Process Improvements and Adaptation of *Spathaspora passalidarum* to AFEX hydrolysate

By

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Abstract

The main object of this study is to characterize a new isolated native xylose-fermenting yeast, *Spathaspora passalidarum* NN235 (=NRRL Y27907). Extraordinarily, this yeast is capable of cofermenting glucose and xylose when glucose concentration is below 30 g/L, and it also can simultaneously co-utilize cellobiose, glucose, and xylose for ethanol production with a yield of 0.42 g/g. The specific ethanol production rate on xylose is higher than that of the corresponding rate on glucose. Metabolome analysis showed that the flux of glycolytic intermediates is significantly higher on xylose than on glucose. These unusual capacities might be attributed to the high affinity of its xylose reductase activities for NADH and xylose combined with allosteric activation of glycolysis. In the subsequent study, the effects of aeration on ethanol and polyol accumulation of *S. passalidarum* were compared with those of *Scheffersomyces stipitis*. The results suggested a higher flux in glycolytic pathway in *S. passalidarum* compared to *S. stipitis*. Later, we applied adaptation and cell-recycle to increase the tolerance of yeast cells to AFEX hydrolysate. The adapted population (YK105.6) was subjected to high throughput screening for selecting promising candidates. The evolved strain YK208-E11 showed a 3-fold increase in specific fermentation rate compared to the parental strain and an ethanol yield above 0.45 g per gram substrate by co-utilizing cellobiose, glucose, and xylose. YK208-E11 also exhibited less cell yield compared to WT under fully aerobic condition, which implied a possible deficiency in either its respiration system and/or its anabolism pathway. Four respiration inhibitors, including rotenone, antimycin A, salicylhydroxamic acid and KCN were used with a combination of tetrazolium overlay to investigate the possible mutation location in YK208-E11's respiration chain. The results first

confirmed our previous study that glucose-grown cells were more respirative compared to xylose-grown cells. Our findings revealed that YK208-E11 was more subjected to respiration inhibitors compared to WT strain, suggesting that a mutation might occur at its alternative oxidase pathway. This data also implied that the xylose-induced fermentation could be related to the action site of antimycin A.

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Chapter I

Introduction

1.1 Abstract

To solve the need for alternative environmentally friendly fuel to replace traditional fossil fuel, research on developing lignocellulosic biofuels such as ethanol derived from agriculture residues and paper industry waste by the means of saccharification and fermentation has been intensively conducted in the past two decades. In this chapter, the D-xylose assimilation pathway, the metabolic engineering of *S.* cerevisiae, and the physiological differences between glucose-fermenting, Crabtree-positive *S. cerevisiae* and native-xylose-fermenting Crabtree-negative yeasts are reviewed. In the latter part, the methods of adaptation and evolutionary engineering and the mechanism of inhibition by toxic materials during hydrolysate fermentation are also briefly discussed. In the end of this chapter, a newly isolated, native-pentose fermenting yeast, *Spathaspora passalidarum*, is introduced.

1.2 Global warming and second generation biofuels

Petroleum is vital to human society and civilization because of the intense demand of petroleum derivatives for plastics and the high energy density of petroleum for liquid transportation fuels. The latter is particularly critical for aircraft, automobiles and trucks (Ragauskas et al. 2006). However, using petroleum as the sole transportation energy source causes serious problems including, but not limited to, energy unsustainability and insecurity (Graham-Rowe 2011, Tilman et al. 2009), global warming with the rise of sea level (Allison et al. 2009, Petit et al. 1999, Rahmstorf 2010, Rahmstorf and Coumou 2011, Rahmstorf and Coumou 2012), and ecosystem imbalance (Boyd 2011, Fuhrer 2003, Garrett et al. 2006, Hoegh-Guldberg et al. 2007). One promising approach to alleviating these problems is to replace petroleum with renewable biofuels, such as bioethanol or advanced "drop-in" fuels. Currently the U.S. ethanol production has topped out at around 13 billion gallons of bioethanol from corn ("Annual Energy Review " 2014, Entrix 2010), most of which is blended with gasoline at a 9:1 gasoline-to-ethanol ratio in order to improve the fuel's octane rating. Even if all of the domestically available cornstarch were converted to ethanol, it could only replace less than 10% of the gasoline demand for US. (Service 2007)

Converting lignocellulosic feedstocks into biofuels such as ethanol also can temper the demand for gasoline and decrease CO_2 accumulation when properly integrated with cultivation of diverse native grasses (Tilman et al. 2006). Furthermore, studies suggest that replacing gasoline with lignocellulose-derived ethanol can reduce greenhouse gas emissions by 88% compared to only 18% from corn ethanol. The net energy gain of producing lignocellulosic ethanol is much higher than that of producing gasoline (Farrell et al. 2006). Bioethanol derived from lignocellulosic biomass can also serve as an alternative biofuel. Advantages of using this

feedstock are its potentially low price, availability from agriculture residues and forest wastes rather than food grains, and much lower greenhouse gas emissions as compared to corn ethanol (Balat 2011, Bessou et al. 2011)

1.3 The composition of lignocellulosic materials

Cellulose (20-50% of the dry weight), hemicellulose, and lignin (10-30%) make up the cell wall of lignocellulosic biomass (Chundawat et al. 2011), and on average about 25% of the total dry weight in the lignocellulosic biomass is hemicellulose (Jeffries 1983, Sjöström 1993). Hemicelluloses are a class of branched heterogeneous polymers comprised of hexoses (α-D-galactose, β-D-mannose, β-D-glucose), pentoses (α-L-arabinose, β-D-xylose), and sugar acids (the terminal carbon's hydroxyl group being oxidized to a carboxylic acid), such as uronic acid (Chundawat et al. 2011). In some cases the hydroxyl groups of sugars are partially substituted with acetyl groups (Girio et al. 2010).

Xylan (the anhydride polymer form of xylose) is the main hemicellulosic component in angiosperms (flowering plants), such as birch, maple and oaks, in which the dry weight of xylan content constitutes about 20-30% of the biomass of hardwood (Girio et al. 2010). Xylan can comprise up to 32-50% of the dry biomass in herbaceous plants, such as rye grass (Walker 2011) or in agriculture residues, such as corn fiber (Hahn-Hagerdal et al. 2001), sugar cane bagasse (Girio et al. 2010, Tsao et al. 1982), or cereals (Girio et al. 2010). Substantial volumes of wood residues are used for energy production in US, and wood and bagasse supplied $4\sim6\times10^{14}$ KJ of energy in the United States in 1980s. While hemicellulose comprises 30% of the dry weight in the plant biomass, it only provides two-thirds of the heat value compared to an equal mass of lignin. Thus, it is possible to extract the hemicellulose for use as alternative feedstock for

bioenergy production without a proportionate loss of the energy value of the original feedstock. Moreover, the lignin fraction is difficult to convert to other products, and hence can better be used as fuel for process heat (Jeffries 1983).

1.4 D-Xylose assimilation and metabolism

After pretreatment, the xylan content in hemicellulose will be broken down as xylose which can be further converted to ethanol by fungi (Anasontzis et al. 2011, Singh et al. 1992, Suihko et al. 1983), bacteria (Jarboe et al. 2007, Mortlock et al. 1965), or yeast. Fermentation of xylose to ethanol by yeasts was first observed with *Candida tropicalis* in 1959 (Karczewska et al.). This work was not widely recognized until the early 1980s when the capacity of yeasts, including *Pachysolen tannophilus*, *Scheffersomyces* (*Candida*) *shehatae*, and *Scheffersomyces* (*Pichia*) *stipitis*, to convert xylose directly to ethanol was discovered in several laboratories (Debus et al. 1983, Dellweg et al. 1984, Dupreez and Vanderwalt 1983, Gong et al. 1981b, Jeffries 1981, Maleszka et al. 1981, Schneider et al. 1981).

The theoretical ethanol yield from xylose is 0.51 g per g of xylose or every 5 mol of ethanol per 3 mol of xylose consumed, and overall reaction for converting xylose to ethanol can be presented by the following equation:

$$3C_5H_{10}O_5\ (\text{xylose}) \rightarrow 5C_2H_5OH\ (\text{ethanol}) + 5CO_2$$

Conversion of xylose to ethanol first requires effective xylose uptake. It has been suggested that the rate of xylose uptake may be a rate-limiting factor for xylose fermentation. Transporting xylose can be carried out by either facilitated diffusion or by active transporters in

yeasts and fungi (Jeffries 1983, Kilian et al. 1993, Kilian and Vanuden 1988, Lucas and Vanuden 1986, Nobre et al. 1999). A facilitator transports sugars from high concentration to low concentration and exhibits different specificity depending on the substrate. On the other hand, an active transporter not only exhibits specificity toward different substrates but it can move sugar against a concentration gradient by expending metabolic energy, such as ATP. Figure 1-1 shows a simplified diagram of facilitator and active transporters for transporting xylose. Yeast may synthesize merely a few or several sugar transporters with affinity for xylose. The sugar transport system differs among yeast species, but usually can be categorized into two classes: high-affinity symporters and low-affinity facilitators. In *S. stipitis*, both the high and low-affinity transport systems are xylose/H^+ symporters. Glucose enters cells by the low-affinity transporter and inhibits the high affinity xylose/H+ symporter system (Kilian and Vanuden 1988). In *S. shehatae*, the low-affinity transport system transports both glucose and xylose through facilitated diffusion, but glucose and xylose are transported by different high affinity H^+ symporters. (Lucas and Vanuden 1986). Under most conditions, glucose inhibits the xylose high-affinity transport system and competes with xylose for the low-affinity facilitator. In most cases, H^+ symporters are expressed at low-sugar environment, and low-affinity facilitators are expressed when sugar concentration is high in the medium (Leandro et al. 2009)

