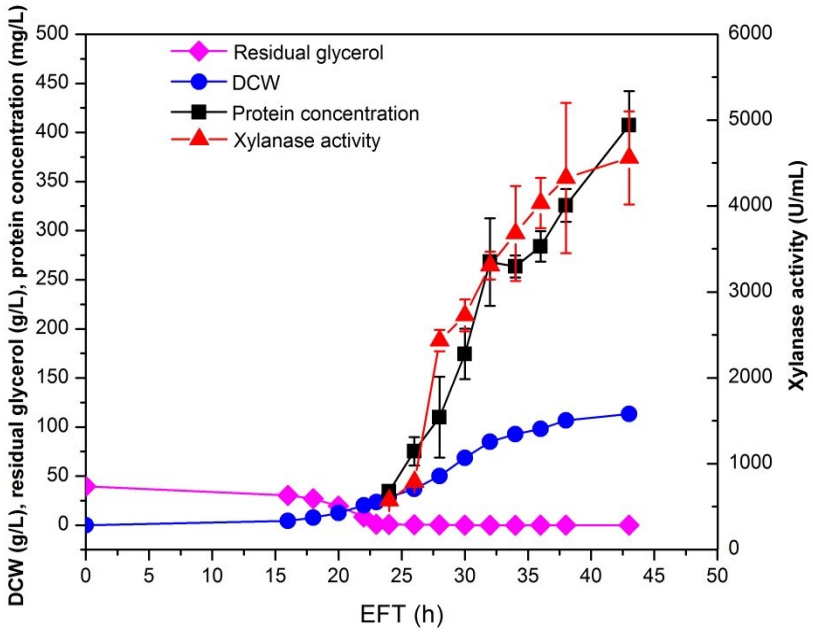
Feeding strategy	Fermentation time (h)	Glycerol consumed (g)	DCW (g/L)	Protein concentration (mg/L)	Xylanase activity (U/mL)
Constant feeding (0.6 mL/min)	94	1080	126.8	211.9	2490.5
Stepwise (0.3-0.6 mL/min)	94	1080	113.8	366.4	1139.4
Stepwise (0.9-1.7 mL/min)	43	1080	113.2	407.5	4560.4


Protein concentration
DCW
Xylanase activity
DCW (g/L) and protein concentration (mg/L)
Xylanase activity (U/mL)
EFT (h)
0
50
100
150
200
250
300
350
400
200
400
600
800
1000
1200
1400
0
20
40
60
80
100

(a)


Protein concentration
DCW
Xylanase activity
DCW (g/L) and protein concentration (mg/L)
Xylanase activity (U/mL)
EFT (h)
0
40
80
120
160
200
240
280
0
400
800
1200
1600
2000
2400
2800
0
20
40
60
80
100

(b)



(c)

Fig. 5-1 Time course of fed-batch cultivations of *P. pastoris* X33-Xyn11A. EFT indicates elapsed fermentation time. Bioreactor contained 2 L of initial fermentation media. 2 L of 500 g/L glycerol was used as feeding solution. (a) Glycerol feed rate was 0.3-0.6 mL/min. (b) Glycerol feed rate was 0.6 mL/min. (c) Glycerol feed rate was 0.9-1.7 mL/min. Error bars indicate duplicate experiments for protein concentration and xylanase activity assay.

5.4.2.2 Exponential feeding strategy using glycerol

Protein production is highly associated with cell growth, which depends on carbon source supply. In constant or stepwise feeding, glycerol feed rate was adjusted based on DO level. Specific growth rate was found to be an important factor affecting recombinant protein production. Maintaining a proper μ is critical to achieving high yield of protein production. In this work, exponential feeding was used to evaluate its performance. Two pre-determined μ were tested. As was shown in Fig. 5-2, glycerol was depleted at 22 h. Glycerol was exponentially fed to bioreactor. Feed rate was initiated with 0.47 mL/min and increased until 2.45 mL/min was

reached due to maximum limit of agitation speed and aeration rate. The final biomass reached 105.8 g/L. Xylanase activity was found to be 2590.7 U/mL. When a higher μ (0.2 h^{-1}) was investigated (Fig. 5-3), DCW was enhanced to 171 g/L and xylanase activity reached 5904 U/mL, which was 61.6% and 2.27-fold higher than that of μ=0.15 h^{-1}, respectively. Xylanase activity achieved under μ=0.2 h^{-1} was 29.4% higher than the stepwise feeding strategy (0.9-1.7 mL/min). The maximum feed rate was 2.54 mL/min. Residual glycerol was detected below 0.5 g/L during the feeding phase, indicating that no accumulation of glycerol occurred. The mean μ was found to be 0.11 h^{-1} in the first 12 h of feeding when μ_{set}=0.15 h^{-1}. Similarly, the mean μ of the first 13 h of feeding when μ_{set}=0.2 h^{-1} was 0.13 h^{-1}. The determined μ was lower than the nominal μ. The mean μ during the overall glycerol feeding phase was even lower, which could be explained by the feed rate limited by the bioreactor.

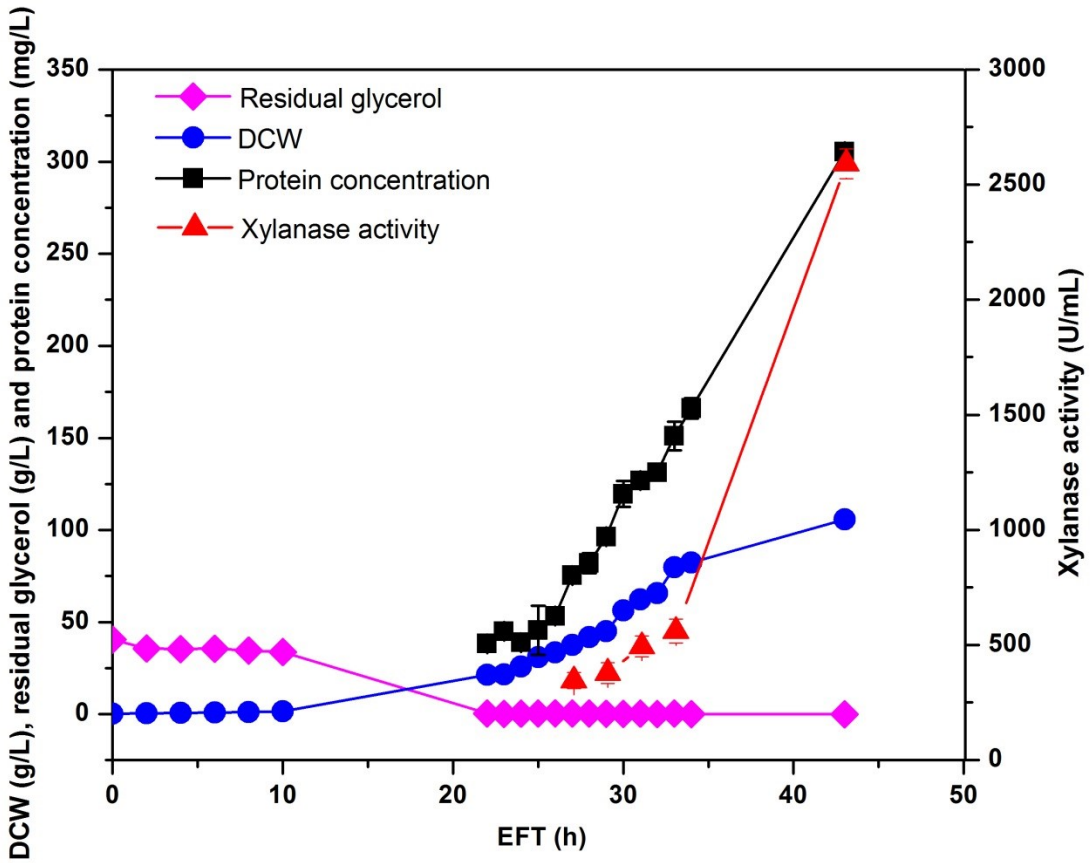


Fig. 5-2 Time course of fed-batch cultivation of *P. pastoris* X33-Xyn11A using glycerol exponential feeding. μ was set to be 0.15 h^{-1}. Bioreactor contained 2 L initial fermentation media. 2 L of 50% glycerol was fed to the bioreactor upon depletion of glycerol in batch phase. Error bars indicate duplicate experiments for protein concentration and xylanase activity assay.

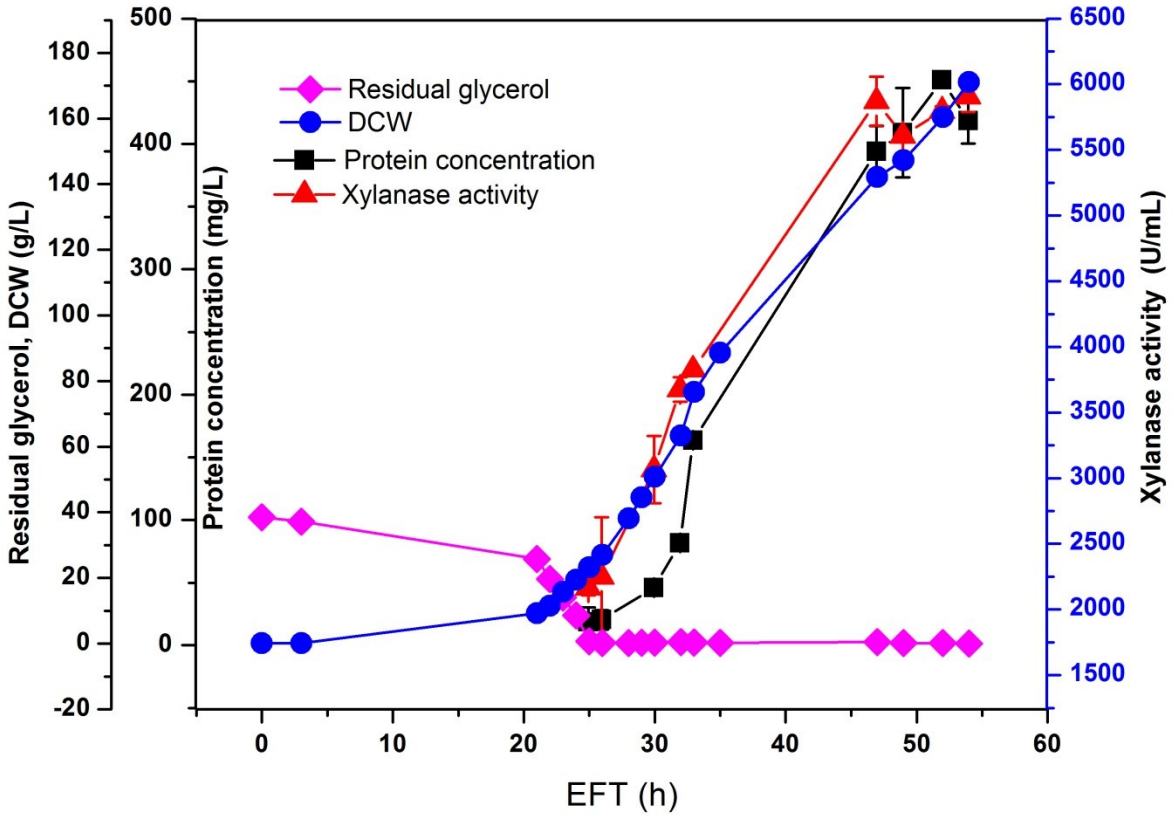


Fig. 5-3 Time course of fed-batch cultivation of *P. pastoris* X33-Xyn11A using glycerol exponential feeding. μ was set to be 0.2 h^{-1}. Bioreactor contained 2 L initial fermentation media. 2 L of 500 g/L glycerol was fed to the bioreactor upon depletion of glycerol in batch phase. Error bars indicate duplicate experiments for protein concentration and xylanase activity assay.

5.4.2.3 Exponential glucose feeding

Glucose and glycerol are routinely used as carbon source for P_{GAP}-based cultivation. In some reports, glycerol outperformed glucose in terms of protein titer and enzyme activity. In other studies, the opposite was observed. Fed-batch fermentations using glucose were performed to evaluate its performance. Two pre-determined specific growth rates were used: μ=0.15 h^{-1} and μ=0.2 h^{-1}. As was shown in Fig. 5-4, at lower μ_{set}, the DCW reached 135 g/L. Protein concentration was 359 mg/L at the end of fermentation. Xylanase activity was detected at 3271

U/mL. The mean μ was calculated to be 0.12 h^{-1} during the first 14 h of glucose feeding. When μ_{set}=0.2 h^{-1}, the DCW was significantly boosted to 175 g/L (Fig. 5-5). The mean μ in the first 12 h of glucose feeding was 0.18 h^{-1}. Protein concentration was 516 mg/L and xylanase activity was 14914 U/mL. Fed-batch fermentation was repeated to confirm the results obtained under μ_{set}=0.2 h^{-1}. Similar results were obtained. Xylanase activity achieved 15000 U/mL. These results suggest that glucose could achieve better process performance for xylanase production in this system.

Process engineering of *P. pastoris* has mainly focused on the development of various methanol feeding strategies for the P_{AOX1}-based system. More efforts need to be made to the P_{GAP} system which could be used as an efficient alternative to P_{AOX1}. The effect of μ on the production of human antigen-binding fragment (Fab) was investigated recently. The authors studied three values of μ: 0.05 h^{-1}, 0.1 h^{-1} and 0.15 h^{-1}. It was found that similar yields were achieved under 0.1 h^{-1} and 0.15 h^{-1}. Higher volumetric productivity favours higher μ, which was consistent with the results obtained in this work. μ-stat strategy was also used in the production of lipase using P_{GAP} (Zhao et al., 2008). The authors investigated four different μ ranging from 0.1 h^{-1} to 0.25 h^{-1} and found that the highest product yield was achieved at μ=0.15 h^{-1}. The variations between mean μ and nominal μ could be attributed to the fact that substrate feed rate cannot be excessively increased as calculated due to the requirement of maintaining the DO above 20% air saturation.

High level production of xylanase using in *P. pastoris* has been recently reported. The highest reported xylanase activity was 52940 U/mL with 8.1 g/L secreted protein (Fan et al., 2012). To enhance the recombinant protein production in *P. pastoris*, many process engineering strategies could be used such as optimization of medium composition, pH, temperature and DO (Çalık et

al., 2015). Investigation of feeding strategies remains an effective way to achieve high level production of protein. More values of μ could be tested in future work to find the optimal value.

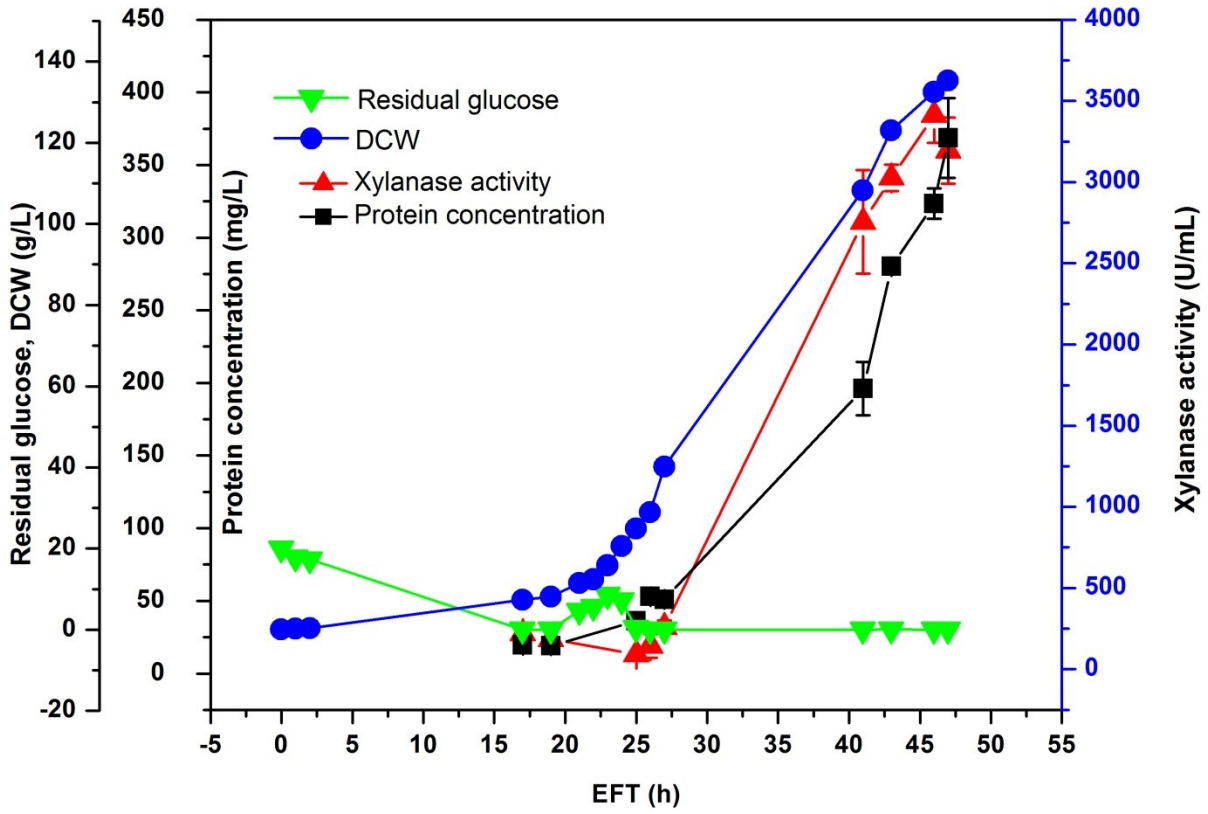


Fig. 5-4 Time course of fed-batch cultivation of *P. pastoris* X33-Xyn11A using glucose exponential feeding. μ was set to be 0.15 h^{-1}. Bioreactor contained 2 L initial fermentation media. 2 L of 500 g/L glucose was fed to the bioreactor upon glucose depletion in batch phase. Error bars indicate duplicate experiments for protein concentration and xylanase activity assay.

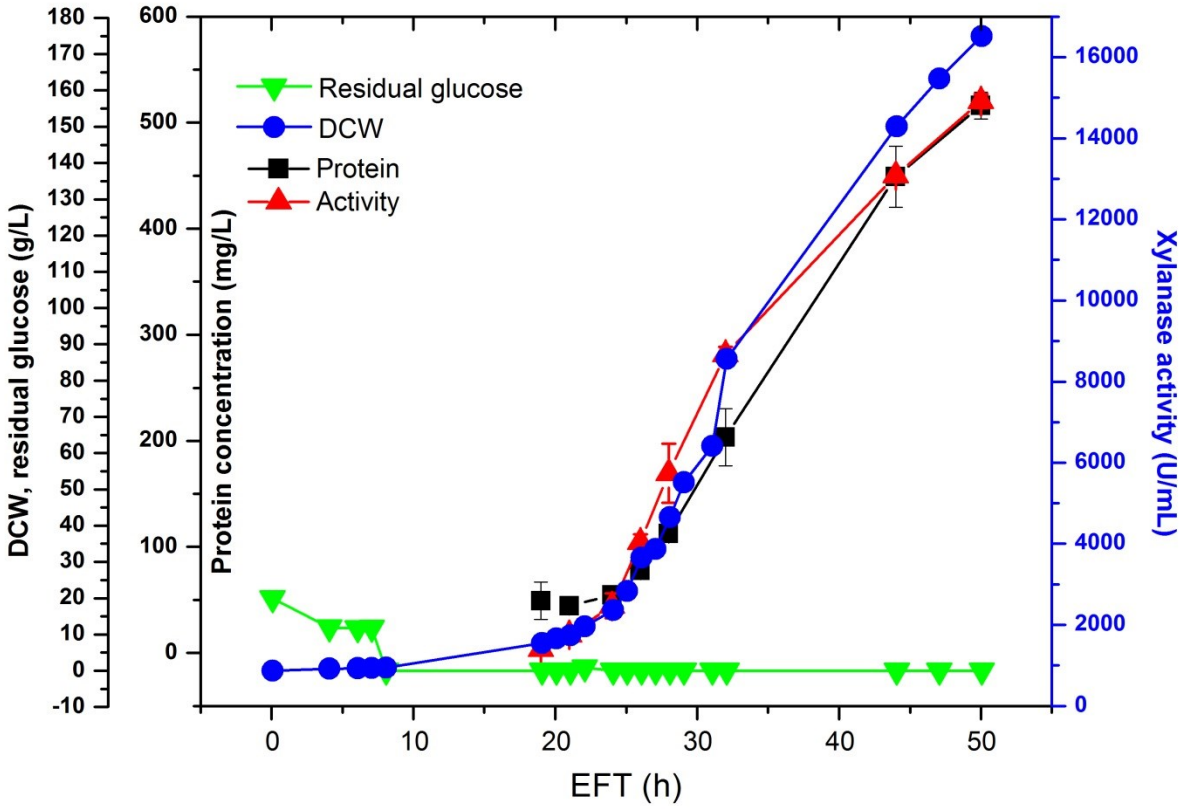


Fig. 5-5 Time course of fed-batch cultivation of *P. pastoris* X33-Xyn11A using glucose exponential feeding. μ was set to be 0.2 h^{-1}. Bioreactor contained 2 L initial fermentation media. 2 L of 500 g/L glucose was fed to the bioreactor upon glucose depletion in batch phase. Error bars indicate duplicate experiments for protein concentration and xylanase activity assay.

5.5 Conclusions

P. pastoris has become a popular platform for heterologous production of xylanase. Xylanase production using P_{GAP}-based systems in *P. pastoris* offers several advantages. Optimization of carbon sources and feeding strategies could lead to enhanced production of xylanase. In this study, glucose was proven to be a better substrate in terms of higher biomass concentration, higher protein titer and xylanase activity. Final xylanase activity was significantly improved to

15000 U/mL. To the best of our knowledge, this is the highest xylanase activity achieved using a P_{GAP}-based system.

5.6 Acknowledgements

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5.7 References

Bajpai P. Application of enzymes in the pulp and paper industry. Biotechnology progress. 1999;15:147-57.

Birijlall N, Manimaran A, Kumar KS, Permaul K, Singh S. High level expression of a recombinant xylanase by *Pichia pastoris* NC38 in a 5 L fermenter and its efficiency in biobleaching of bagasse pulp. Bioresource technology. 2011;102:9723-9.

Çalık P, Ata Ö, Güneş H, Massahi A, Boy E, Keskin A, et al. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochemical Engineering Journal. 2015;95:20-36.

Elgharbi F, Hmida-Sayari A, Zaafouri Y, Bejar S. Expression of an Aspergillus niger xylanase in yeast: Application in breadmaking and in vitro digestion. International journal of biological macromolecules. 2015;79:103-9.

Fan G, Katrolia P, Jia H, Yang S, Yan Q, Jiang Z. High-level expression of a xylanase gene from the thermophilic fungus *Paecilomyces thermophila* in *Pichia pastoris*. Biotechnology letters. 2012;34:2043-8.

Juturu V, Wu JC. Microbial xylanases: engineering, production and industrial applications. Biotechnology advances. 2012;30:1219-27.

Mellitzer A, Weis R, Glieder A, Flicker K. Expression of lignocellulolytic enzymes in *Pichia pastoris*. Microbial cell factories. 2012;11:61.

Polizeli ML, Rizzatti AC, Monti R, Terenzi HF, Jorge JA, Amorim DS. Xylanases from fungi: properties and industrial applications. Applied microbiology and biotechnology. 2005;67:577-91.

Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal. 2012;64:91-105.

Potvin G, Zhang Z, Defela A, Lam H. Screening of alternative carbon sources for recombinant protein production in *Pichia pastoris*. International Journal of Chemical Reactor Engineering. 2016;14:251-7.

Tang S, Boehme L, Lam H, Zhang Z. *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochemical Engineering Journal. 2009;43:157-62.

Xu Y, Wu J, Zheng K, Wu D. A xylanase from Streptomyces sp. FA1: heterologous expression, characterization, and its application in Chinese steamed bread. Journal of industrial microbiology & biotechnology. 2016;43:663-70.

Yang Z, Zhang Z. Codon-optimized expression and characterization of a pH stable fungal xylanase in *Pichia pastoris*. Process Biochemistry. 2017;53:80-7.

Zhao W, Wang J, Deng R, Wang X. Scale-up fermentation of recombinant *Candida rugosa* lipase expressed in *Pichia pastoris* using the GAP promoter. Journal of industrial microbiology & biotechnology. 2008;35:189-95.

Chapter 6: Production of (2R, 3R)-2, 3-butanediol using engineered *Pichia pastoris:* Strain construction, Characterization and Fermentation

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6.1 Abstract

2, 3-butanediol (2, 3-BD) is a bulk platform chemical with various potential applications such as aviation fuel. 2, 3-BD has three optical isomers: (2R, 3R)-, (2S, 3S)- and meso-2, 3-BD. Optically pure 2, 3-BD is a crucial precursor for the chiral synthesis and it can also be used as anti-freeze agent due to its low freezing point. 2, 3-BD has been produced in both native and non-native hosts. Several pathogenic bacteria were reported to produce 2, 3-BD in mixture of its optical isomers including *Klebsiella pneumoniae* and *Klebsiella oxytoca*. Engineered hosts based on episomal plasmid expression such as *Escherichia coli*, *Saccharomyces cerevisiae* and *Bacillus subtilis* are not ideal for industrial fermentation due to plasmid instability. *Pichia pastoris* is generally regarded as safe and is a well-established host for high level heterologous protein production. To produce enantiomerically pure (2R, 3R)-2, 3-BD, we developed a *P. pastoris* strain by introducing a synthetic pathway. The *alsS* and *alsD* gene from *B. subtilis* were codon-optimized and synthesized. The BDH1 gene from *S. cerevisiae* was cloned. These three pathway genes were integrated into the genome of *P. pastoris* and expressed under the control of GAP promoter. Production of (2R, 3R)-2, 3-BD was achieved using glucose as feedstock. The optical purity of (2R, 3R)-2, 3-BD was more than 99%. The titer of (2R, 3R)-2, 3-BD reached 12 g/L with 40 g/L glucose as carbon source in shake flask fermentation. The fermentation conditions including pH, agitation speeds and aeration rates were optimized in batch cultivations. The highest titer of (2R, 3R)-2, 3-BD achieved in fed-batch fermentation using YPD media was 45 g/L. The potential of engineering *P. pastoris* into a microbial cell factory for biofuel production was evaluated in this work using (2R, 3R)-2, 3-BD as an example.

Keywords: *Pichia pastoris*; 2, 3-butanediol; metabolic engineering; fermentation;

6.2 Introduction

Driven by depleting fossil fuels and growing environmental concern, production of bulk chemicals from renewable sources via biosynthesis is becoming increasingly desirable (Ji et al., 2011). 2, 3-butanediol (2, 3-BD) is a crucial platform chemical with enormous applications. It can be converted to other platform chemicals such as methyl ethyl ketone, gamma-butyrolactone and 1, 3-butadiene (Bialkowska, 2016). 2, 3-BD has three stereoisomers: (2R, 3R)-, (2S, 3S)- and meso-2, 3-BD (Celinska and Grajek, 2009). Enantiopure isomers of 2, 3-BD could serve as the precursor for asymmetric synthesis and synthetic rubber. It is also used as anti-freeze agent due to its low freezing point. 2, 3-BD is also a good alternative liquid fuel owing to its high energy capacity. Biosynthesis of 2, 3-BD has been reported in many microorganisms. Native producers belonging to *Enterobacteriaceae* family such as *Klebsiella pneumoniae*, *Klebsiella oxytoca* and *Enterobacter aerogenes* were able to produce high titer of 2, 3-BD in mixture of its optical isomers (Ji et al., 2011). Despite the high titer achieved in those hosts, chiral purity of 2, 3-BD was not satisfactory. Moreover, the use of risk group 2 microorganisms projects a safety concern for industrial fermentation. Production of 2, 3-BD via fermentation of GRAS (generally regarded as safe) bacteria has also been reported. Several *Bacillus* species including *B. subtilis* (Fu et al., 2014), *B. amyloliquefaciens* (Yang et al., 2011) and *B. licheniformis* (Ge et al., 2016) are effective producers of 2, 3-BD. Jurchescu and coworkers reported 144.7 g/L 2,3-BD production using B. *licheniformis* DSM 8785, a risk group 1 bacterium (Jurchescu et al., 2013). *Paenibacillus polymyxa* ZJ9 was reported to produce 36.92 g/L (2R, 3R)-2, 3-BD with 98% purity from Jerusalem artichoke tubers under optimized conditions (Gao et al., 2010). Engineered hosts based on plasmid expression of heterologous pathway genes from the aforementioned microorganisms were reported. The use of non-native hosts such as *Escherichia*

coli (Ji et al., 2015, Tong et al., 2016) and *Saccharomyces cerevisiae* (Kim et al., 2013, Kim et al., 2014, Yamada et al., 2017) offers several advantages due to their well-established genetics, higher metabolic rate, simpler process control and lack of competing pathways. Lian and coworkers reported the production of 100 g/L 2, 3-BD in *S. cerevisiae* using glucose and galactose as carbon source (Lian et al., 2014).

The methylotrophic yeast *Pichia pastoris* has been recognized as an excellent host for heterologous protein production. Its success as a workhorse for recombinant protein production was attributed to the ability to perform post-translational modifications, high secretion efficiency of proteins and simple nutrition requirements (Potvin et al., 2012). It's also ideal for industrial fermentation due to the GRAS status. *P. pastoris* can be grown to high cell density on defined medium using crude glycerol, a by-product of biodiesel production, as carbon source (Tang et al., 2009). *P. pastoris* has been engineered into microbial cell factory for the production of biofuel in recent years. *P. pastoris* was used as whole cell catalysts to produce biodiesel through surface display of lipases (Liu et al., 2014, Liu et al., 2016b). Other bio-products produced by engineered *P. pastoris* included lycopene (Bhataya et al., 2009), β-carotene (Araya-Garay et al., 2012b), xanthophylls (Araya-Garay et al., 2012a), (+)-nootkatone (Wriessnegger et al., 2014), dammarenediol-II (Liu et al., 2015) and lactic acid (de Lima et al., 2016). In this work, we aim to produce chiral pure (2R, 3R)-2, 3-BD in *P. pastoris* using metabolic and process engineering strategies. The potential of *P. pastoris* as a promising host for 2, 3-BD production was demonstrated for the first time.

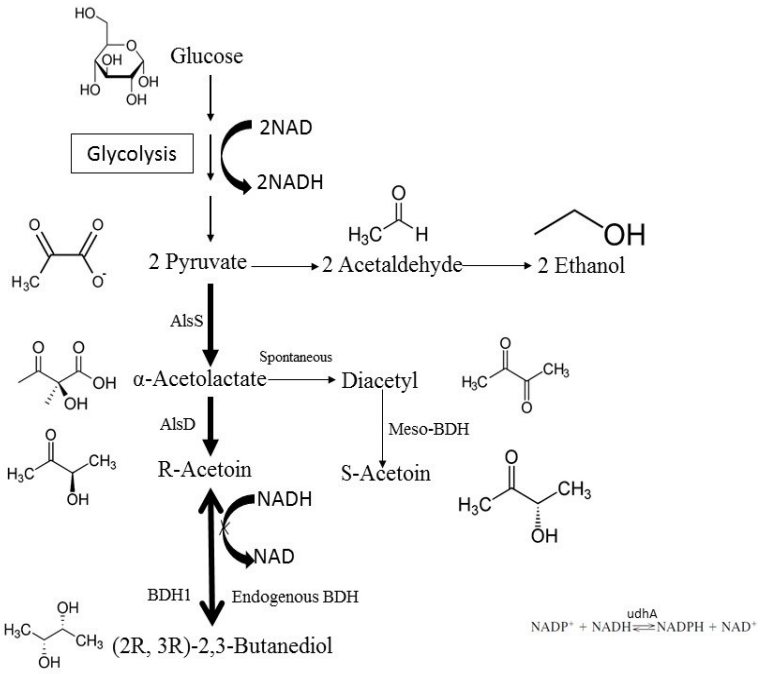


Fig. 6-1 Engineered metabolic pathway for 2, 3-BD synthesis in this work. The glycolysis pathway enzymes were omitted for simplicity. The 2, 3-BD synthesis pathway was indicated by bold arrows.