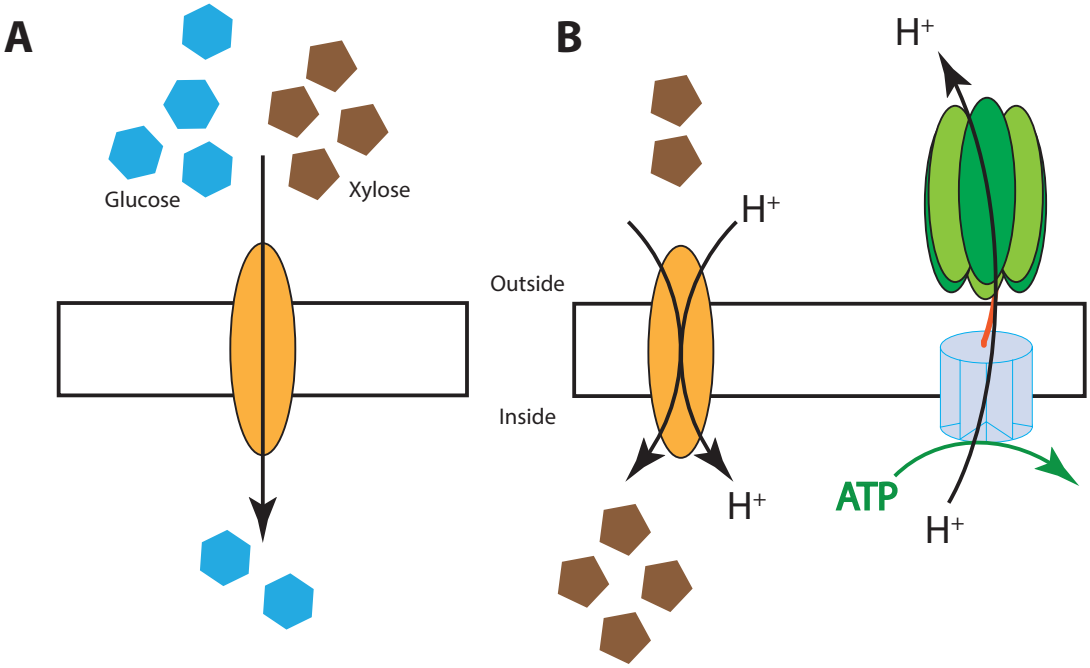

Figure 1-1 The two mechanisms of xylose uptake in yeast. A facilitated diffusion – the concentration gradient between the medium and the cytosol able the sugar uptake, these transporters, also called facilitators generally have a broad substrate range; B proton-xylose symport system – xylose is transported the proton motive force with the expense of ATP, able to transport xylose against the concentration gradient.

After xylose enters the cell, it cannot be used directly. Instead, it has to be converted to xylulose via one of two different biochemical pathways: (i) a two-step reduction and oxidation which converts xylose to xylulose by two oxidoreductases, aldose (xylose) reductase (XR) and xylitol dehydrogenase (XDH), or (ii) direct isomerization which converts D-xylose to D-xylulose by xylose isomerase (XI) (see Figure 1-2). In the two-step reaction, the first step of converting (Chiang and Knight 1960) xylose to xylitol by XR requires NADPH or NADH as a cofactor; in the second step from xylitol to xylulose by XDH, NAD^+ is exclusively used as the cofactor (Kim et al. 2013a). Most bacteria use the first pathway but the vast majority of filamentous fungi, yeasts, and other eukaryotes use the second pathway. This distinction has been recognized since 1960 (Chiang and Knight 1960). Summaries of yeast physiological properties show that approximately 50% of yeasts can assimilate xylose under aerobic conditions but only few can produce ethanol from xylose under oxygen-limited conditions (Jeffries 1983, Kurtzman et al. 2011).

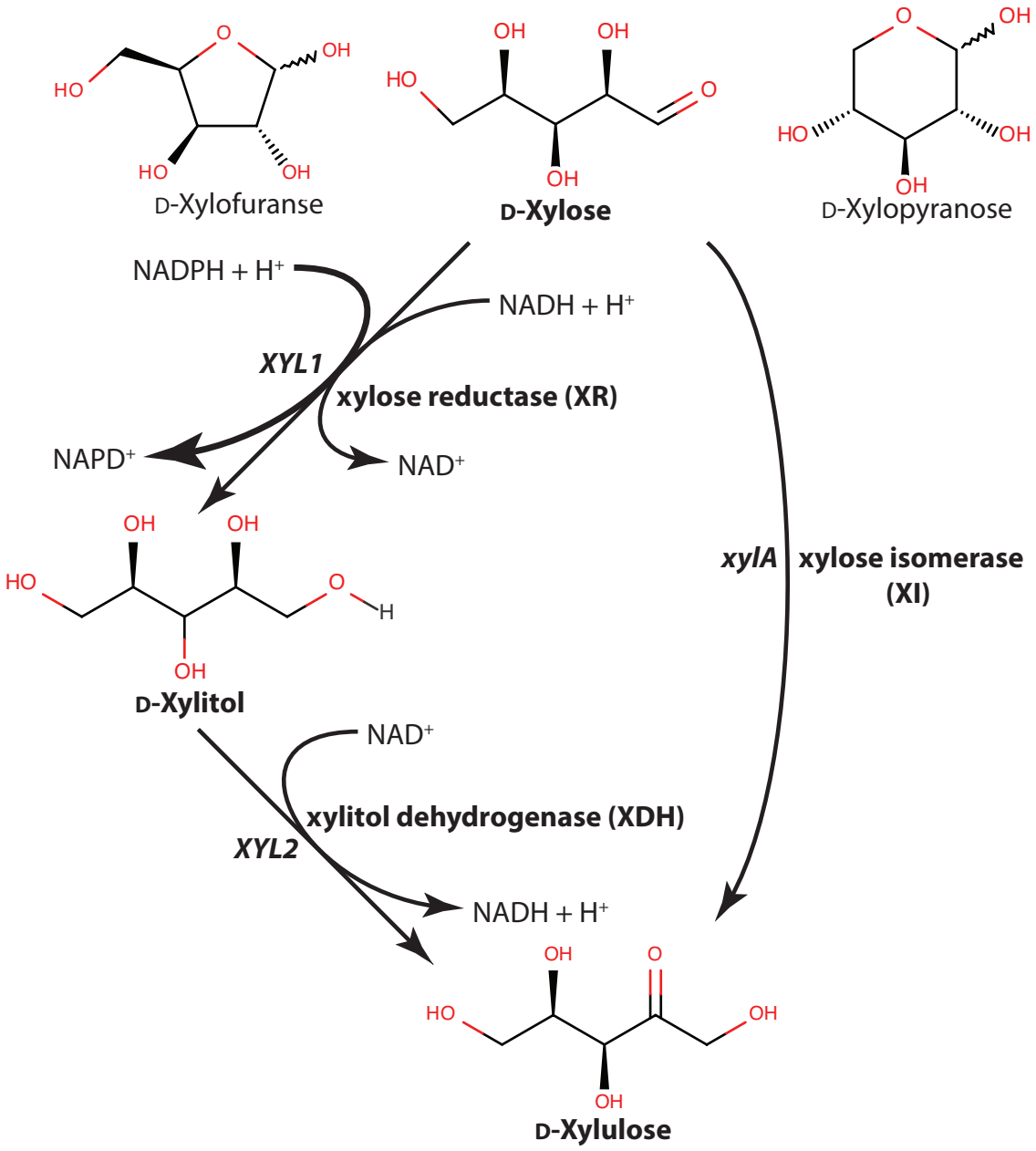

Figure 1-2 The conversion of xylose to xylulose via two different biochemical pathways. The two-step reduction-oxidation via xylose reductase and xylitol dehydrogenase (left) was widely used by yeast and fungi. The one step conversion by xylose isomerase (right) mainly exists in bacteria.

In the first pathway, XR first reduces xylose to xylitol, and XDH further converts xylitol to xylulose. D-Xylulose is converted to xylulose-5-phophate by xylulokinase (XK) and then enters into the pentose phosphate pathway (PPP). The pentose phosphate pathway is also referred to as the phosphogluconate pathway or the hexose monophosphate pathway (HMP). PPP includes an oxidative phase that generates NADPH, which provides reducing power for cell synthesis (anabolism), and a non-oxidative phase producing ribose-5-phosphate, precursors for DNA and RNA synthesis. In the oxidative phase, glucose-6-phosphate is converted to ribulose-5-phophsate, which generates NADPH and CO_2; in the non-oxidative phase, ribulose-5-phosphate and xylulose-5-phosphate are recycled and converted to fructose-6-phosphate and glyceraldehye-3-phospahte by transketolase and transaldolase. When pure xylose is the carbon source, 6 moles of xylulose-5-phosphate will be converted into 4 moles of fructose-6-phosphate and 2 moles of glyceraldehyde-3-phosphate to enter the Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) for ATP production. Since no ATP is involved in the PPP in either the oxidative or non-oxidative phase, converting ribulose-5- phosphate and ribose-5-phosphate from glucose-6-phosphate is carefully adjusted in the cytosol to maintain the flux balance.

In glycolysis, 1 mole of glucose is converted to 2 moles of pyruvate, generating 2 moles of ATP and NADH. Under an anaerobic or oxygen limited environment, 2 moles of acetaldehyde are produced by pyruvate decarboxylase (PDC), and then acetaldehyde is reduced to ethanol by aldehyde dehydrogenase (ADH) to replenish NAD+. NADH generated from glycolysis will be recycled in the second step from acetaldehyde to ethanol. Figure 1-3 illustrates the basic biochemical pathway, including PPP and glycolysis, for ethanol production from xylose and glucose in native xylose-fermenting yeasts.