6.3 Materials and methods

6.3.1 Strains, plasmids and reagents

E. coli XL1-Blue was used for plasmid cloning and propagation. *P. pastoris* X33 was used as host for metabolic engineering. *E. coli* was grown in low salt LB broth (1% peptone, 0.5% yeast extract and 0.5% sodium chloride) supplemented with 25 μg/mL of Zeocin where appropriate. *P. pastoris* was cultivated in YPD medium (1% yeast extract, 2% peptone and 2% dextrose) with 100 μg/mL of Zeocin. YPDS plates consisting of 1% yeast extract, 2% peptone, 2% dextrose, 18% sorbitol and 2% agar were used for yeast transformant screening. Plasmid pGAPZαA (Invitrogen, USA) was used for gene cloning. Chemicals of analytical grade and restriction enzymes used in this work were purchased from Sigma-Aldrich (USA) or Fisher scientific (Canada). DNA primers were ordered from Invitrogen (USA) and nucleotide sequences of primers were listed in

Table 6-1. *E. coli* or yeast genomic DNA was purified using Gentra Puregene Yeast/Bac. Kit (Qiagen, Germany). Polymerase chain reaction (PCR) was performed using KAPA HiFi Hotstart Readymix PCR Kit (Kapa Biosystems, USA).

Table 6-1 Primers used in this study

Primer name	Sequence (5'-3')
AlsS-f	CGGGGTACCATGTTGACTAAGGCTACTAAGGAACAAA
AlsS-r	TCCCCGCGGTTACAAAGCTTTAGTCTTCATCAAC
AlsD-f	CGGAATTCAAAATGAAGAGAGAGTCCAACATCCAAG
AlsD-r	CGGGGTACCTTATTCTGGTGATCCCTCGGTTGTT
BDH1-f	CGGAATTCAAAATGAGAGCTTTGGCATATTTCAAGA
BDH1-r	CGGGGTACCTTACTTCATTTCACCGTGATTGTTA
udhA-f	CGGGGTACCAAAATGCCACATTCCTACGATTACGATG
udhA-r	TGCTCTAGATTAAAACAGGCGGTTTAAACCGTTT
HIS4-f	GAAGATCTATGACATTTCCCTTGCTACCTGC
HIS4-r	CGGGATCCTTAAATAAGTCCCAGTTTCTCCATACG

Restriction sites were underlined

6.3.2 DNA manipulation

To clone the 2, 3-BD biosynthesis genes under the control of GAP promoter, pGAPZαA was digested with *Bsp*119 I and *Eco*R I to remove the α-signal. The large fragment was gel-purified and blunted by DNA blunting enzyme (CloneJET PCR cloning kit, Thermo scientific, USA). The treated fragment was self-ligated to result in a plasmid designated pGAPZ which is used for gene expression in this work. DNA sequences of biosynthesis pathway genes *B. subtilis* 168 *alsS* and *alsD* were retrieved from NCBI. Coding sequences of *alsS* and *alsD* were codon-optimized according to the *P. pastoris* codon usage to achieve better expression. The optimized genes were synthesized by Genscript (USA) and inserted into vector pUC57, resulting in pUC57-*alsS* and pUC57-*alsD*, respectively. *AlsS* gene was amplified from pUC57-*alsS* using AlsS-f and AlsS-r. The PCR product was digested with *Kpn* I and *Sac* II and ligated into pGAPZ digested with the same enzymes to obtain pGAPZ-*alsS*. *AlsD* was cloned into pGAPZ to result in pGAPZ-*alsD*

with the same manner using *Eco*R I and *Kpn* I. 2, 3-butanediol dehydrogenase gene BDH1 was amplified from *S. cerevisiae* genomic DNA using BDH1-f and BDH1-r and cloned into pGAPZ between *Eco*R I and *Kpn* I to obtain pGAPZ-BDH1. pGAPZ-*alsD* was digested with *Bam*H I and *Bgl* II to obtain a fragment containing P_{GAP}-*alsD*-tAOX1. pGAPZ-*alsS* was linearized with *Bam*H I and ligated with P_{GAP}-*alsD*-tAOX1 to result in plasmid pGAPZ-SD. BDH1 was cloned into pGAPZ-SD in the same manner to result in pGAPZ-SDB. To integrate the pathway genes into HIS4 locus of the *P. pastoris* genome, HIS4 gene was PCR amplified using primers HIS4-f and HIS4-r and *P. pastoris* X33 genomic DNA as template. HIS4 was cloned into pGAPZ-SD and pGAPZ-SDB to result in pGAPZ-SDH and pGAPZ-SDBH, respectively. *E. coli udhA* gene was amplified from *E. coli* XL1-blue genomic DNA using udhA-f and udhA-r. Gene *udhA* was first cloned into pGAPZ to result in pGAPZ-*udhA* and then inserted into pGAPZ-SD to obtain pGAPZ-SDU. HIS4 was inserted into pGAPZ-SDU to result in pGAPZ-SDUH (Table 2). All plasmid constructs were confirmed by PCR and sequencing.

6.3.3 Yeast transformation and screening

P. pastoris X33 was made competent and transformed with various plasmids using an electroporator (Eppendorf, Canada) according to the manufacturer′s instructions. Briefly, 5 μg of pGAPZ-SDH, pGAPZ-SDBH and pGAPZ-SDUH were linearized with *Nhe* I and transformed into competent *P. pastoris* X33 cells to obtain strain X33-SD, X33-SDB and X33-SDU, respectively (Table 6-2). Yeast transformants were screened on YPDS plates by incubating at 30˚C for 2-3 days. Yeast colonies were picked up and grown in YPD media. Genomic DNA was extracted and used as PCR templates for the confirmation of pathway gene integration.

Table 6-2 Plasmids and strains used in this study

Strains or plasmids	Description	Source
E. coli XL1-blue	recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F′ proAB lacIq ZΔM15 Tn10 (Tetr)]	Lab stock
P. pastoris strains		
X33	Wilde type	Lab stock
X33-SD	*P. pastoris* X33 harbouring codon-optimized *alsS* and *alsD* gene	This study
X33-SDB	*P. pastoris* X33 harbouring codon-optimized *alsS* and *alsD* and *S. cerevisiae* BDH1 gene	This study
X33-SDU	*P. pastoris* X33 harbouring codon-optimized *alsS* and *alsD* and *E. coli udhA* gene	This study
Plasmids		
pGAPZαA	GAP promoter, α-signal, Zeocin resistance	Invitrogen
pGAPZ	GAP promoter, Zeocin resistance	This study
pGAPZ-AlsS	pGAPZ harbouring *alsS* gene	This study
pGAPZ-AlsD	pGAPZ harbouring *alsD* gene	This study
pGAPZ-BDH1	pGAPZ harbouring BDH1 gene	This study
pGAPZ-udhA	pGAPZ harbouring *udhA* gene	This study
pGAPZ-SD	pGAPZ harbouring *alsS* and *alsD* genes	This study
pGAPZ-SDB	pGAPZ harbouring *alsS*, *alsD* and BDH1 genes	This study
pGAPZ-SDU	pGAPZ harbouring *alsS*, *alsD* and *udhA* genes	This study
pGAPZ-SDH	pGAPZ harbouring *alsS*, *alsD* and HIS4 genes	This study
pGAPZ-SDBH	pGAPZ harbouring *alsS*, *alsD*, BDH1 and HIS4 genes	This study
pGAPZ-SDUH	pGAPZ harbouring *alsS*, *alsD*, *udhA* and HIS4 genes	This study

6.3.4 Shake flask cultivation

Single yeast colony was inoculated into 10 mL YPD medium in 50 mL tube and grown overnight. Shake flask cultivation was performed by inoculating 1 mL of overnight culture into 100 mL YPD media containing various concentrations of glucose in a 500 mL shake flask. Aliquots were taken every 4-6 h. Samples were centrifuged at 13000 rpm for 5 min. Supernatant was filtered through 0.22 μm filter and used for further analysis.

6.3.5 Batch and fed-batch cultivation

Fermentation of *P. pastoris* strain was performed in 5 L bioreactors (Bioflo 320, Eppendorf, Canada). YP media (10 g/L yeast extract and 20 g/L peptone) containing various concentrations

of glucose were used for cultivation. Bioreactor inoculum was prepared by streaking a single colony from the plate and growing in a 500 mL shake flask containing 100 mL YPD media overnight (30 °C and 250 rpm).

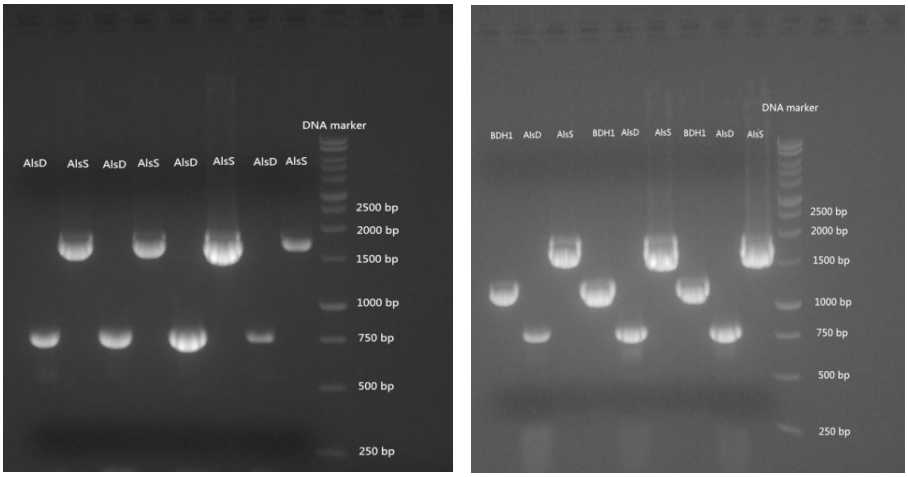
6.3.6 Analytical methods

Biomass was monitored by measuring optical density at 600 nm (OD_{600}) with Ultraspec 60 (UK). Glucose concentration was measured using YSI2900 Bio-analyzer (Mandel, Canada) installed with a glucose membrane. 2, 3-BD isomers were differentiated using gas chromatography (GC, Agilent 6850 series, Santa Clara, CA, USA) equipped with flame ionized detector (FID). For GC-FID analysis, yeast cultures were centrifuged at 13000 rpm for 5 min. The supernatant was extracted with equal volume of ethyl acetate. Organic phase was dehydrated with sodium sulfate before GC analysis. The capillary GC column (Supelco Astec CHIRALDEX^{TM} B-PM, 35 m × 0.25 mm × 0.12 μm) was used in this study. Helium was used as carrier gas with a flow rate of 2 mL/min. The injector and detector were kept at 250 °C. Injection volume was 1 μL. Oven temperature program (Liu et al., 2016a) was as follows: 1.5 min at 50°C, programmed to increase to 160 °C at a rate of 8.8 °C/min and hold for 5 min at 160 °C. Extracellular metabolites were determined by high performance liquid chromatography (HPLC, Agilent 1200 series, Santa Clara, CA, USA) equipped with a Shodex Sugar SH1011 column (8 mm ID × 300 mm, 6 μm; Showa Denko, Tokyo, Japan). The column was maintained at 60 °C and eluted with 5mM sulfuric acid at a flow rate of 0.6 mL/min. Acetoin was detected with variable wavelength detector at 214 nm. Other metabolites were detected with refractive index detector.

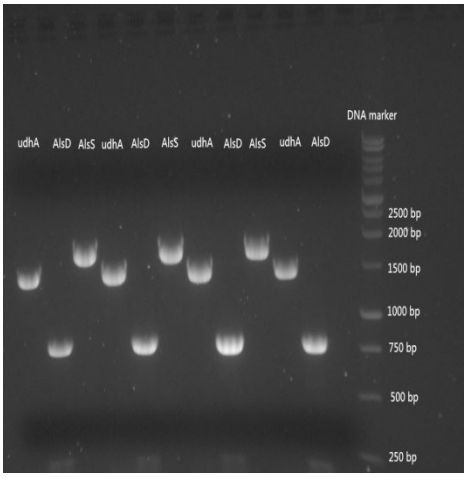
6.4 Results and discussion

6.4.1 Construction of P. pastoris strains for the production of (2R, 3R)-2, 3-BD

P. pastoris is an industrially relevant host for recombinant protein production and has not been reported to produce 2, 3-BD from glucose up to date. Previous study described the conversion of acetoin to 2, 3-BD using *P. pastoris* through NADH regeneration (Schroer et al., 2010). 2, 3-BD is produced from pyruvate via three enzymatic steps (Fig. 6-1). To introduce a synthetic route for 2,3-BD production in *P. pastoris*, *B. subtilis* α-acetolactate synthase AlsS, *B. subtilis* α-acetolactate decarboxylase AlsD and *S. cerevisiae* (2R, 3R)-2,3-BD dehydrogenase BDH1 were selected to direct the carbon flux towards 2,3-BD synthesis. Constitutive promoter P_{GAP} is commonly used for protein expression in *P. pastoris* due to its high level constitutive expression and growth-associated product formation (Çalık et al., 2015). The three pathway genes were cloned under the control of P_{GAP} for constitutive expression. Codon optimization of *alsS* and *alsD* was performed to achieve better expression in *P. pastoris*. The pathway genes were assembled into a single plasmid for chromosome integration at the HIS4 locus. As shown in Fig. 6-2, *alsS*, *alsD*, *udhA* and BDH1 were cloned into vector pGAPZ and successfully integrated into *P. pastoris* genome via homologous recombination. The constructed strains were grown in YPD media to verify 2, 3-BD production. The optical purity of 2, 3-BD is determined by the stereo-specificity of 2, 3-BD dehydrogenase (Fig. 6-3). As shown in Fig. 6-3, recombinant *P. pastoris* strains X33-SD, X33-SDB and X33-SDU were able to produce entianopure (2R,3R)-2,3-BD using glucose as feedstock. Optical purity was determined to be over 99%. Meso-2, 3-BD and (2S, 3S)-2, 3-BD were not detected, indicating the stereo-specificity of endogenous 2, 3-butanediol dehydrogenase (2, 3-BDH) is specific for (2R, 3R)-2, 3-BD synthesis.



(a) (b)



(c)

Fig. 6-2 PCR confirmation of constructed strains. (a) PCR verification of X33-SD. Genomic DNA of four yeast colonies were extracted and used as templates for PCR. (b) PCR verification of X33-SDB. Genomic DNA of three yeast colonies were extracted and used as templates for PCR. (c) PCR verification of X33-SDU. Genomic DNA of four yeast colonies were extracted and used as templates for PCR. DNA bands and DNA markers were indicated on the pictures.

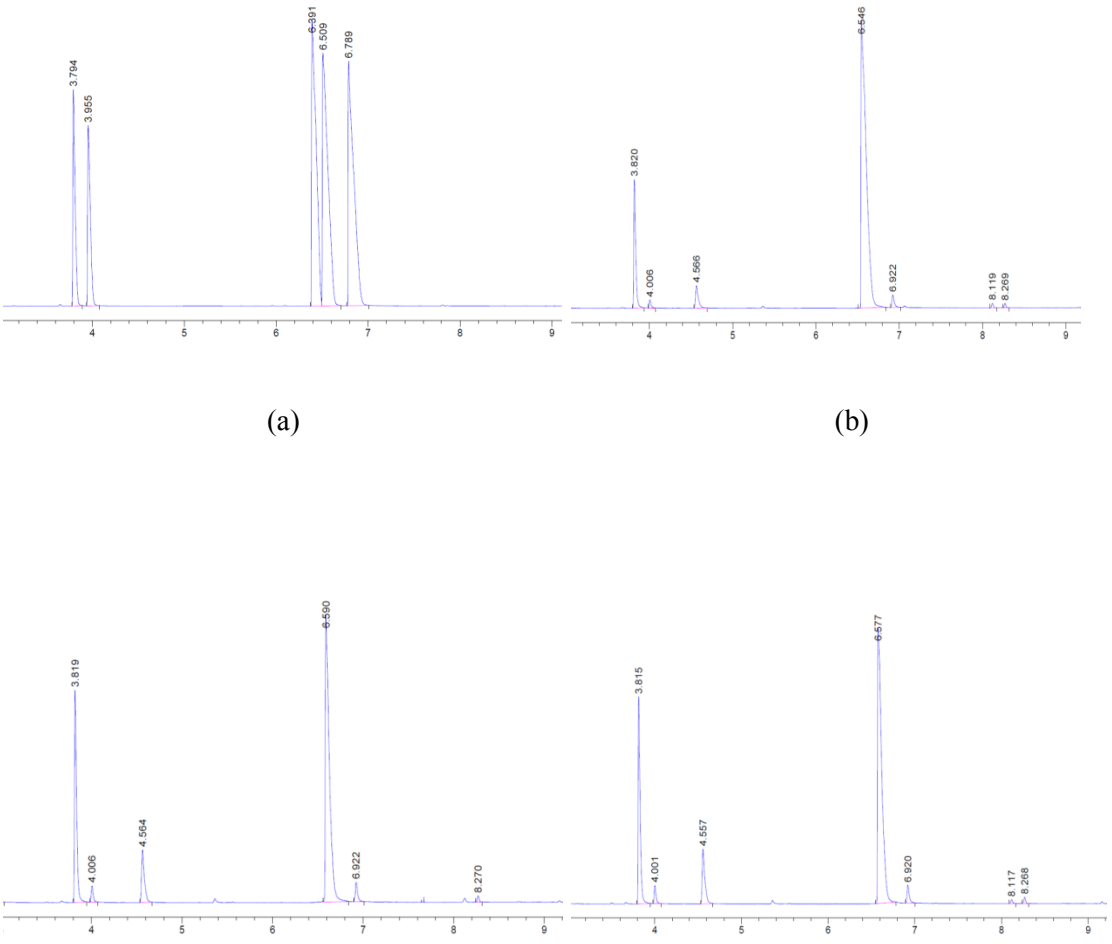


Fig. 6-3 GC profiles of metabolites in different strains. (a) GC profile of standards. Retention times: 3S-acetoin, 3.8 min; 3R-actoin, 4.0 min; (2S, 3S)-2, 3-BD, 6.4 min; (2R, 3R)-2, 3-BD, 6.5 min; Meso-2, 3-BD, 6.8 min. (b) GC profile of extract of culture of X33-SD. (c) GC profile of extract of culture of X33-SDB. (d) GC profile of extract of culture of X33-SDU.

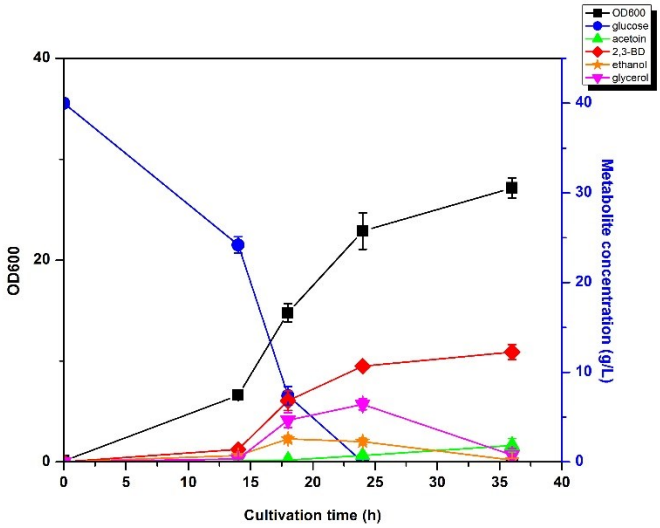
6.4.2 Screening of different strains for high 2, 3-BD production

Three strains constructed in this work were cultivated to evaluate their potential for 2, 3-BD production in shake flasks containing 40 g/L glucose (Fig. 6-4). Yeast cultures were grown for 36 h and aliquots were analyzed. As shown in Fig. 6-4, glucose was depleted at 24 h. Major by-products detected in the broth were glycerol, acetoin and ethanol. No acetic acid was detected in

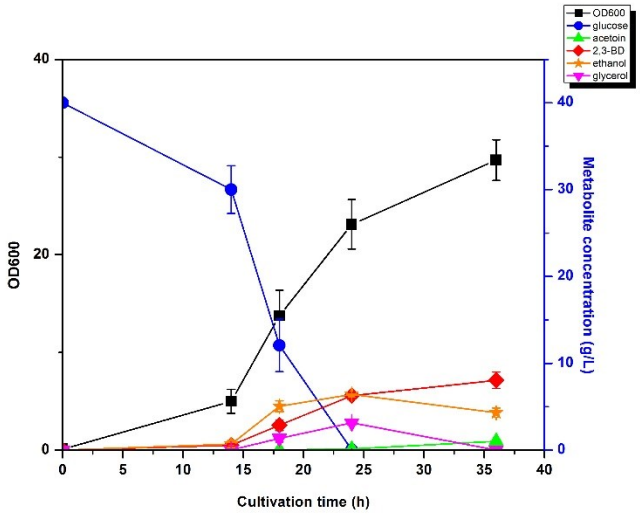
all three strains. Cell growth was not significantly affected with the synthetic pathway. Strain X33-SD, X33-SDB and X33-SDU produced 2, 3-BD with a titer of 12.24 g/L, 8.04g/L and 7.44 g/L, respectively. Surprisingly, overexpression of *S. cerevisiae* BDH1 was not beneficial to boost 2, 3-BD production. This result is contradictory with previous study (Schroer et al., 2010). The *E. coli udhA* gene for NADH regeneration from NADPH did not improve 2, 3-BD titer as well. The highest acetoin titer was observed in strain X33-SD, reaching 1.84 g/L. Acetoin consists mainly of S-acetoin with minor R-acetoin detected based on the GC analysis (Fig. 6-3). Despite *P. pastoris* is generally recognized as Crabtree effect-negative, ethanol is produced as a by-product under anaerobic condition. Wild type *P. pastoris* X33 produced 15 g/L ethanol after 36 h cultivation (data not shown). The highest ethanol titer of X33-SD, X33-SDB and X33-SDU was detected at 24 h, with 2.5 g/L, 6.4 g/L and 4.9 g/L, respectively. Strain X33-SD produced 0.21 g/L ethanol after 36 h, in contrast with 4.3 g/L and 1.99 g/L obtained with X33-SDB and X33-SDU, respectively. Ethanol titer decreased at the end of cultivation because it could be consumed after glucose depletion. Compared with wild type *P. pastoris* X33, the heterologous 2, 3-BD synthetic pathway effectively redirected the carbon flux from ethanol formation towards 2, 3-BD synthesis.

Glycerol was produced as a major by-product as a channel for the regeneration of NAD^+ in engineered *S. cerevisiae* for the production of 2, 3-BD (Kim and Hahn, 2015). Notably, 6.42 g/L, 3.14 g/L and 2.94 g/L glycerol was accumulated in strain X33-SD, X33-SDB and X33-SDU, respectively. Glycerol has been commonly used as carbon source in the high cell density fermentation of *P. pastoris*. Production of glycerol in *P. pastoris* has never been reported in literature. Analysis of the *P. pastoris* genome sequence could offer a possible mechanism for glycerol formation. Two putative glycerol-3-phosphate dehydrogenases (GPDs) were present in

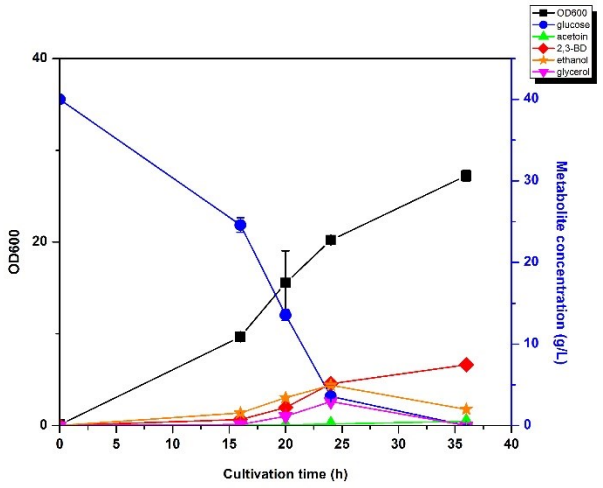
P. pastoris genome. Putative GPD (PAS_ch2_2_0111) shares 57% amino acid similarity with GPD2 in *S. cerevisiae*. Further investigation is imperative to verify the enzyme activity of the putative GPDs in *P. pastoris*. Strain X33-SD is the most promising strain in terms of highest 2, 3-BD production and least ethanol accumulation and is thus used for further study.



(a)



(b)



(c)

Fig. 6-4 Screen of yeast strains in shake flask cultivation. (a) Time course of X33-SD cultivation in 500 mL shake flask containing 100 mL YPD media. (b) Time course of X33-SDB cultivation in 500 mL shake flask containing 100 mL YPD media. (c) Time course of X33-SDU cultivation in 500 mL shake flask containing 100 mL YPD media. Error bars indicate the standard deviation of three replicate experiments.

6.4.3 Effect of glucose concentration

Yeast strain X33-SD was cultivated in shake flasks with YPD medium containing different initial concentrations of glucose. As shown in Fig. 6-5, higher titer of 2,3-BD was obtained at higher glucose concentration, reaching 5.64 g/L, 9.22 g/L and 12.24 g/L with glucose concentration of 20 g/L, 30 g/L and 40 g/L, respectively. Higher concentrations of ethanol and glycerol were also observed with higher glucose concentrations. Acetoin was detected below 1 g/L for all three glucose concentrations. The 2, 3-BD yield on glucose remained about 0.3 g/g for the three glucose concentrations tested, which is 60% of the theoretical yield (0.5 g/g).

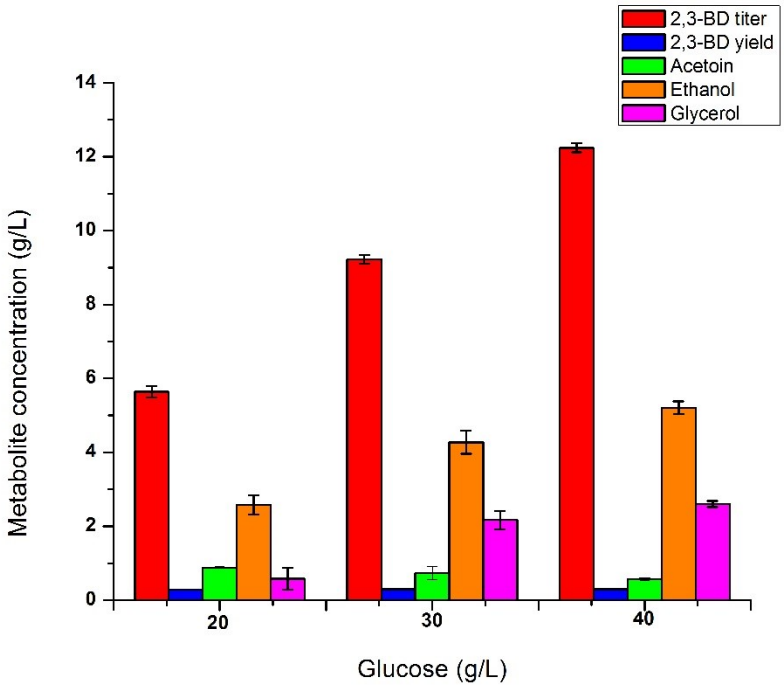


Fig. 6-5 Effect of glucose concentration. Strain X33-SD was cultured in 500 mL shake flask containing 100 mL YP media containing 20 g/L, 30 g/L and 40 g/L glucose. The flasks were incubated at 30°C and 250 rpm. Error bars indicate standard deviation of three replicate experiments.

6.4.4 Effect of agitation

Bioreactor cultivations of strain X33-SD were performed to investigate its potential for large-scale production of (2R, 3R)-2, 3-BD. Fermentation conditions such as agitation speeds, aeration rates and pH were optimized under controlled conditions. Effect of agitation was illustrated in Fig. 6-6. Three agitation speeds were examined: 300 rpm, 400 rpm and 500 rpm. It was found that metabolite profile was closely related to agitation speed. Production of 2, 3-BD was detected under 300 rpm and 400 rpm but not detected under 500 rpm. The highest titer of 2, 3-BD was achieved at 300 rpm, reaching 8.34 g/L. Acetoin was the major product at 500 rpm with a titer of 9.27 g/L. Glycerol and ethanol were accumulated only at 300 rpm. The highest biomass concentration was obtained at 500 rpm with an OD_{600} of 42. 35, compared with 23.65 and 34.8 at

300 rpm and 400 rpm, respectively. It was reported that lower dissolved oxygen (DO) level favours the reduction of acetoin to 2, 3-BD. DO is normally controlled by cascading agitation and aeration where agitation is more prominent to DO control. Glycerol formation is associated with the synthesis of 2, 3-BD. Optimization of agitation speed for improved production of 2, 3-BD has been reported in previous studies. Xu and coworkers optimized the agitation speed within the range of 200 rpm to 500 rpm using engineered *E. coli* and found that 400 rpm was optimal in terms of high biomass accumulation and 2, 3-BD production (Xu et al., 2014). Two stage agitation control strategy has been implemented to cultivate *K. oxytoca* to achieve a balance between biomass production and 2, 3-BD synthesis. Agitation was maintained at 300 rpm in the first 15 h for biomass growth and lowered to 200 rpm for the production of 2, 3-BD (Ji et al., 2009). Two stage agitation improved the titer by 6.2% compared with constant speed of 200 rpm. The agitation-associated metabolite profile suggests that strain X33-SD could be used to produce acetoin under higher agitation speed.

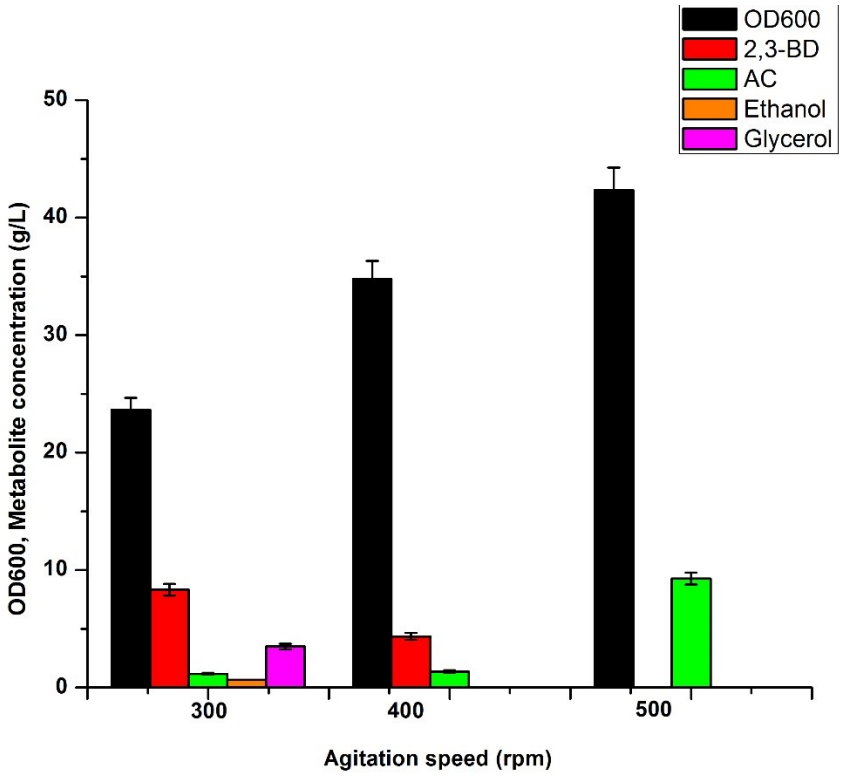


Fig. 6-6 Effect of agitation. Strain X33-SD was cultivated in 3 L YPD media in 5 L Bioflo320 reactors. Glucose concentration was 40 g/L. Cultivation was performed at 30 °C, 1.5vvm and pH 5.0. Error bars indicate standard deviation of duplicate experiments.