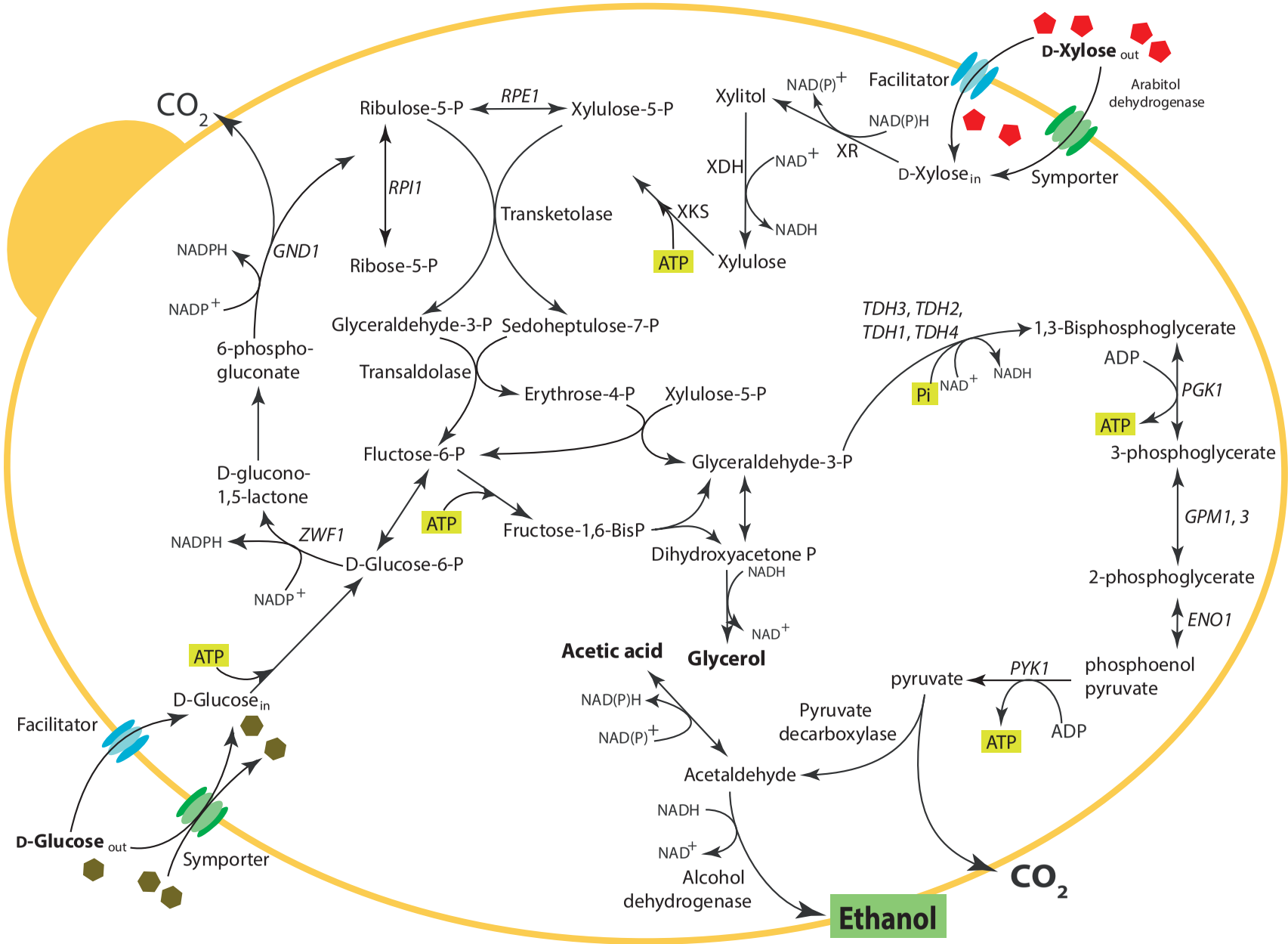
CO2
Ribulose-5-P
RPE1
Xylulose-5-P
RPI1
Ribose-5-P
Transketolase
NADPH
GND1
NADP+
6-phospho-
gluconate
Glyceraldehyde-3-P
Sedoheptulose-7-P
Transaldolase
Erythrose-4-P
Xylulose-5-P
Fluctose-6-P
D-glucono-
1,5-lactone
NADPH
ZWF1
NADP+
D-Glucose-6-P
ATP
Fructose-1,6-BisP
Dihydroxyacetone P
NADH
NAD+
Glyceraldehyde-3-P
TDH3, TDH2,
TDH1, TDH4
1,3-Bisphosphoglycerate
Pi
NAD+
NADH
ADP
PGK1
ATP
3-phosphoglycerate
GPM1, 3
2-phosphoglycerate
ENO1
phosphoenol
pyruvate
PYK1
pyruvate
ATP
ADP
Pyruvate
decarboxylase
Acetaldehyde
NADH
NAD+
Alcohol
dehydrogenase
Ethanol
CO2
Acetic acid
Glycerol
NAD(P)H
NAD(P)+
Xylitol
NAD(P)+
NAD(P)H
XR
XDH
NAD+
NADH
XKS
ATP
Xylulose
D-Xylose out
Facilitator
Arabitol
dehydrogenase
D-Xylose in
Symporter
ATP
D-Glucose in
Facilitator
D-Glucose out
Symporter

Figure 1-3 Metabolic pathways include xylose assimilation, pentose phosphate pathway (PPP), and Embden-Meyerhof-Parnas (EMP) pathway (glycolysis) in native xylose-fermenting yeast. XR, xylose reductase; XDH, xylitol dehydrogenase; XKS, xylulose kinase; *ENO1*, enolase (2-phosphoglycerate dehydratase); *GND1*, 6-phosphogluconate dehydrogenase; *GPM*, phosphoglycerate mutase; *PGK1*, phosphoglycerate kinase; *PYK1*, pyruvate kinase; *RPE1*, D-ribulose-5-phosphate 3-epimerase; *RPI1*, ribose-5-phophate isomerase ; *TDH*, glceraldehyde-3-phosphate dehydrogenase; *ZWF1*, glucose-6-phosphate dehydrogenase.

1.5 Engineering *Saccharomyces cerevisiae* for xylose fermentation

Despite the fact that native pentose-fermenting yeasts, such as *S. stipitis* and *S. shehatae*, are able to ferment xylose efficiently (ethanol yield ~ 0.4 g ethanol/g xylose), their performance (e.g. ethanol productivity, ethanol yield) is still not sufficient for industrial commercialization when compared to glucose fermentation by brewer's yeast, *Saccharomyces cerevisiae,* which has been employed in alcohol-producing industry for thousands of years. A modern industrial corn ethanol plant can produce about 12-14% (v/w) ethanol within 72 hours with an ethanol productivity of 2 g/L·h using robust industrial *Saccharomyces cerevisiae*; however, the xylose fermentation by native xylose fermenting yeasts at the laboratory scale can only reach ≈ 0.95 g/L·h from pure xylose medium and 0.6 g/L·h in maple hydrolysate (Long et al. 2012). Additionally, the maximum specific ethanol productivity of *S. stipitis* or *S. shehatae* from xylose could be 5-fold less than that of *S. cerevisiae* from glucose (Hahn-Hägerdal et al. 1994, Jeffries 1985).

However, native *S. cerevisiae* neither breaks down xylan nor ferments xylose. To overcome this barrier, various strain development approaches have been attempted. Early research showed *S. cerevisiae* could ferment xylulose to ethanol under certain conditions (Chiang et al. 1981, Wang et al. 1980). Thus, converting xylose to ethanol by *S. cerevisiae* could be achieved by using purified XI from other microorganism to convert xylose into xylulose first and then subsequent fermentation with *S. cerevisiae* (Gong et al. 1981a). Nevertheless, the unstable enzyme activity during hemicellulose fermentation and the high cost of purifying xylose isomerase posed major limitations on this approach (Hahn-Hägerdal et al. 1991, Jeffries 1983). Another strategy is to express xylose-assimilating genes from other pentose-utilizing microorganism in *S. cerevisiae* through metabolic engineering.

The initial attempt was to heterologously express an XI gene from bacteria so that *S. cerevisiae* could ferment xylose (Amore et al. 1989, Moes et al. 1996, Sarthy et al. 1987, Walfridsson et al. 1996). However, these attempts were not successful, possibly due to incorrect protein folding (Gardonyi and Hahn-Hagerdal 2003) or different codon preferences (van Maris et al. 2007). Overexpression of XI from an anaerobic fungus strain *Piromyces* sp. E2 (Harhangi et al. 2003) in *S. cerevisiae* enables anaerobic xylose fermentation in a chemostat with a glucose/xylose mixture, but the initial engineered strain only showed extremely slow growth (doubling time ~140 h) in shake flasks when xylose was the sole carbon source (Kuyper et al. 2003, Kuyper et al. 2004). Using other fungal xylose isomerase genes from organisms such as *Orpinomyces* sp. (Madhavan et al. 2009) or bacterial xylose isomerase from *Clostridium phytofermentans* (Brat et al. 2009), *Clostridium cellulovorans* (Ota et al. 2013) and *Burkholderia cenocepacia* (Vilela et al. 2013) has also protein expression in host *S. cerevisiae* strains.

However, using XI for xylose assimilation may encounter two potential challenges: first, XI favors xylose formation: At equilibrium at 40°C, only about 16% of 1 M D-xylose is isomerized to D-xylulose (Hsiao et al. 1982). *In vivo*, xylulose will be further phosphorylated and enter glycolysis via the PPP pathway, but a small amount of residual xylose will remain due to product inhibition and intracellular accumulation of xylulose, which suggests that another driving force is essential to promote the reaction (Jeffries and Jin 2004). Secondly, a non-specific NADPH-linked aldoreductase (Gre3p) exists in *S. cerevisiae* (Garay-Arroyo and Covarrubias 1999, Kuhn et al. 1995, Traff et al. 2002), and this enzyme is capable of converting xylose into xylitol, which not only reduces the ethanol yield but also competitively inhibits XI for xylulose formation (Hahn-Hagerdal et al. 2001). The deletion of *GRE3* can reduce xylitol accumulation in engineered *S. cerevisiae* when an XI-assimilation pathway is introduced, and this enzyme

converts xylose into xylitol, which not only reduces the ethanol yield but also competitively inhibits XI for xylulose formation (Hahn-Hagerdal et al. 2001). The deletion of *GRE3* can reduce xylitol accumulation in engineered *S. cerevisiae* when the XI-assimilation pathway is introduced (Karhumaa et al. 2005, Kuyper et al. 2005, Traff et al. 2001).

Another approach for xylose assimilation in *S. cerevisiae* is to overexpress the two-step reduction-oxidation pathway (Figure 1-2 B) by introducing XR and XDH from native-xylose fermenting yeasts, such as *S. stipitis* (Amore et al. 1991, Takuma et al. 1991, Tantirungkij et al. 1993). The reason for using *S. stipitis'* XR (*XYL1*) and XR (*XYL2*) is because *S. stipitis* has much higher ethanol yield and lower xylitol production as compared to *S. shehatae* and *P. tannophilus*. This is possibly due to the kinetic properties of its enzymes for oxidoreductase assimilation. Most XRs found in fungi have a much higher affinity for NADPH than for NADH as a cofactor and some of them use NADPH exclusively, such as the unspecific aldose reductase (Gre3p) in *S. cerevisiae* (Kuhn et al. 1995) and XR from *C. utilis* (Bruinenberg et al. 1983). On the other hand, XRs from *S. stipitis* (Rizzi et al. 1988, Verduyn et al. 1985b), *S. shehatae* (Ho et al. 1990) and *C. tenuis* (Neuhauser et al. 1997) can also use NADH as a cofactor (but prefer NADPH). Two isoenzymes of XRs were found in *P. tannophilus*, one of which only uses NADPH as a cofactor and the other uses both NADPH and NADH (Verduyn et al. 1985a). At pH 7, the thermodynamic equilibrium constant was about 0.575×10^3 (M^{-1}), which favors xylitol production (Rizzi et al. 1988). Studies on XDH showed that NAD^+ was exclusively used as a cofactor in native xylose-assimilating yeast (Kotter et al. 1990, Maleszka et al. 1983). The thermodynamic equilibrium constant at pH 7 was about 6.9×10^{-4} (M), which also favors xylitol production (Rizzi et al. 1989a).