6.4.5 Effect of aeration

The effect of agitation on the production of 2, 3-BD was conducted with an aeration rate of 1.5 vvm. Aeration is another major factor to determine DO level. We tested three aeration rates: 0.1 vvm, 0.5 vvm and 1 vvm. As was shown in Fig. 6-7, higher aeration resulted in higher biomass production. OD_{600} reached 25.1 at 1vvm compared with 17.6 at 0.1 vvm. The titer of 2, 3-BD obtained at 0.1 vvm was slightly higher than that at 0.5 vvm, reaching 11.07 g/L and 10.78 g/L, respectively. Only 8.87 g/L 2, 3-BD was detected at 1 vvm. Acetoin was produced at 0.97 g/L, 1.6 g/L and 1.89 g/L, respectively. The highest glycerol concentration of 4.95 g/L was observed

at 0.1 vvm. Ethanol was not detected at 1 vvm while 0.46 g/L and 2.43 g/L was produced at 0.5 vvm and 1 vvm, respectively. Aeration rate of 0.5 vvm was used further study for high production of biomass and 2, 3-BD.

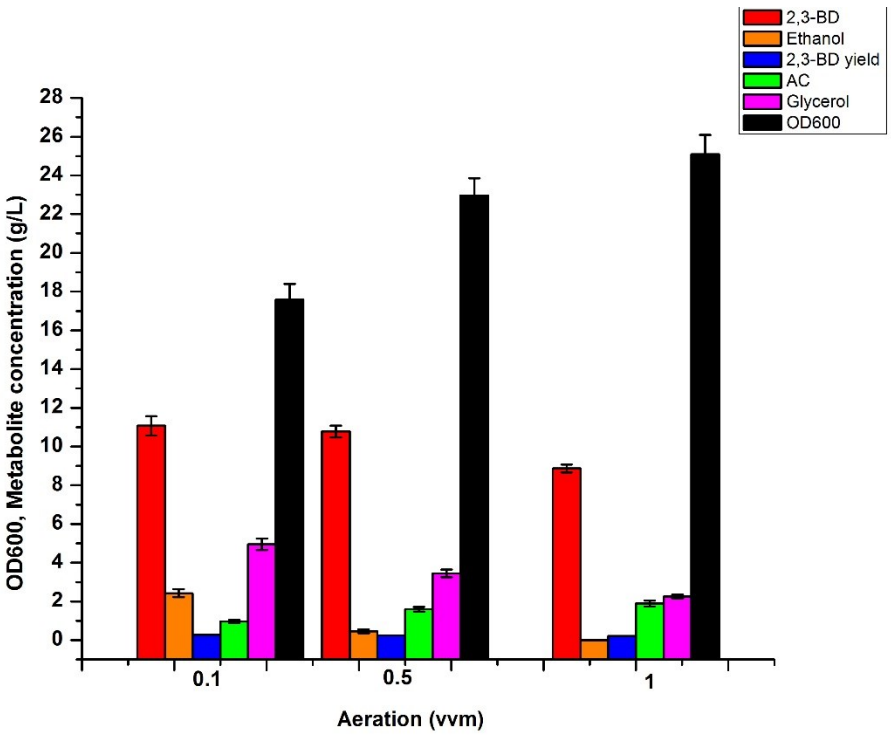


Fig. 6-7 Effect of aeration. Strain X33-SD was cultivated in 3L YPD media in 5L Bioflo320 reactors. Glucose concentration was 40 g/L. Cultivation was performed at 30 °C, 300 rpm and pH 5.0. Error bars indicate standard deviation of duplicate experiments.

6.4.6 Effect of pH

The acidity of fermentation is critical to the cell growth and metabolite production. In the case of 2, 3-BD production, it was reported that the formation of neutral 2, 3-BD could be a countermeasure against acidification. It was found that the production of 2, 3-BD was triggered by pH drop in *K. pneumoniae* G31 (Petrov and Petrova, 2010). Forced pH fluctuation was implemented to increase the titer of 2, 3-BD to 70 g/L in comparison with 52.5 g/L without pH

control. In this work, we examined the effect of pH through cultivation ranging from pH4 to pH6 and without pH control. As was illustrated in Fig. 6-8, the production of 2, 3-BD was not affected by the pH, reaching a final titer of about 10.8 g/L. The highest acetoin titer was observed at pH6. The lowest glycerol concentration was detected at pH6, reaching 0.46 g/L. At pH4, cell growth was significantly retarded (data not shown). When pH was not controlled, pH value dropped from initial pH6.8 to pH5.2 at the end of the batch. In previous studies, pH was reported to have an impact on the activity of acetoin reductase. Through a two-stage pH control strategy, cell growth of *K. oxytoca* was favoured in the first stage where pH was uncontrolled and maintained at a set pH for 2, 3-BD production (Cho et al., 2015). The results obtained in this study indicate that *P. pastoris* could be a robust host for the production of 2, 3-BD within a wide range of pH. We selected pH5 for fed-batch cultivation as optimal cell growth was achieved under this pH value.

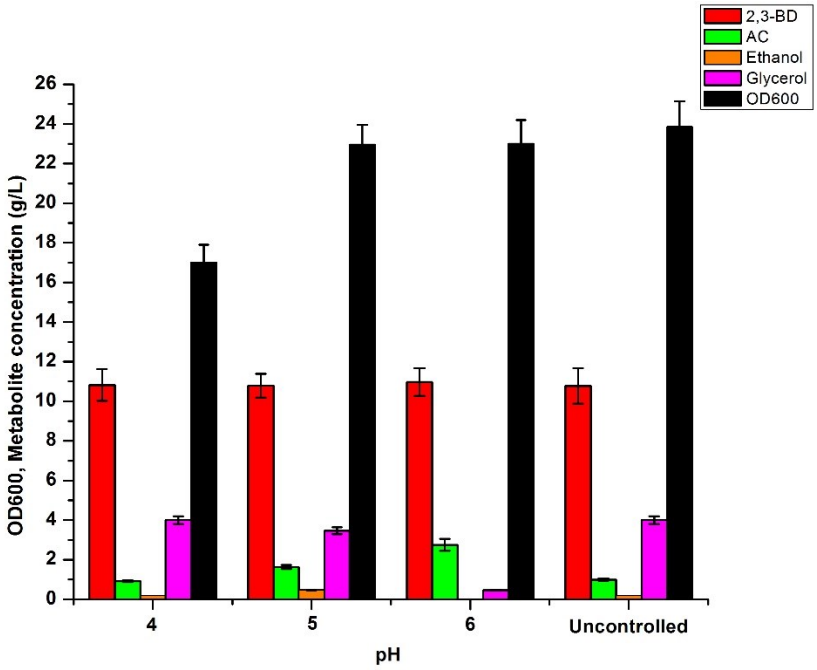


Fig. 6-8 Effect of pH. Strain X33-SD was cultivated in 3 L YPD media in 5 L Bioflo320 reactors. Glucose concentration was 40 g/L. Cultivation was performed at 30 °C, 0.5vvm and 300 rpm. Error bars indicate standard deviation of duplicate experiments.

6.4.7 Fed-batch cultivation

To scale up the production of 2, 3-BD using strain X33-SD, fed-batch cultivation was performed. Batch cultivation was started in 2 L YPD media with initial glucose concentration of 40 g/L. The time course of fed-batch fermentation was shown in Fig. 6-9. Glucose was depleted at 19 h and feeding solution consisting of 500 g/L glucose was fed to the bioreactor. Fermentation lasted for 135 h with a final volume of approximately 2.5 L. Biomass and the titer of 2, 3-BD steadily increased. The final OD_{600} reached 42.5 at the end of fed-batch. The production of 2, 3-BD stagnated after 100 h and a final titer of 45.8 g/L was achieved. Ethanol was detected below 1 g/L. Final acetoin concentration was found to be 15.9 g/L. Glycerol production was 17.5 g/L at 66 h and remained unchanged at the end of cultivation. The yield of 2, 3-BD on glucose of the

whole fed-batch cultivation was 0.197 g/g, which is 39.4% of the theoretical yield. The productivity achieved in fed-batch fermentation was 0.34 g L^{-1} h^{-1}. Compared with shake flask cultivation, 2, 3-BD yield obtained in fed-batch cultivation was relatively low. This could be due to the inhibitory effect of much higher concentration of glycerol and 2, 3-BD in fed-batch cultivation. Moreover, YPD might not be a suitable medium for long time fermentation. Medium optimization could be performed to boost the titer and yield of 2, 3-BD in future work.

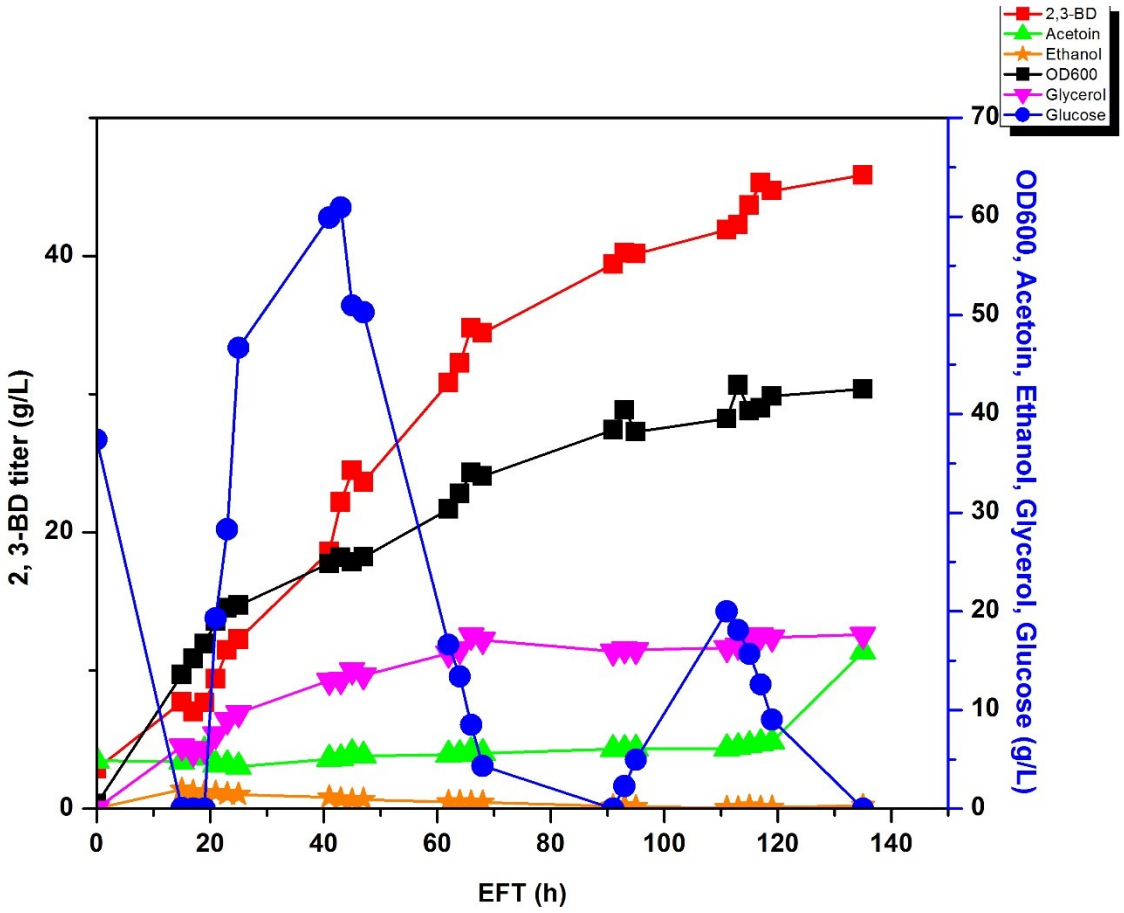


Fig. 6-9 Time course of fed-batch cultivation. Fermentation was carried out with 2 L YPD media at 30 °C, 0.5vvm, 300 rpm and pH 5.0. 1 L of 500 g/L glucose was fed to the bioreactor upon the depletion of initial glucose.

A comparison of the production of (2R, 3R)-2, 3-BD was summarized in Table 3. Recent efforts on the production of (2R, 3R)-2, 3-BD has achieved great success. Titer up to 150 g/L has been reported in engineered *S. cerevisiae*. However, those systems were mainly plasmid-dependent and thus might cause concern of plasmid instability. Gene expression in *P. pastoris* was based on genome-targeting cassette and eliminated the need of episomal plasmids. Combined with the high growth rate, high alcohol tolerance and GRAS status, engineered *P. pastoris* could become a robust host for the production of 2, 3-BD.

Table 6-3 Comparison of (2R, 3R)-2,3-BD production in various microorganisms

Species	Titer (g/L)	Operation strategy	Entiano purity (%)	Feed stock	2,3-BD productivity (g/L/h)	Reference
S. cerevisiae	43.6	Fed-batch	97	Xylose	0.15	(Kim et al., 2014)
S. cerevisiae	96.2	Fed-bacth	NA	Glucose	0.39	(Kim et al., 2013)
S. cerevisiae	154.3	Fed-batch	NA	Glucose	1.97	(Kim et al., 2016)
S. cerevisiae	100	Fed-bacth	98	Glucose and galactose	0.33	(Lian et al., 2014)
E. coli	6.9	Shake flask	99	Glucose	0.14	(Yan et al., 2009)
E. coli	115	Fed-batch	99	Glucose	1.44	(Ji et al., 2015)
E. aerogenes	152	Fed-batch	97.5	Glucose and xylose	3.5	(Li et al., 2015)
B. subtilis	49.29	Shake flask	99	Glucose	0.224	(Fu et al., 2014)
P. polymyxa	36.92	Batch	98	Raw inulin extract	0.88	(Gao et al., 2010)
P. pastoris	45	Fed-batch	99	Glucose	0.34	This study

6.5 Conclusions

P. pastoris was engineered into a microbial cell factory to produce bulk chemical (2R, 3R)-2, 3-BD using glucose as feedstock for the first time. The endogenous 2, 3-BDH could support efficient conversion of acetoin to 2, 3-BD. The titer of (2R, 3R)-2, 3-BD reached 45.8 g/L in a fed-batch cultivation under optimized fermentation conditions. *P. pastoris* was proved a versatile platform for biofuel production other than heterologous protein production. Medium optimization could be conducted to further boost the titer of 2, 3-BD.

6.6 Acknowledgements

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6.7 References

Araya-Garay JM, Ageitos JM, Vallejo JA, Veiga-Crespo P, Sánchez-Pérez A, Villa TG. Construction of a novel *Pichia pastoris* strain for production of xanthophylls. AMB Express. 2012a;2:24.

Araya-Garay JM, Feijoo-Siota L, Rosa-dos-Santos F, Veiga-Crespo P, Villa TG. Construction of new *Pichia pastoris* X-33 strains for production of lycopene and beta-carotene. Applied microbiology and biotechnology. 2012b;93:2483-92.

Bhataya A, Schmidt-Dannert C, Lee PC. Metabolic engineering of *Pichia pastoris* X-33 for lycopene production. Process Biochemistry. 2009;44:1095-102.

Bialkowska AM. Strategies for efficient and economical 2,3-butanediol production: new trends in this field. World journal of microbiology & biotechnology. 2016;32:200.