Earlier attempts to introduce *XYL1* and *XYL2* showed that transformed *S. cerevisiae* could assimilate xylose under aerobic conditions. However, the recombinant yeast produced mainly xylitol (9.88 g) from 19.68 g/L of xylose, and the ethanol yield was only 0.16 g/g (Kotter and Ciriacy 1993). A similar observation was confirmed by Tantirungkij et al. (1993), in which about 30 g/L of xylitol and less than 10 g/L of ethanol were produced from 50 g/L of xylose under an oxygen-limited condition. The observation of low ethanol and high xylitol accumulation could be attributed to the cofactor imbalance between XR (use both NADPH and NADH) and XDH (which uses only NAD^+). The depletion of NAD^+ caused xylitol secretion by passive diffusion in recombinant *S. cerevisiae* (Jeffries 2006, Kotter and Ciriacy 1993, Tantirungkij et al. 1993). Another explanation for the high xylitol accumulation could be poor expression of the xylulokinase (XK). Overexpressing an endogenous xylulokinase (*XKS1*) in *S. cerevisiae* harboring *XYL1* and *XYL2* significantly improved ethanol production and ethanol yield under various aerations (anaerobic, 2% O_2, 20% O_2) when xylose was the sole carbon source (Toivari et al. 2001). But, later research reported that overexpression of *XKS1* also led to a decrease in xylose consumption by 50-80% (Johansson et al. 2001), and xylose utilization could decline by almost an order of magnitude when aeration decreased (Jeffries and Jin 2004). Overexpression of a heterologous xylulokinase from *S. stipitis* (*XYL3)* showed similar results (Jin et al. 2002, Jin et al. 2003), suggesting that a fine-tuned moderate level of xylulokinase would be necessary for improving the flux into PPP in engineered xylose-fermenting *S. cerevisiae* (Hahn-Hägerdal et al. 2007, Matsushika and Sawayama 2008).

Further study showed that overexpression of *SsXYL3* along with high levels of *XYL1* and *XYL2* would kill *S. cerevisiae* when plated onto xylose (Jin et al. 2005). However, mutations soon arose that would show improved growth on xylose. This led to the discovery of two

independent paths that would further improve xylose metabolism in *S. cerevisiae*: (1) overexpression of *TAL1*, which had been previously recognized (Jin et al. 2005) and (2) the deletion or inactivation of *PHO13* (Fujitomi et al. 2012, Kim et al. 2013b, Ni et al. 2007, Tomitaka et al. 2013, Van Vleet et al. 2008). The basis for enhanced xylose metabolism in a *pho13* background is still not fully understood, but since this protein has been characterized as a protein phosphatase in *S. cerevisiae* (Tuleva et al. 1998) it is possible that its activity reverses the effect of a protein kinase regulatory cascade. More recent work, however, suggests that the activity of Pho13 is that of a phosphoglycolate phosphatase (Blake et al. 2012). It exhibits rather high specificity for phosphoglycolate, (Suzanne O'Handley, personal communication), which is known to be a strong inhibitor of triosephosphate isomerase (Zhai et al. 2014)

Measurement of intermediate metabolites in an engineered xylose-fermenting *S. cerevisiae* revealed accumulation of sedoheptulose-7-phosphate but not fructose-1, 6-bisphosphate during xylose assimilation, which indicates that the initial XR/XDH/XK was not the limiting step in an engineered *S. cerevisiae*; instead, the subsequent non-oxidative phase in PPP was limiting (Kötter and Ciriacy, 1993). Thus, overexpression of genes to increase throughput in the non-oxidative PPP was a rational target. Overexpression of endogenous genes involved in the non-oxidative pathway of PPP in *S. cerevisiae*, including endogenous XK (*XKS1*) (Deng and Ho 1990), ribulose 5-phosphate isomerase (*RKI1*), ribulose 5-phosphate epimerase (*RPE1*), transketolase (*TKL1)*, transaldolase (*TAL1*) (Kuyper et al. 2005, Walfridsson et al. 1995) and evolutionary engineering (Zhou et al. 2012), were able to improve strain performance in XI-recombinant yeast. The levels of *TAL1* and *TKL1* were ≈ 2.8- and 1.8-fold higher in a Δ*pho1* mutant than in a corresponding *S. cerevisiae* control (Ni et al. 2007).

As mentioned earlier, different cofactor dependence between XR and XDH was thought to be the possible reason of xylitol secretion during xylose fermentation by recombinant *S. cerevisiae* (Krahulec et al. 2012). Protein-engineering by either site-directed mutagenesis (Bengtsson et al. 2009, Kostrzynska et al. 1998, Lee et al. 2012, Petschacher and Nidetzky 2008, Watanabe et al. 2007) or random mutagenesis with error-prone PCR (Runquist et al. 2010) and evolutionary engineering (Liang et al. 2007) have been used for creating NADH-preferring XR. A 20% increased in ethanol production and 52% decrease in xylitol secretion by alternating *Ss*XDH from strictly NAD^+-dependent to partially $NADP^+$-dependent have been reported by Watanabe et al. (2007, 2005). Matsushika et al. (2008) reported an increased ethanol yield to 0.49 g/g in the non-sulfuric acid hydrolysate wood chip by exercising the similar approach. Recently, Khatta et al. (2013, 2011) reported using strict NADPH-dependent mutated *Ss*XR and $NADP^+$-dependent *Ss*XDH is able to reduce xylitol production 34-50% in recombinant *S. cerevisiae*. Genome-scale modeling projected an increase in ethanol yield by 25% and 70% reduction in fermentation time can be achieved if the cofactor of XR/XDH was balanced (Ghosh et al. 2011).

Sugar transporters have been widely considered another target for metabolically engineered xylose-fermenting *S. cerevisiae* (Leandro et al. 2009, Young et al. 2014). This is because *S. cerevisiae* mainly takes up xylose with its hexose transporters, which have 10-100 times higher affinity for glucose than for xylose (Saloheimo 2007). *Candida intermedia* possesses both low-affinity facilitator (Gxf1) and a high capacity xylose/H+ symporter (Gxs1) (Leandro et al. 2006). Expressing Gxf1 in engineered *S. cerevisiae* was able to increase the xylose uptake rate at low substrate concentration in both synthetic medium and hydrolysate (Fonseca et al. 2011, Runquist et al. 2009).

Other targeted genes for improved cofactor balance and xylose fermentation include genes in the oxidative phase of the PPP (Jeppsson et al. 2002, Johansson et al. 2001) (i.e. disrupting *ZWF1*), overexpressing phosphoketolase (converting D-xylulose-5-phosphate into acetyl phosphate and D-glycerol-3-phosphate), and overexpressing transhydrogenase (Bruinenberg et al. 1984, Jeppsson et al. 2003), and *Pho13* (Fujitomi et al. 2012, Kim et al. 2013b, Van Vleet et al. 2008). Several extensive reviews of strategies to metabolically engineer *S. cerevisiae* for lignocellulosic ethanol production have been performed in recent years and will not be detailed here (Kim et al. 2013a, Laluce et al. 2012, Madhavan et al. 2012, Van Vleet and Jeffries 2009, Wang et al. 2013).

1.6 Oxygen and glucose fermentation by *S. cerevisiae*

The relationship between oxygen uptake and fermentation can vary greatly among microorganisms. Possibly the first correlation was observed and recorded by a French chemist and microbiologist, Louis Pasteur (Barnett 2000). In 1861, he observed that sugar consumption was significantly higher when yeast cells grew anaerobically compared to aerobically, and fermentation (ethanol accumulation) occurred only when oxygen was absent. When air was introduced, fermentation ceased (Pasteur 1861a, Pasteur 1861b). Thus, the term "Pasteur effect" was first used to describe the significantly greater amount and faster rate of sugar consumption by cells under anaerobiosis as compared to aerobically (Barnett and Entian 2005). Later, the term Pasteur Effect was used by Otto Meyehof (1920) to describe an inhibition of fermentation caused by oxygen molecules in yeast, muscle and other tissue (Tejwani 1978). One hypothesis attributed this phenomenon to the induction of respiratory enzymes by molecular oxygen along with an inactivation of an essential glycolytic enzymes (Lipmann 1933, Meyerhof et al. 1942, Wikén et al. 1961). Later research (Engelhardt and Sakov 1943, Engelhardt 1982) provided one explanation for the Pasteur effect by showing that cytochrome *c* and its oxidase (a key enzyme in mitochondria) were repressed by 6-phosphofructokinase (PFK) in the presence of air. PFK catalyzes the reaction from fructose-6-phosphate to fructose-1,6-bisphophate in glycolysis (Fig 1-3). No inhibition was observed when cells were cultivated in the presence of nitrogen gas, but when oxygen was present, the inhibition happened immediately (Engelhardt 1982). Recently, it has been confirmed that in *S. cerevisiae*, ATP and citrate are the *in vivo* allosteric inhibitors for PFK, and AMP, ADP, and fructose-2,6-bisphosphate are the allosteric activators (Schoneberg et al. 2013). Thus even small increases in ATP levels as a result of respiration can rapidly shut down glycolytic activity.

It is now generally accepted that the Pasteur effect describes the occurrence of fermentation in the absence of oxygen (Barker et al. 1964, Wikén et al. 1961), and that the increased consumption sugar accompanied by a more rapid CO_2 accumulation is necessary for ATP synthesis (for cellular proliferation) under anaerobiosis as compared to aerobiosis (Barnett and Entian 2005). Looked at from a different perspective, respiration allows more ATP synthesis than what can be obtained from glycolysis alone. Today, the term describing Pasteur's finding is applied not only to yeast but also to many other living systems.