Çalık P, Ata Ö, Güneş H, Massahi A, Boy E, Keskin A, et al. Recombinant protein production in *Pichia pastoris* under glyceraldehyde-3-phosphate dehydrogenase promoter: From carbon source metabolism to bioreactor operation parameters. Biochemical Engineering Journal. 2015;95:20-36.

Celinska E, Grajek W. Biotechnological production of 2,3-butanediol--current state and prospects. Biotechnology advances. 2009;27:715-25.

Cho S, Kim T, Woo HM, Lee J, Kim Y, Um Y. Enhanced 2,3-Butanediol Production by Optimizing Fermentation Conditions and Engineering *Klebsiella oxytoca* M1 through Overexpression of Acetoin Reductase. PloS one. 2015;10:e0138109.

de Lima PB, Mulder KC, Melo NT, Carvalho LS, Menino GS, Mulinari E, et al. Novel homologous lactate transporter improves L-lactic acid production from glycerol in recombinant strains of *Pichia pastoris*. Microbial cell factories. 2016;15:158.

Fu J, Wang Z, Chen T, Liu W, Shi T, Wang G, et al. NADH plays the vital role for chiral pure D-(-)-2,3-butanediol production in *Bacillus subtilis* under limited oxygen conditions. Biotechnology and bioengineering. 2014;111:2126-31.

Gao J, Xu H, Li Q-j, Feng X-h, Li S. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce R, R-2, 3-butanediol. Bioresource technology. 2010;101:7076-82.

Ge Y, Li K, Li L, Gao C, Zhang L, Ma C, et al. Contracted but effective: production of enantiopure 2,3-butanediol by thermophilic and GRAS *Bacillus licheniformis*. Green Chem. 2016;18:4693-703.

Ji X-J, Huang H, Du J, Zhu J-G, Ren L-J, Hu N, et al. Enhanced 2, 3-butanediol production by *Klebsiella oxytoca* using a two-stage agitation speed control strategy. Bioresource technology. 2009;100:3410-4.

Ji XJ, Huang H, Ouyang PK. Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnology advances. 2011;29:351-64.

Ji XJ, Liu LG, Shen MQ, Nie ZK, Tong YJ, Huang H. Constructing a synthetic metabolic pathway in *Escherichia coli* to produce the enantiomerically pure (R, R)-2,3-butanediol. Biotechnology and bioengineering. 2015;112:1056-9.

Jurchescu IM, Hamann J, Zhou X, Ortmann T, Kuenz A, Prusse U, et al. Enhanced 2,3-butanediol production in fed-batch cultures of free and immobilized *Bacillus licheniformis* DSM 8785. Applied microbiology and biotechnology. 2013;97:6715-23.

Kim JW, Kim J, Seo SO, Kim KH, Jin YS, Seo JH. Enhanced production of 2,3-butanediol by engineered *Saccharomyces cerevisiae* through fine-tuning of pyruvate decarboxylase and NADH oxidase activities. Biotechnology for biofuels. 2016;9:265.

Kim S, Hahn JS. Efficient production of 2,3-butanediol in *Saccharomyces cerevisiae* by eliminating ethanol and glycerol production and redox rebalancing. Metabolic engineering. 2015;31:94-101.

Kim SJ, Seo SO, Jin YS, Seo JH. Production of 2,3-butanediol by engineered *Saccharomyces cerevisiae*. Bioresource technology. 2013;146:274-81.

Kim SJ, Seo SO, Park YC, Jin YS, Seo JH. Production of 2,3-butanediol from xylose by engineered *Saccharomyces cerevisiae*. Journal of biotechnology. 2014;192 Pt B:376-82.

Li L, Li K, Wang Y, Chen C, Xu Y, Zhang L, et al. Metabolic engineering of *Enterobacter cloacae* for high-yield production of enantiopure (2R,3R)-2,3-butanediol from lignocellulose-derived sugars. Metabolic engineering. 2015;28:19-27.

Lian J, Chao R, Zhao H. Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (2R,3R)-butanediol. Metabolic engineering. 2014;23:92-9.

Liu J, Chan SH, Brock-Nannestad T, Chen J, Lee SY, Solem C, et al. Combining metabolic engineering and biocompatible chemistry for high-yield production of homo-diacetyl and homo-(S,S)-2,3-butanediol. Metabolic engineering. 2016a;36:57-67.

Liu XB, Liu M, Tao XY, Zhang ZX, Wang FQ, Wei DZ. Metabolic engineering of *Pichia pastoris* for the production of dammarenediol-II. Journal of biotechnology. 2015;216:47-55.

Liu Y, Zhang R, Lian Z, Wang S, Wright AT. Yeast cell surface display for lipase whole cell catalyst and its applications. Journal of Molecular Catalysis B: Enzymatic. 2014;106:17-25.

Liu Z, Ho SH, Hasunuma T, Chang JS, Ren NQ, Kondo A. Recent advances in yeast cell-surface display technologies for waste biorefineries. Bioresource technology. 2016b;215:324-33.

Petrov K, Petrova P. Enhanced production of 2,3-butanediol from glycerol by forced pH fluctuations. Applied microbiology and biotechnology. 2010;87:943-9.

Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal. 2012;64:91-105.

Schroer K, Peter Luef K, Stefan Hartner F, Glieder A, Pscheidt B. Engineering the *Pichia pastoris* methanol oxidation pathway for improved NADH regeneration during whole-cell biotransformation. Metabolic engineering. 2010;12:8-17.

Tang S, Boehme L, Lam H, Zhang Z. *Pichia pastoris* fermentation for phytase production using crude glycerol from biodiesel production as the sole carbon source. Biochemical Engineering Journal. 2009;43:157-62.

Tong YJ, Ji XJ, Shen MQ, Liu LG, Nie ZK, Huang H. Constructing a synthetic constitutive metabolic pathway in *Escherichia coli* for (R, R)-2,3-butanediol production. Applied microbiology and biotechnology. 2016;100:637-47.

Wriessnegger T, Augustin P, Engleder M, Leitner E, Muller M, Kaluzna I, et al. Production of the sesquiterpenoid (+)-nootkatone by metabolic engineering of *Pichia pastoris*. Metabolic engineering. 2014;24:18-29.

Xu Y, Chu H, Gao C, Tao F, Zhou Z, Li K, et al. Systematic metabolic engineering of *Escherichia coli* for high-yield production of fuel bio-chemical 2,3-butanediol. Metabolic engineering. 2014;23:22-33.

Yamada R, Wakita K, Mitsui R, Nishikawa R, Ogino H. Efficient production of 2,3-butanediol by recombinant *Saccharomyces cerevisiae* through modulation of gene expression by cocktail delta-integration. Bioresource technology. 2017.

Yan Y, Lee CC, Liao JC. Enantioselective synthesis of pure (R,R)-2,3-butanediol in *Escherichia coli* with stereospecific secondary alcohol dehydrogenases. Org Biomol Chem. 2009;7:3914-7.

Yang T, Rao Z, Zhang X, Lin Q, Xia H, Xu Z, et al. Production of 2,3-butanediol from glucose by GRAS microorganism *Bacillus amyloliquefaciens*. J Basic Microbiol. 2011;51:650-8.

Chapter 7: Statistical medium optimization for enhanced production of 2, 3-butanediol in engineered *Pichia pastoris*

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7.1 Abstract

2, 3-butanediol (2, 3-BD) has been recognized as an important platform chemical and drop-in fuel, widely used in fields of chemistry, food, renewable energy, aerospace, etc. Microbial production of 2, 3-BD from renewable feedstock such as lignocellulose has attracted increasing interest due to its sustainability and carbon neutral process. *P. pastoris* is established as one of the most successful protein expression systems owing to its many advantages. Production of optically pure (2R, 3R)-2, 3-BD using engineered *Pichia pastoris* has been developed in our lab. The titer and yield achieved using YPD medium was relatively low compared with previous reports. To enhance the production of 2, 3-BD by *P. pastoris*, we performed optimization of basal salts medium (BSM). Three components including yeast extract, potassium phosphate monobasic (KH_2PO_4) and magnesium sulfate ($MgSO_4$) were identified as key factors affecting the titer of 2, 3-BD. Concentrations of these constituents were further optimized based on Box-Behnken design. Fed-batch cultivation was performed to verify the model. The titer of 2, 3-BD obtained in a fed-batch fermentation reached 75 g/L, which is significantly improved compared with our previous results using YPD medium.

Keywords: Medium optimization, Plackett-Burman design, Box-Behnken design, 2, 3-butanediol.

7.2 Introduction

Increased carbon emission due to burning of fossil fuels has led to serious environmental impact. Biotechnological production of bulk chemicals has been an alternative to petroleum-based approaches. 2, 3-Butanediol (2, 3-BD), also known as 2, 3-dihydroxybutane or dimethylene glycol. It is a colorless and odorless transparent liquid at room temperature with relative molecular weight of 90.12 g/mol, and boiling point of 177-182 ° C (Celinska and Grajek, 2009). It contains two chiral carbon atoms, thus there are three kinds of optical isomers: (2R, 3R)-2, 3-butanediol, (2S, 3S)-2, 3-butanediol and *meso*-2, 3-butanediol. Optically pure 2, 3-BD isomers are crucial precursors for chiral synthesis in the pharmaceutical industry.

2, 3-BD could be used as a fuel additive due to its high heating value (27.2 KJ/g) (Syu, 2001). It can be converted to diacetyl, which is a high-value flavoring agent with certain antibacterial effect; It can be reduced to 3-hydroxy-2-butanone (acetoin), a widely used natural food spice via dehydrogenation; It can react with acetic acid to obtain diacetate-2,3-butylene glycol ester, which is an important ingredient of fruit aroma of melon, banana and other fruits; It is dehydrated forming ethyl ketone, which can be used as a high-value liquid fuel additive and is also an important low boiling solvent for coatings, adhesives, lubricants, fuels, inks, etc. (Bialkowska, 2016). The 1, 3-butadiene produced by dehydration of 2, 3-BD can be used for synthetic rubber, ABS resin and SBS elastomer. And 2, 3-BD can be used as aviation fuel or octane improver for gasoline. (2R, 3R)-2, 3-butanediol has a low freezing point (-60 °C) and can be used as an anti-freeze agent. In addition, 2, 3-BD and its derivatives has seen applications in the production of drug carriers, plasticizers, softeners, etc (Ji et al., 2011).

Native producers have been reported to accumulate high titer of 2, 3-BD. For instance, *K. pneumoniae* SDM was able to produce 150 g/L 2, 3-BD with a productivity of 4.21 g/L/h, which is the highest reported titer among 2, 3-BD producing bacteria (Ma et al., 2009). Metabolically engineered *B. subtilis* can produce 103.7 g/L chiral pure meso-2, 3-BD (Fu et al., 2016). Non-native hosts such as *E. coli* (Ji et al., 2015) or *S. cerevisiae* (Lian et al., 2014) have been engineered into efficient microbial cell factories for the production of optically pure 2, 3-BD with high titer and yield. Medium optimization based on the Plackett-Burman and Box-Behnke design has been an efficient strategy for improving the production of 2, 3-BD. Production of 2, 3-BD using *Paenibacillus polymyxa* ICGEB2008 was enhanced using optimized level of manganese, potassium and yeast extract (Adlakha and Yazdani, 2015). Gao and coworkers optimized the concentration of inulin, KH_2PO_4 and NH_4Cl in the fermentation of *P. polymyxa* ZJ-9 and achieved 36.92 g/L 2, 3-BD in batch fermentation (Gao et al., 2010).

Pichia pastoris is a kind of methylotrophic yeast that can use methanol as the sole carbon source and energy source. It is generally employed as a host for recombinant protein production due to its advantages (Potvin et al., 2012). We have developed an engineered *P. pastoris* strain X33-SD which was able to produce 45 g/L of (2R, 3R)-2, 3-BD in YPD media. However, the titer and yield was not satisfactory compared with engineered *S. cerevisiae* (Kim et al., 2016). In this work, we performed statistical medium optimization based on the basal salts medium (BSM). In fed-batch cultivation, titer of 2, 3-BD reached 75 g/L, which is the highest level achieved in engineered *P. pastoris*. This strain could be a promising host for efficient production of 2, 3-BD.

7.3 Materials and Methods

7.3.1 Strain and media

P. pastoris strain X33-SD harbouring the *B. subtilis* α-acetolactate synthase gene *alsS*, and α-acetolactate decarboxylase gene *alsD* was constructed previously. The zeocin-resistant strain was kept by streaking yeast culture on YPD agar plates supplemented with 100 mg/L zeocin. All chemicals are of analytical grade and purchased from Fisher scientific (Canada) or Sigma-Aldrich (USA).

YPD media containing 10 g/L yeast extract, 20 g/L peptone and 20 g/L glucose was used to prepare seed culture. The fermentation media included YPD (10 g/L yeast extract, 20 g/L peptone and 40g/L glucose), BSM consists of per liter: 0.6 g/L $CaSO_4 \cdot 2H_2O$, 42.9 g/L KH_2PO_4, 14.33 g/L K_2SO_4, 5.7 g/L $MgSO_4 \cdot 7H_2O$ and 4 ml/L PTM1. The trace element solution PTM1 consists of per liter: BSMY (BSM + 5 g/L yeast extract) and BSMYP (BSM + 10 g/L yeast extract + 20 g/L peptone), and the concentration of contents of those media varied in specific experiments according to different considerations and noted where necessary.

7.3.2 Shake flask cultivation

A single colony of the yeast strain was streaked from agar plate and inoculated into 10 ml YPD and incubated in a rotary shaker at 250 rpm for 20-24 h at 30 °C. Then the seed culture was inoculated into 100 ml fermentation media with appropriate carbon sources and concentrations. Samples were collected periodically, and measurements of OD_{600}, residual substrates and metabolites were carried out subsequently.

7.3.3 Batch and Fed-batch fermentation

Batch and fed-batch fermentations were conducted in 5 L bioreactors (Bioflo320, Eppendorf, Canada) equipped with Rushton impellers. Bioreactor was started with 2 L media. Overnight culture (5% v/v) was inoculated into the fermentation media. Bioreactors were kept at 30 °C. The

cultivation was performed at the optimal condition with pH 5.0 (±0.02), 300 rpm for the agitation speed and filtered air was sparged to keep 0.5 vvm aeration rate. The pH was maintained constant by adding 30% ammonia hydroxide through on-line pH monitoring. Feeding solution containing 1 L of 500 g/L glucose and 12 mL/L PTM1 trace metal solution was fed to the bioreactor once the glucose was consumed. Glucose feed rate was adjusted between 0.2-0.8 mL/min to maintain a low concentration of glucose to avoid substrate inhibition. In specific experiments, the concentration of fermentation media varied for different research objectives. Aliquots of fermentation culture were taken periodically and used for further analysis.

7.3.4 Analytical methods

Optical density was measured at 600 nm using a Biochrom Ultrospec 60 spectrophotometer (UK). Samples were centrifuged for 5 min at 13000 rpm and supernatant was used for analysis of substrate and metabolites. Glucose concentration was determined using a YSI 2900 Biochemical Analyzer (Mandel, Canada). Metabolite concentrations were determined using high performance liquid chromatography (HPLC, Agilent 1200 series, Santa Clara, CA, USA), equipped with a Shodex Sugar SH1011 column (8 mm ID × 300 mm, 6 μm; Showa Denko, Tokyo, Japan). The column was maintained at 60 °C and eluted with 5mM sulfuric acid at a flow rate of 1 mL/min.