Even though the Pasteur effect could be demonstrated widely, subsequent research showed contradictory results. In some cases *S. cerevisiae's* rate of glucose fermentation was either rapid in the early exponential phase of cell growth or independent of oxygen - depending on the sugar concentration (Brown 1892). Similar results were confirmed by Otto Meyehof (1925) as he concluded that Pasteur effect (meaning the activation of fermentation or sugar utilization by anaerobiosis) did not occur with *S. cerevisiae* when a high concentration of glucose was present (Barnett and Entian 2005). Swanson et al. (1948) showed that *S. cerevisiae* performed fermentative assimilation and produced ethanol even under aerobic condition when glucose (1%) is the carbon source. A switch from aerobic to anaerobic environment also had no effect on the rate of glycolysis and no observation of Pasteur effect (Lagunas 1979).

De Deken (1966) performed a comprehensive study on the respiration rates by measuring O_2 uptake and CO_2 accumulation under aerobic and anaerobic condition with various yeasts on four different carbon sources (glucose, fructose, mannose, and galactose). He found that respiration rate of *S. cerevisiae* was low on glucose and fructose (4.8~6.1 μL O_2/10 min/10^7 cells) but could be four times higher on galactose (20 μL O_2/10 min/10^7 cells) under aerobic conditions. These values also corresponded to the CO_2 accumulation rate, which was 78 μL CO_2/10min/10^7

cell for glucose and only 15.3 μL CO_2/10min/10^7 for galactose. These results suggested that it was possible to observe a respiratory system on *S. cerevisiae* when galactose was used as the carbon source, and the fermentation of glucose accumulated CO_2 faster (because sugar was utilized faster to generate more ATP) as compared to aerobic respiration of galactose, in which slower glycolysis (slower CO_2 evolved) is followed by an active TCA cycle.

De Deken (1966) also compared the CO_2 accumulation rate of *S. cerevisiae* under various growth conditions. He found that the fermentative pathway was constitutively employed when glucose was the carbon source under either aerobic or anaerobic conditions, even when cells were cultivated aerobically and then transferred into another aerobic condition. In other words, with *S. cerevisiae,* glucose was fermented rather than respired regardless of whether or not the cells were aerated. However, when transferring an anaerobic growing *S. cerevisiae* (on glucose) into a medium contains lactose (a non-fermentable substrate), the CO_2 accumulation rate decreased to only 25% compared to glucose-growing culture. In the same publication, he introduced the term "Crabtree effect" –named after Herbert Crabtree's finding to describe a repression of the respiration system by another energy-producing system. In this case, the system is the induction of fermentation by addition of glucose. Herbert Crabtree (1929) found that addition of glucose in tumor cells reduced the oxygen uptake rate by about 10%; but in normal tissue cells, adding glucose increased the oxygen uptake rate. The term "Crabtree effect" in yeast microbiology is used to describe the repression of aerobic respiration by glucose - even in the presence of oxygen. In this mode, the inhibition of respiration by glucose causes the flux of metabolites through glycolysis to overflow into fermentation (Barford and Hall 1979a, Beck and von Meyenburg 1968).

De Deken (1966) categorized yeasts into two classes based on the Crabtree effect: Crabtree positive and Crabtree negative. Crabtree positive yeast displayed a stronger or complete inhibition of respiration when glucose was present under aerobic conditions, such as *S. cerevisiae*. On the other hand, Crabtree negative yeasts manifested little or no repression of their respiration pathway when glucose was present. This included yeasts such as *C. utilis* and *C. tropicalis*, because the O_2-uptake pathway was dominant when oxygen was present (Dedeken 1966). Around the same time, Polakis et al. (1965) reported that glucose caused greater repression of both activity and the synthesis of the enzymes in TCA cycle as compared to galactose during aerobic growth, and the presence of glucose (>0.9%) could also inhibit mitochondria formation unless the glucose concentration was extremely low (<0.09%).

However, De Deken's finding on aerobic respiration triggered by galactose was not sufficient to explain the Pasteur's earlier contrary findings that the presence of oxygen decreased fermentation in *S. cerevisiae*. Later, Lagunas et al. (1982) revealed that even though air decreased sugar utilization in the resting *S.* cerevisiae after growing in nitrogen-limited medium, air had very little effect on fermentation when *S. cerevisiae* was freshly-grown on nitrogen-rich medium as long as sugar was present. The observations with resting *S. cerevisiae* could be explained by to the inactivation of sugar transporters or at least the need for energy to activate transport or certain enzymes necessary for anaerobiosis when switching from aerobic to anaerobic environments (Lagunas et al. 1982)

Growing *S. cerevisiae* with galactose or using resting *S. cerevisiae* caused by nitrogen starvation was able to demonstrate Pasteur effect (much faster CO_2 accumulation rate under anaerobic condition), while growing *S. cerevisiae* in exponential phase with excess glucose or fructose triggers Crabtree effect. To account for these contradictory findings, intensive chemostat

studies with metabolite measurement and enzyme assay were conducted in order to understand the mechanism behind this regulation system and to calculate the metabolic flux at branching point (Barford and Hall 1979a, Beck and von Meyenburg 1968, Fiechter and von Meyenburg 1966, Postma et al. 1989, Rieger et al. 1983, Verduyn et al. 1992, Vonstockar and Auberson 1992).

Aerobic chemostat studies with *S. cerevisiae* under glucose-limited condition revealed that when the specific growth rate (μ) or dilution rate (D) (in a chemostat study, μ equals to D when the system reaches the steady-state) was higher than a critical limit, approximately 0.31(values reported ranged from 0.2 to 0.38 h^{-1}, depending on the strain), cell yield dropped accompanied by ethanol accumulation and enhanced CO_2 accumulation, and the glycolytic pathway was predominately used for energy production. When cells were growing at this critical D, maximum specific O_2 uptake rates (qO_2) ranging from 7.6-12 mmol O_2/g cell dry weight/ h were observed, and the increasing dilution rate will cause the respiration quotient (RQ), the ratio of specific CO_2 production rate to qO_2, to be greater than 1 due to the significantly increased specific CO_2 evolved rate. When the specific growth rate drops below this critical limit (e.g. 0.05-0.30 h^{-1}), fully aerobic respiration accompanied by increased cell yield ranging from 0.47 to 0.49 can be observed, and RQs were reported around 1 (Barford and Hall 1979a, Beck and von Meyenburg 1968, Fiechter and von Meyenburg 1966, Postma et al. 1989, Rieger et al. 1983, von Meyenburg 1969). Several important findings based on the above chemostat studies are summarized as follows:

1. Medium composition used in the chemostat can have a significant influence on the yeast physiology and the reported critical dilution rate for *S. cerevisiae*. Reiger (1983)

suggested that the maximum respiration rate and critical dilution rate reported by von Meyenburg (1969) could be a result of using yeast extract.

2. Alcoholic fermentation under aerobic conditions can be triggered during continuous operations when D is above the critical limit (long-term effect). Alcoholic fermentation can also be triggered by a sudden change in dilution rate of more than 0.02 h^{-1} (Fiechter and Seghezzi 1992, Postma et al. 1989, Verduyn et al. 1992).
3. Addition of weak acid (i.e. benzoic acid, acetic acid) in glucose-limited conditions (at D=0.1 h^{-1}) reduced cell yield but increased qO_2 and specific ethanol production rate. This was assumed to be due to the uncoupling effect of weak acid (Postma et al. 1989, Verduyn et al. 1992).
4. Based on metabolic flux analyses and enzymatic assays, it was the glucose flux or the *in vivo* concentration of the metabolite that was responsible for the repression of respiration, and it was the presence of glucose that caused repression of enzymes in the TCA cycle (Postma et al. 1989, Verduyn et al. 1992). Data suggested the glucose-induced repression of oxidative metabolism was attributed to the lower respiration capacity of *S. cerevisiae* compared to Crabtree negative yeast (i.e. *C. utilis*), as well as the reduced capacity of acetaldehyde dehydrogenase (converts acetaldehyde to acetic acid) and acetyl-CoA synthetase (converts acetic acid to acetyl-CoA for entering the TCA cycle with the expense of 1 mole of ATP per mole of acetic acid). Pyruvate decarboxylase (converts pyruvate to acetaldehyde) had a much higher Km value (6 mM) when compared to pyruvate dehydrogenase (the first enzyme of the pyruvate dehydrogenase complex, which converts pyruvate to acetyl-CoA for later oxidative phosphorylation). It was assumed that when the dilution rate increases, the saturated acetaldehyde to acetic

acid pathway and respiration pathway cannot provide sufficient energy, and the fermentation pathway needs to step in to provide more ATP, which increases the glycolysis flux and *in vivo* pyruvate concentration. These trigger the pyruvate decarboxylase and alcohol dehydrogenase for ethanol accumulation for redox balance (Barford and Hall 1979b, Fiechter and Seghezzi 1992, Postma et al. 1989, Vandijken and Scheffers 1986, Verduyn et al. 1992).

5. Pyruvate is located at the branching point of metabolic flux in *S. cerevisiae.* As soon as pyruvate is formed, cells need to decide to either convert pyruvate to ethanol for redox balance (only make 2 mole of ATP/mole glucose) or to convert pyruvate to acetyl-CoA and into TCA cycle for oxidative phosphorylation (make 30 ATP/mole of glucose).

It has been found that the lower respiration capacity of *S. cerevisiae* compared to Crabtree negative yeast is due to the loss of mitochondria complex 1 in *S. cerevisiae*, which accounts for about half of energy production in oxidative phosphorylation. Research has also shown that when glucose fermentation initiates, overexpression of certain key enzymes in the glycolytic pathway, such as hexokinase (Hks2), PFK, and pyruvate decarboxylase (Pyk1) is observed, and the overexpression of Hks2 is insensitive to the product inhibition caused by glucose-6-phosphate (Diaz-Ruiz et al. 2011, Frohlich et al. 1985). Glucose also inactivates certain enzyme in mitochondria (Polakis et al. 1965, Takeda 1981) Also, glucose regulates the expression level of several enzymes by different mechanisms such as decreasing the concentration of corresponding mRNA, decreasing the mRNA translation rate, or accelerating the protein degradation (Gancedo 1998).

The fermentation of glucose by *S. cerevisiae* occurs regardless of the presence of oxygen (due to Crabtree effect); thus, theoretically this reaction should happen in strict anaerobic

conditions. However, under a strict anaerobic environment, the fermentation of glucose in minimal defined medium by *S. cerevisiae* cannot proceed unless certain nutrients, such as ergosterol, tween 80, or oleic acid are added as a supplement (Andreasen and Stier 1953, Andreasen and Stier 1954, Andreasen and Stier 1956). During ecological fermentation (wine-making) and brewery fermentation, addition of oxygen is permitted and it is also a common practice for yeast cells to synthesize sterols and unsaturated fatty acid for membrane integrity (Rosenfeld et al. 2003).

Possible confusion could arise due to the use of the term Crabtree effect in yeast microbiology, and use of the terms Crabtree effect as well as Warburg effect in oncology in several review articles and book chapters (Barnett and Entian 2005, Dakubo 2010, Diaz-Ruiz et al. 2011, Diaz-Ruiz et al. 2009). The author considers it necessary to clarify these terms when writing this chapter because of the difference of cellular physiology between tumor cells and yeasts. Based on personal communication with Prof. Chris Hittinger (Department of Genetic, University of Wisconsin-Madison), modern authors are often interpreting these findings (usually from other language) through a modern or discipline-specific lens. Thus, the definition of Pasteur effect and Crabtree could vary.

1.7 Oxygen and xylose fermentation by native pentose-fermenting yeasts

Despite tremendous work with engineering *S. cerevisiae* for lignocellulosic ethanol production, most engineered *S. cerevisiae* showed slower xylose uptake and lower ethanol yield as compared to native xylose-fermenting yeast when a moderate amount of xylose (40~60 g/L) was the sole carbon. Also, most engineered *S. cerevisiae* produce ethanol from the glucose-utilization phase instead of the xylose-consuming phase, and exhibit respiratory response and Crabtree negative effect when fermenting xylose. (Jin et al. 2004, Souto-Maior et al. 2009). Therefore, using native xylose-fermenting yeasts has also been considered a possible alternative route for lignocellulosic ethanol production (Hickert et al. 2013, Jin et al. 2004, Silva et al. 2012, Watanabe et al. 2012).

One major challenge for ethanol production from xylose by native pentose-fermenting yeasts is to control the requirement of oxygen for ethanol production. Too much oxygen induces aerobic cell growth and causes low ethanol yield, whereas low oxygen concentration triggers xylitol accumulation, low ethanol titers, and stalls fermentation.

Native xylose-fermenting yeasts belong to the group of Crabtree negative yeasts. The first reported xylose-fermenting yeast, *C. tropicalis*, even requires aeration for ethanol production (Jeffries 1981). The reason for the oxygen requirement by native-xylose fermenting yeasts has not been fully understood so far, but it was first thought to be associated with the cofactor imbalance between XR and XDH in native xylose-fermenting yeasts (Bruinenberg et al. 1983).

The first investigation on oxygen requirement on xylose fermentation with native xylose-fermenting yeasts was possibly done by Ligthelm et al. (1988), in which *P. tannophilus*, *S. shehatae*, *and S. stipitis* were examined under three different aeration conditions (aerobic,

oxygen limited, anoxic). The result suggested that oxygen stimulates not only the xylose fermentation rate but also the rate of glucose fermentation by these yeasts. The improved ethanol production under aerobic conditions was similar to what has been observed as the "Custers effect" with yeast *Brettanomyces intermedius* (Wijsman et al. 1984). The Custers effect was first discovered by Custers (Custers 1940) with *Brettanomyces spp.* and the term was introduced in the 1960s (Scheffer.Wa 1966, Wikén et al. 1961). The Custers effect describes an inhibition of alcoholic production during anaerobic condition when yeast cells are transferred from an aerobic environment to anaerobic conditions (Scheffers 1979). These observations were very different from what has been observed with *S. cerevisiae*.

The Custers effect was attributed to an initial shortage of NAD^+ and could be abolished by addition of a carbonyl hydrogen acceptor such as acetoin, to re-oxidize NADH to NAD+, or with a small amount of oxygen (Scheffer.Wa 1966). Fermentation of xylose (20 g/L) by *C. utilis* ceased immediately when transferred from aerobic to anaerobic condition. When growing *S. stipitis* and *S. shehatae* under strictly anaerobic conditions, similar results were observed (Du Preez 1994, Slininger et al. 1991). But, the fermentation by *C. utilis* can be restored by the addition of acetoin (100 mM) with formation of ethanol, acetic acid and concomitant reduction of acetoin to 2,3-butanediol (Bruinenberg et al. 1983). The studies on the effect of addition of hydrogen acceptor later were performed on *P. tannophilus* (Ligthelm et al. 1989) and *S. stipitis* (Delgenes et al. 1991). When acetaldehyde (100 mM), acetone (50 mM), and acetoin (50 mM) were added, *P. tannophilus'* molar ethanol yield increased from 1.03 to 1.63, 1.43, and 1.24 respectively at the expense of the molar xylitol yield (the theoretical molar ethanol yield is 5/3). Delgenes et al. (1991) performed a fermentation of 20 g/L xylose with *S. stipitis* NRRL Y-1724 in the presence of an initial ethanol concentration varying from 0 to 40 g/L of ethanol. Ethanol

stimulated xylitol production linearly and reached a ratio of 0.2 mol xylitol produced/mol xylose consumed with an initial ethanol of 40 g/L. But upon the addition of 85 mM of acetoin in the fermentation medium in the presence of 10 g/L ethanol, the xylitol concentration decreased about 60%. The result also suggested that *S. stipitis* utilized acetoin as an external NAD^+-generating system since the conversion rate of acetoin to 2,3 butanediol was about 96% (Delgenes et al. 1991).

Oxygen could also be necessary for inducing or activating a transport system (Hahn-Hägerdal et al. 1994). Another possible explanation for the oxygen requirement for xylose fermentation could be the difference of cellular physiology that was favorable in the particular environments in which native pentose-fermenting yeasts and *S. cerevisiae* evolved. For example, glucose induces a strong carbon catabolite repression in *S. cerevisiae*, which not only shuts down the respiration pathway but also overexpresses key enzymes in glycolysis (Gancedo 1998). Additionally, the loss of mitochondrial complex I during evolution possibly gave *S. cerevisiae* an advantage as it evolved high levels of several enzymes in the glycolytic pathway. These included pyruvate decarboxylase and alcohol dehydrogenase with much higher capacity compared to the levels observed in *S. stipitis* and *S. shehatae*. Moreover by being deficient in NADH/ubiquinone reductase, more NADH is available for reduction of acetaldehyde to ethanol. The phenomenon of overflowing metabolite flux induced by glucose in *S. cerevisiae* does not exist in native pentose-fermenting yeasts either.

Besides, the sugar transporter systems are also very different between *S. cerevisiae* and native-xylose-fermenting yeasts. Glucose is only transported by facilitators in *S. cerevisiae* and has at least 17 hexose transporters (Hxt1p-Hxt17p), which is very different compared to *S. stipitis*. Xylose is mainly transported by active H+ symporter in *S. stipitis*, which requires energy.

S. stipitis also has a smaller number of sugar transporters than S. cerevisiae, and these fall into the SUT/HXT/XUT designations (Jeffries et al. 2007, Jeffries and Jin 2004, Leandro et al. 2009).

Visser et al. (1990) screened of 75 type strains of yeast genera and found that only 18 out of 75 showed positive fermentation in a serum-flask anaerobic growth experiment, and most of them exhibited a long lag time (5-10 day). Further characterization showed that *S. cerevisiae* stood out as a yeast able to grow rapidly (less than 24 h) at low dissolved oxygen condition (less than 0.005% DOT [100% DOT is equal to 7.54 mg O_2/L at 30°C, 0.005% DOT = 0.038 mg O_2/L ≈ 1 μmol O2/L]).

A number of studies on the oxygen requirements for native xylose-ferment yeasts have been carried out since 1986. A wide range of oxygen transfer rates (OTR) from 0.7 to 8.6 mmol O_2/L·h has been investigated in various studies on *S. stipitis* and *S. shehatae* to understand the correlation between aeration and fermentation performance (Delgenes et al. 1989, Dupreez 1994, Skoog and Hahnhagerdal 1990, Sreenath et al. 1986). The OTR values ranging from 1.75 to 5 mmol/L·h were suggested as optimal for *S. stipitis* with 5% sugar (Grootjen et al. 1990, Guebel et al. 1991, Laplace et al. 1991). Other studies suggested that no ethanol accumulation occurred when dissolved oxygen tension (DOT) is higher than 3 μmol O_2/L (Rizzi et al. 1989b), and the optimal DOT of *S. stipitis* and *S. shehatae* are below the detection limit of the dissolved oxygen sensors (<0.08 mg/L = 2.5 μmol O_2/L) (Skoog and Hahnhagerdal 1990). At such low DOT levels, the physiological responses of these yeasts could vary (Dellweg et al. 1989, Du Preez et al. 1989).

Nonetheless, the optimal value of OTR, at which cells exhibit the maximum ethanol productivity, can be affected by medium composition and operation conditions as well as cell density. A study on *S. stipitis* with xylose (10g/L) or a glucose/xylose sugar mixture (40/10 g/L) revealed that addition of glucose can relieve the oxygen demand (Grootjen et al. 1990).

Computational calculation suggested that less oxygen was needed for cell maintenance during glucose fermentation than xylose (Dellweg et al. 1989). Specific oxygen uptake rate (qO_2), which was the overall oxygen utilization rate (OUR) divided by the cell concentration, was suggested to be more meaningful as compared to OTR since it took into consideration the amount oxygen required per cell during xylose assimilation and the redox balance (Dupreez 1994). The value of qO_2 can be calculated by combing ATP yield, stoichiometry, and material balance, and assuming growth is coupled with energy production. (Cooney et al. 1977, Jansen et al. 1984, Rizzi et al. 1989b). A similar approach by Dellweg et al. (1989) suggested that a constant qO_2 of 0.3 mmol O_2/g CDW·h can raise the ethanol yield to 0.44 g/g with xylose concentration not higher than 60 g/L.