7.4 Results and discussions

7.4.1 Effect of yeast extract on 2, 3-BD production in BSM

In our previous study, complex media YPD was used to cultivate strain X33-SD. High yield can be achieved in shake flasks but could not be obtained in fed-batch cultivation. In order to develop a media suitable for long time fermentation, we first tested the BSM media which is a commonly used industrial media for high cell density cultivation of *P. pastoris*. BSM media

consists of mineral salts without complex carbon or nitrogen source. It was reported that addition of complex nitrogen sources such as yeast extract or corn steep liquor could enhance the production of 2, 3-BD because they provide more NADH for the conversion of acetoin to 2, 3-BD. Increased yeast extract resulted in higher titer of 2, 3-BD in the fermentation of *P. polyxyma* DSM 365 (Hassler et al., 2012). Yeast extract and casamino acid was used in the cultivation of *K. oxytoca* and it was found that addition of complex nitrogen source could enhance the titer of 2, 3-BD by 1.6-fold in shake flask (Cho et al., 2015). BSM supplemented with 10 g/L yeast extract and YPD was used as control groups. As was shown in Fig. 7-1, production of 2, 3-BD was detected at 0.5 g/L in BSM. Cultivation in YPD resulted in the highest titer, reaching 9.8 g/L. BSMY led to 4.5 g/L of 2, 3-BD. Therefore it was reasonable to factor in yeast extract to further optimize BSM media.

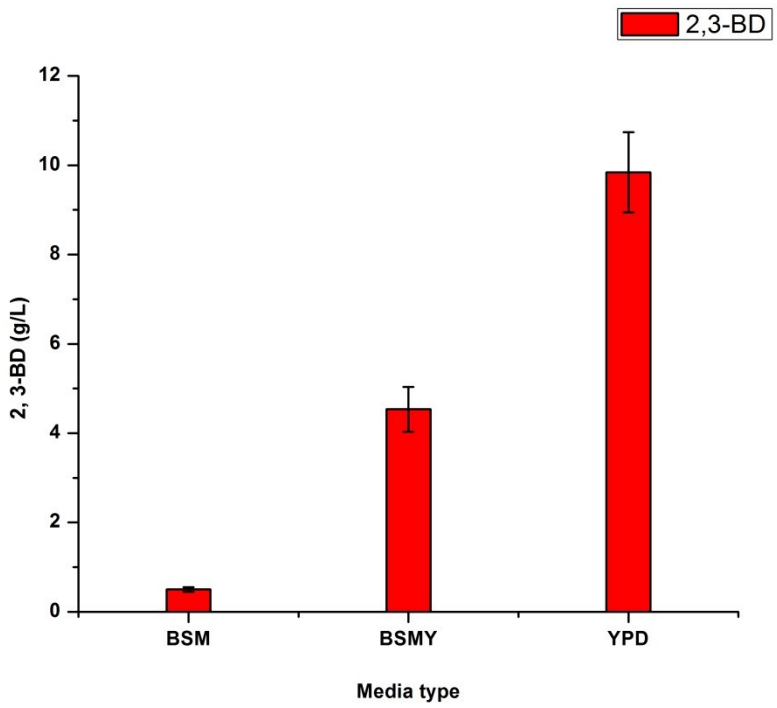

Fig. 7-1 Effect of media type on the production of 2, 3-BD. 100 mL media containing 40 g/L glucose were used to cultivate strain X33-SD. Error bar indicate standard deviation of three replicate experiments.

7.4.2 Plackett-Burman design

The Plackett-Burman design was first employed to determine the significant factors of medium components on 2, 3-BD production. The 12-run experiments were carried out according to the matrix generated by Minitab 15 (Table 7-1). Seven variables were screened including yeast extract, $CaSO_4$, KH_2PO_4, $MgSO_4$, K_2SO_4, $(NH_4)_2SO_4$ and PTM1. Each variable was investigated by high (+1) and low (-1) level respectively, which represents two different component concentrations. The equation based on the first-order model was expressed as follows:

$$Y = \beta_0 + \Sigma\beta_i x_i \quad (i = 1,..., k) \quad (7\text{-}1)$$

where Y is the response (2, 3-BD concentration); β_0 is the model intercept and βi is the regression coefficient, and x_i is the independent variable. The most important factor was determined by the p value ($p < 0.05$) and t value ($t > 0$) evaluation of each individual effect. The p value is the probability of magnitude of contrast coefficient due to random process variability and serves as a tool for checking significance of each coefficient. The components were screened at the confidence level of 95 % based on their effects.

Table 7-1 Level code for variables based on Plackett-Burman design

Run	Variable	Yeast extract X1	$CaSO_4$ X2	KH_2PO_4 X3	K_2SO_4 X4	$MgSO_4$ X5	$(NH4)_2SO_4$ X6	PTM1 X7
1		1	-1	1	-1	-1	-1	1
2		1	1	-1	-1	-1	1	-1
3		-1	1	1	1	-1	-1	-1
4		1	-1	1	-1	1	1	-1
5		1	1	-1	1	-1	1	1
6		1	1	1	1	1	-1	-1
7		-1	1	1	-1	1	1	1
8		-1	-1	1	1	-1	1	1
9		-1	-1	-1	1	1	1	-1
10		1	-1	-1	1	1	-1	1
11		-1	1	-1	-1	1	-1	1
12		-1	-1	-1	-1	-1	-1	-1

Table 7-2 Responses of Plackett-Burman design

Run	1	2	3	4	5	6	7	8	9	10	11	12
2,3-BD (g/L)	5.066	7.545	3.242	3.444	6.280	2.998	3.474	2.685	4.935	7.029	5.415	5.490

The 2, 3-BD concentrations based on Plackett-Burman design were presented in Table 7-2. Statistical analysis of results was shown in Table 7-3. It was found that the significance of each medium component affecting the 2, 3-BD production was in the following order: KH_2PO_4 > yeast extract > $MgSO_4$ > PTM1 > K_2SO_4 > $(NH_4)_2SO_4$ > $CaSO_4$. Apparently, KH_2PO_4, yeast extract and $MgSO_4$ were the major factors affecting the 2, 3-BD production based on the Pareto graph (Fig. 2). KH_2PO_4 showed a strong negative effect while yeast extract exhibited a strong positive effect on the 2, 3-BD production. In a previous report optimizing the medium composition for enhanced phytase production in *P. pastoris*, KH_2PO_4 was also found to negatively influence the phytase yield and should be reduced in the standard BSM media (Potvin et al., 2015). The positive effect of yeast extract was consistent with the results obtained in shake flask cultivation. The following expression of Yi could be obtained by applying the coefficients of each variable:

$$Y=4.800+1.187X_1+0.051X_2-2.631X_3-0.544X_4-0.502X_5-0.146X_6+0.383X_7 \quad (7\text{-}2)$$

The F-value for this equation is 6.93 and P=0.04, indicating the good fitness of this equation.

Table 7-3 Analysis of results from Plackett-Burman design

Code	Variables	Low level (g/L) (-1)	High level (g/L) (+1)	Effects	Standard error	T value	P > |T|
X1	Yeast extract	5	15	1.187	0.2159	2.75	0.051
X2	$CaSO_4$	0.3	0.9	0.051	0.2159	0.12	0.912
X3	KH_2PO_4	21.45	64.35	-2.631	0.2159	-6.09	0.004
X4	K_2SO_4	7.165	21.495	-0.544	0.2159	-1.26	0.276
X5	$MgSO_4$	2.855	8.565	-0.502	0.2159	-1.16	0.309
X6	$(NH4)_2SO_4$	2.585	7.755	-0.146	0.2159	-0.34	0.752
X7	PTM1	2 ml/L	6 ml/L	0.383	0.2159	0.89	0.426

S = 0.747792 PRESS = 20.1310
R^2 = 92.39% R^2 (ADJ) = 79.06%

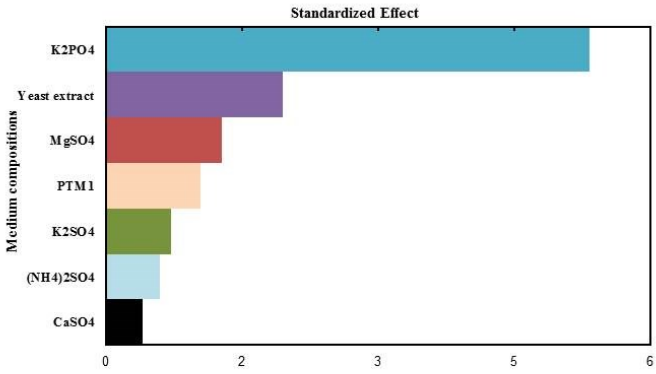

Fig. 7-2 Standardized Pareto graph of variables

7.4.3 Box-Behnken design

The three most significant variables (KH_2PO_4, yeast extract and $MgSO_4$) were selected from results of Plackett-Burman design to further determine their optimal level through response surface methodology. The Box-Behneken design (Table 7-3) consists of a set of points lying at the midpoint of each edge and the replicated center point of the multidimensional cube (Table 7-4). Three levels denoted by -1, 0 and +1 (Table 7-5) were used for each variable. The equation generated by this model is given as follows:

$$Yi = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{23}X_2X_3 + b_{11}X_2^2 + b_{22}X_2^2 + b_{33}X_3^2 \quad (7\text{-}3)$$

where Yi is the independent variable; b_0 is the intercept; b_1–b_{33} are the regression coefficients; and X_1, X_2 and X_3 are the independent variables that were selected from the preliminary experiments. The responses of Box-Behnken design were shown in Table 7-6. Variance analysis

was used to evaluate the effect of independent variables on the response and significant results were identified by a p value of < 0.05. The F value is the ratio of the mean square due to regression to the mean square due to error and indicates the influence of each controlled factor on the tested model. Multiple correlation coefficient (R^2) and adjusted R^2 were used as quality indicators to evaluate the fitness of the second order polynomial equation. Contour plots were employed to demonstrate the relationship and interaction between the coded variables and the response.

Table 7-4 Desgin of Box-Behnken experiments

Number Variable	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Yeast extract	-1	-1	1	1	0	0	0	0	-1	1	-1	1	0	0	0
KH_2PO_4	-1	1	-1	1	-1	1	-1	1	0	0	0	0	0	0	0
$MgSO_4$	0	0	0	0	-1	-1	1	1	-1	-1	1	1	0	0	0

Table 7-5 Concentration level for Box-Behnken design

Factors	Level (g/L)		
	-1	0	+1
KH_2PO_4	21.45	42.9	64.35
Yeast extract	5	10	15
$MgSO_4$	2.855	5.7	8.565

Table 7-6 Responses from Box-Behnken design

Number	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
2,3-BD (g/L)	4.09	4.66	7.90	5.18	8.73	5.51	8.37	5.64	5.31	7.93	5.55	7.04	7.58	7.48	7.41

Statistical analysis of results based on Box-Behnke design (Table 7-7) was performed by using Minitab 15. The following equation of the quadratic model can be found by applying the coefficients of each term:

$$Y = 7.4900 + 1.0425X_1 - 1.0250X_2 - 0.1100X_3 - 0.7975X_1X_2 - 0.2825X_1X_3 + 0.1225X_2X_3 - 1.30625X_1^2 - 0.70125X_2^2 + 0.27375X_3^2$$

The regression model was highly significant confirmed by determination coefficient (R^2=94.2%), indicating that 94.2% of the variability of the response could be explained by the model. Significant terms include X_1, X_2, X_1X_2 and X_2^2. The significance of this model was also confirmed by the high value of R^2 (ADJ) =0.8376. The maximum value of Y could be obtained when the concentration of yeast extract, KH_2PO_4 and $MgSO_4$ was at 15 g/L, 21.5 g/L and 2.85 g/L respectively.

Table 7-7 Analysis of results from Box-Behnken design

Factors	Coefficient	Standard error	T	P
Constant	7.4900	0.3415	21.930	0.000
KH_2PO_4	-1.0250	0.2091	-4.901	0.004
Yeast extract	1.0425	0.2091	4.985	0.004
$MgSO_4$	-0.1100	0.2091	-0.526	0.621
KH_2PO_4 * KH_2PO_4	-0.7013	0.3079	-4.243	0.008
Yeast extract * Yeast extract	-1.3062	0.3079	-2.278	0.072
$MgSO_4$ * $MgSO_4$	0.2737	0.3079	0.889	0.415
KH_2PO_4 * Yeast extract	-0.7975	0.2958	-2.696	0.043
KH_2PO_4 * $MgSO_4$	0.1225	0.2958	-0.955	0.383
Yeast extract * $MgSO_4$	-0.2825	0.2958	0.414	0.696

S = 0.591557 PRESS = 27.7945
R^2 = 94.20% R^2 (ADJ) = 83.76%

7.4.4 Fed-batch fermentation using optimized BSMY medium

The optimized BSMY media consists of 15 g/L yeast extract, 21.5 g/L KH_2PO_4 and 2.85 g/L $MgSO_4$. Other medium components were kept the same as the standard BSM. Several fed-batch cultivations were performed to evaluate the performance of optimized medium. Upon the depletion of initial glucose in the batch within about 18h, feeding solution containing 250 g/L (Fig. 7-3) or 500 g/L glucose was fed to the bioreactor. Feed rate was adjusted to maintain a low glucose concentration. In a fed-batch with 40 g/L initial glucose concentration and 1 L of 250 g/L glucose feeding solution, the fermentation was completed within 49 h. The final titer of 2, 3-

BD was 41 g/L with a yield on glucose of 0.31 g/g. To boost the titer, higher concentrations of initial glucose and feeding glucose were studied. As can be seen from Fig. 7-4 and Fig. 7-5, fed-batch cultivation was completed within 90 h, which was significantly shortened in comparison with YPD. The highest titer of 2, 3-BD was achieved when 60 g/L of initial glucose and 1 L of 500 g/L glucose feeding solution was used, reaching 74.5 g/L. In contrast, the highest titer obtained in YPD was 45 g/L in fed-batch fermentation with a much longer cultivation time. Comparison of the fed-batch cultivation results were summarized in Table 7-8. Compared with YPD media, optimized BSMY could support faster glucose consumption without accumulating excessive glucose in the culture. Glycerol was the major by-product, reaching about 29.4 g/L in the final culture. Ethanol and acetoin was detected at low titer.

Yeast extract as a complex nitrogen source was used in this work. Other cheaply available nitrogen sources derived from lignocellulose such as corn steep liquor and cassava powder could be alternatively used to replace the expensive yeast extract for economic considerations. The optimization strategy described in this work could be easily applied for similar purposes.

Table 7-8 Comparison of fed-batch cultivations

Cultivation media	Duration (h)	Titer of 2,3 –BD (g/L)	Productivity (g/L/h)	Yield (g/g)	Reference
YPD	135	45	0.33	0.194	Chapter 6
BSMY	49	41	0.84	0.31	This work
BSMY	90	57.3	0.64	0.246	This work
BSMY	92	74.5	0.81	0.3	This work

Theoretical yield is 0.5 g/g.

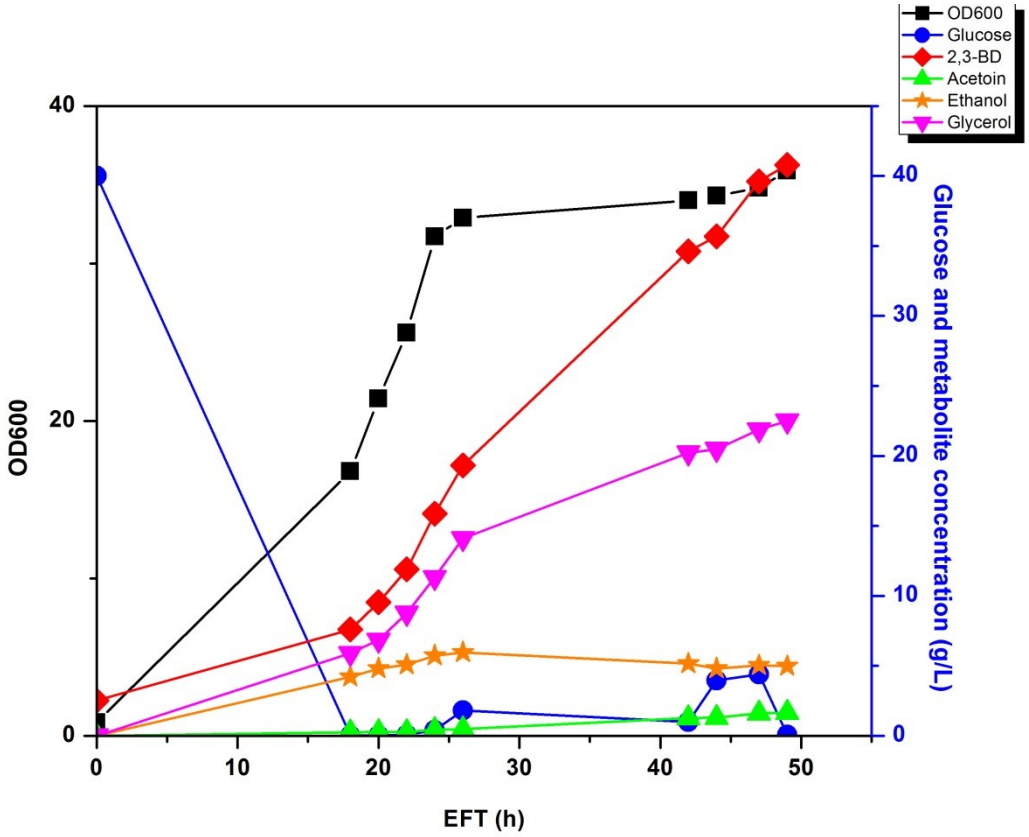

Fig. 7-3 Fermentation profile of 2-L fed-batch fermentation using optimized BSMY medium with 40 g/L glucose as substrate and 1 L of 250 g/L glucose solution as feeding solution.