A study on *S. shehatae* showed that a qO_2 of 1.19 mmol O_2 /g cell dry weight·h was able to maximize ethanol yield (0.327 g/g) with an average ethanol productivity of 2.2 g ethanol/L·h in a fed-batch fermentation containing 10% xylose (Fromanger et al. 2010). Recently, an oxygen transfer coefficient (K_La) within the range between 2.3 to 4.9 h^{-1} was suggested for *S. stipitis* with 90 g/L of xylose, and the K_La corresponds to an OTR between 0.55 to 1.17 mmol O_2/L·h at 30°C. (Silva et al. 2012). Unrean and Nguyen (2012), using an elementary mode analysis, predicted that an OTR of 1.8 mmol/L·h was optimal for *S. stipitis* in a 1-L fermentor containing 15 g/L of glucose and 5 g/L of xylose. This model is validated with batch fermentation, which showed an ethanol yield of 0.4 g/g and ethanol productivity of 0.25 g/L·h. Slininger et al. (2014) suggested that ethanol production with *S. stipitis* is growth associated, and ethanol production can occur even when the growth rate is as high as 0.5 h^{-1}; the requisite dissolve oxygen to support that rate of growth would be at least 1-3 mg O_2/L (or 31 to 93 μmole O_2 /L) at 25°C.

1.8 New isolated native xylose-fermenting yeast*s*

Metabolic engineering or evolutionary engineering on *S. cerevisiae* or native xylose-assimilating yeast has been successfully increased xylose fermentation performance. There is also a continuing effort in isolation of new native xylose-fermenting yeasts, which include genus of *Spathaspora* and *Candida* (Barbosa et al. 2009, Cadete et al. 2012, Cadete et al. 2013, Cadete et al. 2009, Nguyen et al. 2006, Watanabe et al. 2012)

Spathaspora passalidarum NN245 (=NRRL Y-27907=ATCC MYA4345=CBS 10155=11-Y1), isolated from passalid beetle *Odontotaenius disjunctus* (Patent-leather beetle) in Louisiana by Nguyen et al. (2006), was recently reported to have the capacity for 'anaerobic' xylose fermentation in 100 ml sealed vials containing 50 ml medium (Hou 2012). (Note that the conditions were not truly anaerobic as the medium and headspace contained air.) Normally, The XR of native xylose-fermenting yeast exhibits $NADP^+$-preferring rather than NAD^+; however, the XR activity in crude extracts of *S. passalidarum* exhibits a higher affinity for NAD^+ (17.3±1.8 μM) than for $NADP^+$ (31.7±2.6 μM). The genome sequence of *S. passalidarum* reveals the presence of two genes that appear to encode XR.

S. passalidarum and *S. stipitis* are closely related based on phylogenic analyses, and both are found in the hindgut of *O. disjunctus*. They both belong to the "CUG" clade of yeasts that utilize an alternative genetic code and introduce serine instead of leucine when encountering a CUG codon (Wohlbach et al. 2011). However, *S. stipitis* (and *S. stipitis*-like) yeast isolates have been constantly isolated from approximately 400 individual *O. disjunctus*, including both adults and larvae (Gross 2010, Nardi et al. 2006, Nguyen et al. 2006, Suh et al. 2003, Suh et al. 2004), but *S. passalidarum* have only been isolated once from an adult *O. disjunctus*. Molecular techniques also confirmed that *S. stipitis*-like yeast was the predominant yeast isolates from *O.*

disjunctus (Zhang et al. 2003). These observations clearly suggested that *S. passalidarum* is not a common associate of *O. disjunctus* (Nguyen et al. 2006). However, their similar evolution in association with beetles living inside of rotting wood probably accounts for their capacities to metabolize a wide range of lignocellulosic sugars and their capacities to grow at low oxygen tensions. In 2009, *Spathaspora arborariae*, the only single teleomorph species of *Spathaspora* clade at that time, was isolated in Brazil (Cadete et al. 2009). In 2013, four new xylose-fermenting yeasts belonging to the clade *Spathaspora* were recovered from a region of Amazonian forest of Northern Brazil (Cadete et al. 2013).

This thesis focuses on the physiology and strain development of *Spathaspora passalidarum* NN245. To better understand the physiology of this novel pentose fermenting yeast, the kinetics of xylose fermentation in the presence and absence of glucose were first investigated, and a metabolite analysis was performed (Chapter 2), which revealed that this novel yeast can co-ferment glucose and xylose under certain conditions and can co-utilize cellobiose, glucose, xylose for ethanol production. In Chapter 3, the effects of aeration on ethanol production are compared with another native pentose-fermenting yeast *Scheffersomyces stipitis.* The findings suggest a faster glycolytic flux in *S. passalidarum* compared to *S. stipitis* presents. In order to increase the utility of *S. passalidarum* for lignocellulosic ethanol production, strains were adapted and evolved for growth in the presence of cellulosic sugar solutions (hydrolysates) from several sources, and one strain, YK208-E11, exhibit improved fermentative activity in AFEX hydrolysate was further characterized (Chapter 4).

1.9 Nomenclature

EMP	Embden-Meyerhof-Parnas (glycolysis)
OTR	Oxygen transfer rate
OUR	Oxygen utilization rate
qO2	Specific oxygen uptake (or utilization) rate
PPP	Pentose phosphate pathway
XDH	Xylitol dehydrogenase
XI	Xylose isomerase
XK	Xylulokinase
XR	Xylose reductase

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Chapter II

Cofermentation of Glucose, Xylose, and Cellobiose by the Beetle-Associated Yeast *Spathaspora passalidarum*

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2.1 Abstract

Fermentation of cellulosic and hemicellulosic sugar from biomass could resolve food-versus-fuel conflicts inherent in the bioconversion of grains. However, the inability to conferment glucose, xylose, and cellobiose is problematic for most microbes because glucose represses utilization of the other saccharides. Surprisingly, the ascomycetous, beetle-associated yeast *Spathaspora passalidarum*, which ferments xylose and cellobiose natively, can also conferment theses two sugars in the presence of 30 g/L glucose. *S. passalidarum* simultaneously assimilates glucose and xylose aerobically, it simultaneously coferments glucose, cellobiose, and xylose with an ethanol yield of 0.42 g/g, and it has a specific ethanol production rate on xylose more than 3 times that of the corresponding rate on glucose. Moreover, an adapted strain of *S. passalidarum* produced 39 g/L ethanol with a yield of 0.37 g/g sugar from a hardwood hydrolysate. Metabolome analysis of *S. passalidarum* before onset and during the fermentation of glucose and xylose showed that the flux of glycolytic intermediates is significantly higher on xylose than on glucose. The high affinity of its xylose reductase activities for NADH and xylose combined with allosteric activation of glycolysis probably account in part of its unusual capacities. These features make *S. passalidarum* very attractive for studying regulatory mechanisms enabling bioconversion of lignocellulosic materials by yeasts.

2.2 Introduction

Cofermentation of glucose (G) and xylose (X) is critical for bioconversion because they are present in virtually all enzymatic hydrolysates of pretreated lignocellulose (Binder and Raines 2010, Zhong et al. 2009). Their sequential utilization extends fermentation times and can result in incomplete substrate consumption if products reach inhibitory levels before slowly utilized sugar are consumed (Himmel et al. 1997). Moreover, Cofermentation of xylose and cellobiose (Cb) could reduce enzyme costs and facilitate simultaneous saccharification and fermentation. Glucose represses utilization of xylose, cellobiose, or other carbon sources in most microbes (Gancedo 1998, Gorke and Stulke 2008). This is a problem that can be addressed through metabolic engineering or strain selection.

Placing genes for xylose or cellobiose assimilation under the regulation of constitutive promoters or altering transcriptional activators (Tyo et al. 2007) can relieve glucose repression (Kim et al. 2012). For example, coutilization of glucose and xylose (Fonseca et al. 2011, Krahulec et al. 2010) and of cellobiose and xylose (Saitoh et al. 2010) by engineering *Saccharomyces cerevisiae* can produced 60 g/L ethanol in 72 h from pure sugars (Ha et al. 2011). However, even when heterologous genes for assimilation are expressed under constitutive promoters, coutilization with glucose is problematic because the affinities of yeast monosaccharide transporters are 8 to 100 times higher for glucose than for xylose (Saloheimo et al. 2007, Salusjarvi et al. 2008). This problem can be addressed by introducing heterologous transporters with a higher affinity for xylose. For example, *S. cerevisiae* TMB34006, engineering with the glucose/xylose facilitator Gxf1 from *Candida intermedia* (Runquist et al. 2009), show 1.2- to 2-fold higher rates of xylose uptake when the initial glucose concentration is less than 5 g/L (Fonseca et al. 2011). To accommodate higher rates of xylose transport, downstream

reactions in the xylose metabolic pathway must be overexpressed, but cofactor imbalances limit xylose assimilation in the presence of glucose (Krahulec et al. 2010) and reduce its contribution to metabolic flux (Fonseca et al. 2011). Cofermentation of xylose and cellobiose is less difficult because cellobiose, a disaccharide, does not compete with xylose transport (Ha et al. 2011).

Systematic modeling and metabolic engineering can modify *S. cerevisiae* to address these problems, but this yeast does not provide a useful model for how overall metabolic flux is natively regulated during cultivation on xylose. In fact, aside from a few studies of genes and enzymes induced during the assimilation of xylose and the induction of pyruvate decarboxylase under oxygen limitation (Passoth et al. 2003), very little is known about how native xylose-fermenting yeast mediate the simultaneous metabolic demands for cell growth and product formation during ethanol production on xylose. Most native xylose-fermenting yeasts such as *Scheffersomyces* (*Pichia*) *stipitis* (Kurtzman and Suzuki 2010) do not coutilize glucose and xylose, largely because glucose represses the genes for xylose assimilation (Jeffries and Van Vleet 2009). However, this does not appear to be the case for all yeasts.