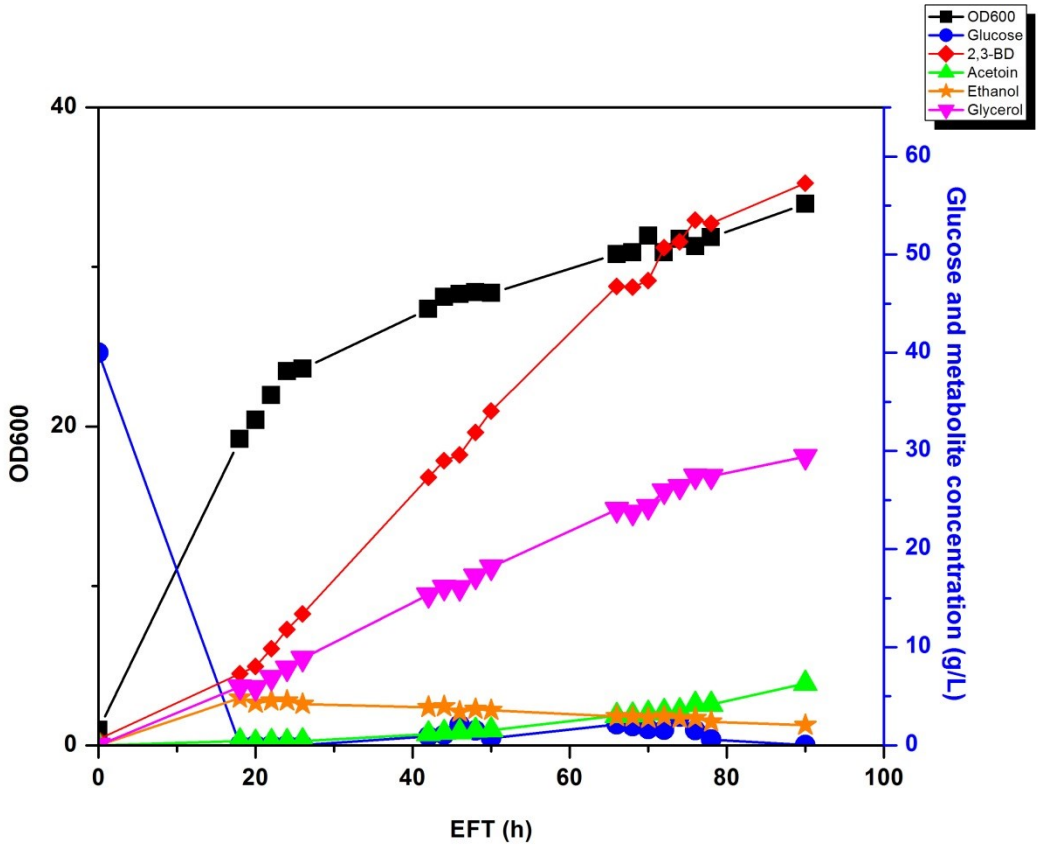

Fig. 7-4 Time course of fed-batch fermentation using optimized BSMY medium. Bioreactor contained 2 L of initial media. 40 g/L glucose was used as initial substrate and 1 L of 500 g/L glucose solution was used as feeding solution in fed-batch.

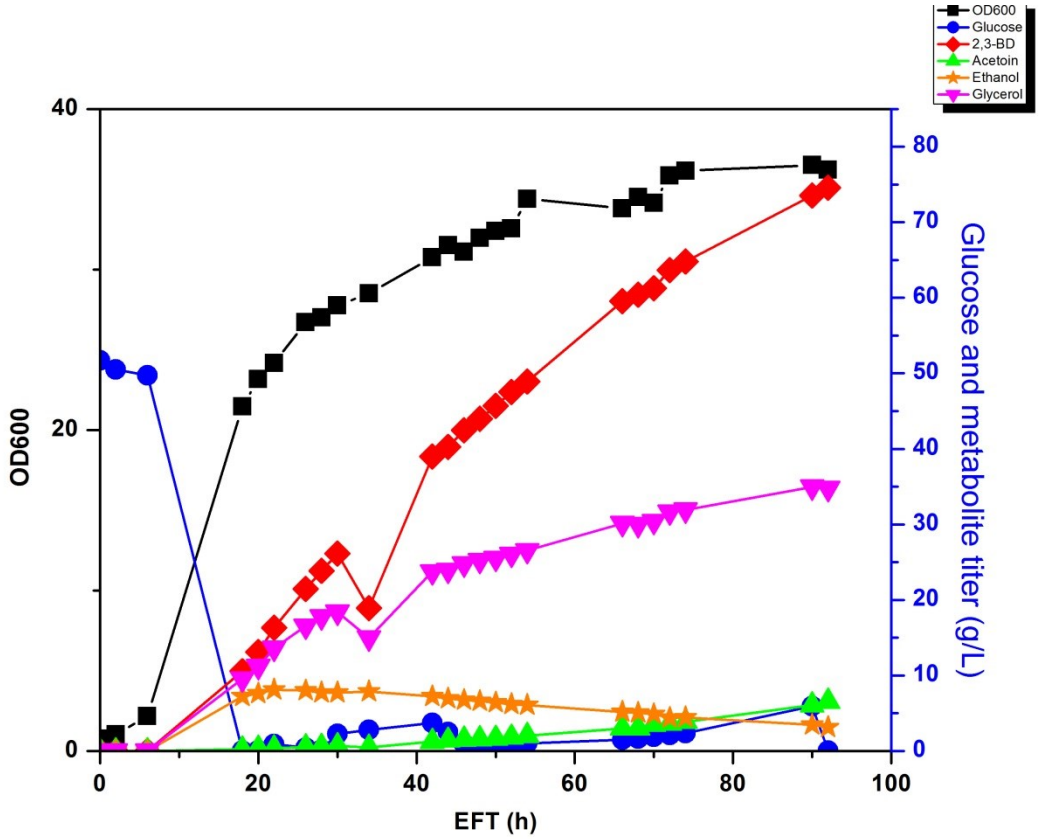

Fig. 7-5 Time course of fed-batch fermentation using optimized BSMY medium. Bioreactor contained 2 L of initial media. 60 g/L glucose was used as initial substrate and 1 L of 500 g/L glucose solution was used as feeding solution in fed-batch.

7.5 Conclusions

Production of value-added chemical (2R, 3R)-2, 3-BD using engineered *P. pastoris* was a promising approach to replace the use of pathogenic microorganisms. However, low titer was the major drawback of this system compared with native hosts or the baker's yeast *S. cerevisiae*. In this work, medium optimization based on Plackett-Burman and Box-Behnken design was performed. In fed-batch fermentation, the highest titer using optimized BSMY was able to reach 74.5 g/L, which was improved by 65.5% over the complex YPD media. The optimized media

could serve as a good starting point for further optimization and engineered strain X33-SD might be a robust platform for the production of 2, 3-BD.

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7.7 References

Adlakha N, Yazdani SS. Efficient production of (R, R)-2, 3-butanediol from cellulosic hydrolysate using *Paenibacillus polymyxa* ICGEB2008. Journal of industrial microbiology & biotechnology. 2015;42:21-8.

Bialkowska AM. Strategies for efficient and economical 2,3-butanediol production: new trends in this field. World journal of microbiology & biotechnology. 2016;32:200.

Celinska E, Grajek W. Biotechnological production of 2,3-butanediol--current state and prospects. Biotechnology advances. 2009;27:715-25.

Cho S, Kim T, Woo HM, Lee J, Kim Y, Um Y. Enhanced 2,3-Butanediol Production by Optimizing Fermentation Conditions and Engineering *Klebsiella oxytoca* M1 through Overexpression of Acetoin Reductase. PloS one. 2015;10:e0138109.

Fu J, Huo G, Feng L, Mao Y, Wang Z, Ma H, et al. Metabolic engineering of *Bacillus subtilis* for chiral pure meso-2,3-butanediol production. Biotechnology for biofuels. 2016;9:90.

Gao J, Xu H, Li Q-j, Feng X-h, Li S. Optimization of medium for one-step fermentation of inulin extract from Jerusalem artichoke tubers using *Paenibacillus polymyxa* ZJ-9 to produce R, R-2, 3-butanediol. Bioresource technology. 2010;101:7076-82.

Hassler T, Schieder D, Pfaller R, Faulstich M, Sieber V. Enhanced fed-batch fermentation of 2,3-butanediol by *Paenibacillus polymyxa* DSM 365. Bioresource technology. 2012;124:237-44.

Ji XJ, Huang H, Ouyang PK. Microbial 2,3-butanediol production: a state-of-the-art review. Biotechnology advances. 2011;29:351-64.

Ji XJ, Liu LG, Shen MQ, Nie ZK, Tong YJ, Huang H. Constructing a synthetic metabolic pathway in *Escherichia coli* to produce the enantiomerically pure (R, R)-2,3-butanediol. Biotechnology and bioengineering. 2015;112:1056-9.

Kim JW, Kim J, Seo SO, Kim KH, Jin YS, Seo JH. Enhanced production of 2,3-butanediol by engineered *Saccharomyces cerevisiae* through fine-tuning of pyruvate decarboxylase and NADH oxidase activities. Biotechnology for biofuels. 2016;9:265.

Lian J, Chao R, Zhao H. Metabolic engineering of a *Saccharomyces cerevisiae* strain capable of simultaneously utilizing glucose and galactose to produce enantiopure (2R,3R)-butanediol. Metabolic engineering. 2014;23:92-9.

Ma C, Wang A, Qin J, Li L, Ai X, Jiang T, et al. Enhanced 2,3-butanediol production by *Klebsiella pneumoniae* SDM. Applied microbiology and biotechnology. 2009;82:49-57.

Potvin G, Ahmad A, Zhang Z. Bioprocess engineering aspects of heterologous protein production in *Pichia pastoris*: A review. Biochemical Engineering Journal. 2012;64:91-105.

Potvin G, Li Z, Zhang Z. Statistical Medium Optimization for the Increased Production of Recombinant Phytase in the Fed-Batch Cultivation of *Pichia pastoris*. International Journal of Chemical Reactor Engineering. 2015;13:427-35.

Syu M-J. Biological production of 2, 3-butanediol. Applied microbiology and biotechnology. 2001;55:10-8.

Chapter 8: Conclusions and recommendations

The work conducted in this thesis examined several aspects of *Pichia pastoris*. The research was focused on the molecular biology level (up-stream) and the bioprocess engineering level (down-stream). As one of the most widely used hosts for heterologous protein, high level expression of a single gene to obtain a desired protein as target product is one of the most common tasks undertaken in *P. pastoris*. The difficulties in developing a strain producing a valuable product are generally specific to the objective of each project. The challenges with regard to protein expression could be mainly categorized into the selection of expression host, the source of gene of interest and the applications of the target protein. In this thesis, xylanase was selected to be expressed due to the large demand of xylanase in industry such as paper and pulp bleaching, animal feed additive, bakery and brewing. Given the potential applications of xylanase in food industry, *P. pastoris* was used to express the fungal xylanase gene due to its GRAS status. The gene of interest was codon-optimized to adjust the codon usage and GC content to those of host *P. pastoris*. High level secreted expression of Xyn11A has been achieved. Biochemical characterization of this xylanase revealed some properties of industrial significance such as wide range pH stability, resistance to most metal ions and protease degradation and remarkable thermal stability at or under 60 °C. Future research could be dedicated to the industrial application such as pulp bleaching or its potential as animal feed supplement.

Production of value-added products such as biofuels or commodity chemicals via biotechnological approaches is of particular interest. 2, 3-BD is a platform chemical with enormous applications and its production using a variety of native and engineered microorganism has been extensively studied. Despite the production of some bio-products using

P. pastoris as a whole cell catalyst has been described in previous reports, metabolic engineering of this host via assembled pathway to achieve production of valuable chemicals using simple carbon source remains limited in literature. This is likely due to the lack of metabolic engineering tools such as promoters, autonomous-replicating plasmid and limited selection markers. In this thesis, we explored the feasibility of production of commodity chemical using 2, 3-BD as an instance to evaluate the potential of engineering *P. pastoris* into hosts to produce industrially important chemicals. Since no 2, 3-BD was detected in wild-type *P. pastoris*, a synthetic pathway consisting of codon-optimized genes was assembled and transformed into *P. pastoris*. The engineered *Pichia* strains were found to synthesize 2, 3-BD at high level. Effects of crucial factors affecting the formation of 2, 3-BD including pH, agitation speed, aeration rate and carbon source concentration were investigated. The highest titer achieved in fed-batch cultivation using complex YPD media was 45 g/L. Although successful production of 2, 3-BD has been achieved in *P. pastoris*, the titer and product yield obtained using YPD media was not competitive compared with other engineered hosts or native producers. Possible strategies to address those issues include but not limited to the knockout of competing pathway genes leading to the formation of glycerol and expression of genes for the regeneration of NADH. The characterization of putative 2, 3-BDHs in *P. pastoris* would benefit the understanding of mechanism of the synthesis optically pure 2, 3-BD and the development of novel *Pichia* strains producing meso-2, 3-BD and (2S, 3S)-2, 3-BD. These projects could be pursued in future work.

Process engineering of *P. pastoris* strains used in this thesis is essential to achieving high level production of the desired products. For xylanase production, maintaining a suitable specific growth rate is key to the enhanced production of xylanase. Effects of carbon source and feeding strategies were investigated. Glucose was found to outperform glycerol in terms of high titer of

protein production and xylanase activity. A higher specific growth rate might be beneficial to the production of xylanase using GAP promoter. The final xylanase activity was detected at 15000 U/mL, which was a significant increase compared with previous work. More experiments could be done to obtain the optimal value of μ. For the production of 2, 3-BD, the production of biomass and protein was not the main objective. To resolve the low titer and yield using YPD media, statistical optimization of medium composition based on the BSM media using Plackett-Burman and Box-Behnken design to identify the key factors influencing the titer and yield of 2, 3-BD. The level of three key factors including KH_2PO_4, yeast extract and $MgSO_4$ were determined. The final titer of 2, 3-BD using optimized media reached 75 g/L in fed-batch cultivation, which enhanced the titer by 66.7%.

In summary, *P. pastoris* was engineered into microbial cell factories for efficient production of xylanase and platform chemical 2, 3-BD. Both metabolic and process engineering strategies were implemented in this thesis. The versatility of *P. pastoris* was demonstrated. The work described in this thesis could provide some insights into the engineering aspects of *P. pastoris* at the molecular or bioprocess level. Potential production of 2, 3-BD using xylan-fermenting *P. pastoris* could be achieved based on the results in this thesis.