In the present work, we report that an unusual native xylose-fermenting yeast, *Spathaspora passalidarum* NN245 (Hou 2012, Nguyen et al. 2006, Wohlbach et al. 2011), coassimilates xylose and glucose aerobically, use xylose faster than glucose when the sugars are presented individually, and coferments glucose, xylose, and cellobiose from mixtures of pure sugar or hydrolysates under oxygen-limiting conditions. These features make *S. passalidarum* potentially useful for simultaneous saccharidification and fermentation (SSF) and for studying enzymatic and regulatory mechanism enabling simultaneous utilization of cellulosic and hemicellulosic sugars,

2.3 Materials and Methods

2.3.1 Organism, medium and culture conditions

Spathaspora passalidarum NN245 (NRRL Y-27907, CB2 10155, MYA-4345) (Nguyen et al. 2006) was used for all experiments and adaptations reported here. Cultures were maintained on YPX agar plates, containing 10 g/L yeast extract, 10 g/L peptone, 20 gr xylose, and 20 g/L agar. Inocula were cultivated overnight in 50 ml of YP broth, containing 20 g/L yeast extract, 10 g/L peptone, plus 20 to 60 g/L G, X, or Cb. The cultivation was then transferred to defined minimal medium and grown to an appropriated cell density. All flask cultivations were carried out at 30C in 125-ml polycarbonate flasks (GeneMate) fitted with membrane filters for aeration. Aerobic and oxygen-limited inoculum prepararion conditions were 200 rpm and ≤ 0.2 g/L cell dry weight (cdw) and 100 rpm and ≥ 1 g/L cdw, respectively. Defined minimal medium (CBS) containing nitrogen, trace metal elements, and vitamins was used in all bioreactor cultivations. It contained 2.4 g/L urea, 3 g/L KH_2PO_4, 0.5 g/L $MgSO_4.7H_2O$, 1 ml/L trace element solution, 1 ml/L vitamin solution, and 0.05 ml/L antifoam 289 (A-8436; Sigma) (modified from the medium described in reference (Verduyn et al. 1992)). Carbon source consisted of D-glucose, D-xylose or a mixture of xylose with glucose and/or cellobiose. Maple hemicelluloses hydrolysate (MHH) medium consisted of a 9:1 (vol/vol) mixture of MHH (Stoutenburg et al. 2008) and 10-fold-concentrated CBS basal defined minimal medium. Supplemental glucose was added to the hydrolysate to attain 65 g/L xylose and 35 g/L glucose. Control medium contained the same sugar in CBS defined minimal medium. The starting inoculum was 0.8 mg cdw/ml. AFEX hydrolysate medium (Lau and Dale 2009, Zhong et al. 2009) consisted of AFEX hydrolysate supplemented with 2.4 g/L urea, 1g/L KH_2PO_4, 1g/L K_2HPO_4, 50 mg/L $FeSO_4.7H_2O$, 5.5 mg/L $ZnSO_4.7H_2O$, and 8 mg/L $CuSO_4.5H_2O$. Modified

vitamin solution (Slininger et al. 2006) deleted riboflavin, folic, and thiotic acid. Bioreactor fermentations were performed in duplicate New Brunswick Scientific Bio Flo 110 3-L reactors with working volumes of 2 liters. The bioreactor temperature was maintained at 25°C (Slininger et al. 1990, Slininger et al. 2006), and the pH was kept constant at 5.0 ± 0.1 by automatic addition of 5 N KOH. All fermentation kinetics and metabolite levels are reported as averages of biological duplicates. The oxygen transfer rate (OTR) was calculated using the gassing out method based on kinetic oxygen measurements (Sikyta 1983). The dissolved oxygen level (dO_2) was monitored using an InPro 6800 oxygen sensor. Oxygen transfer rates were measured independently in four bioreactors at two agitation rates using air and a 90:10 mixture of N_2 and air (Table 2-1). The bioreactor was sparged with air at 0.5 L/min (0.25 volume/volume minute [vvm], 500 rpm). Full air saturation (100% of scale) was set after reaching equilibrium. Under these conditions, the OTR conditions, the OTR was estimated to be 13.56 ± 2.33 mmol O_2/L ·h. The zero level was set following equilibrium when sparging with nitrogen. Fully aerobic cultures (see Figure 2-1) were sparged with 1 vvm air and agitated at 700 rpm. For oxygen-limited fermentation of glucose or xylose, the airflow was set to 0.25 vvm, while a dissolved oxygen (dO_2) controller (New Brunswick) maintained agitation over the range of from 150 to 300 rpm to attain 10% saturation (#2.1% dO_2). Once the growth phase was complete (Figure 2-1, arrow 1), air was replaced with a mixture of 90% N_2 and 10% air to attain a mole fraction of O_2 in the gas phase. The dO_2 controller was set to a range of 300 to 500 rpm, and ethanol production ensued (see Figure 2-2). In the glucose, xylose, and cellobiose

Table 2-1 Oxygen transfer rates (OTR) in New Brunswick Scientific Bio Flo 110 3-L bioreactor at 0.25 vvm (volume [0.5 L] /volume [2 L] per minute), 25°C. The dissolved oxygen (dO_2) was monitored using n InPro 6800 oxygen sensor. OTR was measured independently in four bioreactors using air and a mixture of N_2 and O_2 (9:1).

Gas sparging rate = 0.5 L/min Liquid volume = 2 L	Oxygen transfer rates (mmole O_2/L·h)	
Gas composition	300 rpm	500 rpm
21% O_2 (with house air)	5.74±1.36	13.56±2.33
2.1% O_2 (N_2: Air = 9:1)	0.52±0.06	1.48±0.25

cofermentation (see Figure 2-4) and in the MHH fermentation (see Figure 2-5), the bioreactors were equilibrated with 90% N_2 and 10% air (2.1% O_2) from the start, and the dO_2 controller was set to a range of from 400 to 500 rpm.

2.3.2 Adaptation and fermentation

For cofermentation, *S. passalidarum* was propagated first in four 50-ml aliquots of YPX-Cb medium (45 g/L X, 25 g/L Cb) in a 125-ml Erlenmeyer flask shaken at 200 rpm at 30°C. After 24 h, these cultures were used to inoculate four 200-ml volumes of CBS X-Cb medium in 2-L Erlenmeyer flasks, which were incubated at 150 rpm and 30°C for 24 h. Each bioreactor was inoculated with 200 ml of *S. passalidarum* broth into a final of 2.0 L of CBS X-Cb or CBS G-X-Cb medium. *S. passalidarum* strain AF2 was adapted by two passages (total ~11 doublings) of NRRL Y-27907 in 50 ml wood hydrolysate medium under oxygen limited conditions (Kim et al. 2011). It was then adapted to grow in AFEX corn stover hydrolysate under oxygen-limited conditions for ~ 5 doublings. *S. passalidarum* E7 was derived from NRRL Y-27907 by cultivation in wood hydrolysate and then corn stover hydrolysate for 2 month (54 doublings) under oxygen-limited conditions. Spent broth and cells of strain E7 were directly transferred from an adapted flask containing stover hydrolysate into 500-ml Erlenmeyer flask containing 120 ml MHH plus CBS medium to create inocula for 2-L bioreactors. Inocula were cultured at 150 rpm at 30°C and then cells plus whole broth were added to Bio Flo 3-L bioreactor for a total working volume of 1.2 L. The inoculum growth in MHH was transferred into bioreactors containing CBS medium plus MHH, and the inoculum grown with synthetic medium was transferred into bioreactors containing CBS synthetic medium plus sugar. The bioreactors were sparged at 0.3 L/min (0.25 vvm) with a mixture of nitrogen and air at a 9:1 ratio to attain ~2.1% O_2 saturation. Agitation was controlled between 400 and 500 rpm to maintain saturation. After

12 h, 500-rpm agitation could not meet the oxygen demand of the growing culture, and measureable O_2 levels declined to nearly zero.

2.3.3 Sampling, extraction, and metabolite analysis

Each 5-ml culture samples was rapidly withdrawn and injected by syringe into 20 ml of 60:40 methanol-H_2O in a 50-ml conical centrifuge tube tat had been equilibrate at -80°C. The cell-medium-methanol mixture was immediately vortexed thoroughly, held briefly (<4 min) in an acetone-dry ice bath, and then centrifuged for 12 min at -9°C. The methanol-waster supernatant solution was decanted, and then frozen cell pellet was stored at -80°C until extracted. Metabolite analysis followed the method of Rabinowitz and Kimball (2007). Nucleotide phosphate, organic acid, sugar phosphates, and phosphorylated intermediates of glycolysis were analyzed by ion-chromatography acoupled to electrospray ionization-tandem mass spectrometry (ESI-MS/MS) using minor modification to a previously described method (van Dam et al. 2002). The column (IonPac AS-11HC; Dionex, Inc.) was equilibrate with 0.5 mM NaOH , and metabolites were eluted with 100 mM NaOH. An agilent 1200 high-performance liquid chromatography system with a gradient pump, micro-vacuum degasser, and chilled autosampler (4°C) was employed. The same equipment was used to analyze the extract in positive-ionization mode to quantify 21 amino acids by the method of Bajad et al. (2006). Instrumental precision was estimated by comparing five replicate injections of a mixture of 12 of the reported compounds (pyruvate, malate, succinate, glucose-6-phosphate [G6P], 2-ketoglutarate, fumarate, citrate, isocitrate, fructose-1,6-biosphosphate, AMP, ADP, and ATP). Relative standard deviations ranged from 22.0% to 2.6%, with an average of 8.9% for all 12 compounds.

For routine sugar consumption and end product analysis, 1.0 ml of sample from each bioreactor was used for sugar, xylitol, glycerol, and ethanol determinations by high-performance

liquid chromatography using a refractive index dectctor and Bio-Rad Aminex HPX-87P lead column (300 by 7.8 mm) at 85°C. The mobile phase was distilled deionized H_2O at flow rate of 0.6 ml/min.

2.4 Results

2.4.1 Aerobic coutilization of glucose and xylose

The capacity of *S. passalidarum* of xylose fermentation compared favorably with that of *Schefferosomyces stipitis* in shake flask studies (Du Preez et al. 1986), so we decided to characterize it further. Fully aerobic cultivation in defined minimal medium with a very low initial cell density (0.014 mg cdw/ml) resulted in little growth for the first 20 to 36 h. Subsequently, however, *S. passalidarum* used glucose or xylose at essentially the same rates when present individually or in a 50:50 mixture (Figure 2-1). No ethanol was formed under aerobic condition. Cell yield was ~20% higher on glucose (0.67 g cdw/g) than on xylose (0.56 g cdw/g).

2.4.2 Perferential fermentation of xylose

Prior studies with *S. stipitis* have shown that native xylose-fermenting yeast induce ethanol production under oxygen limitation (Klinner et al. 2005)(Klinner 2005). The very long lag time observed with fully aerobic conditions led us to use higher initial cell densities and lower initial aeration rates. We strove to achieve the same initial oxygen limited fermentative conditions in the bioreactor that were present in the shake flask used for inoculum propagation. We compared sugar consumption and ethanol production rates by *S. passalidarum* on CBS medium in duplicate bioreactors with either glucose or xylose as the carbon source (Figure 2-2). Immediately following inoculation,

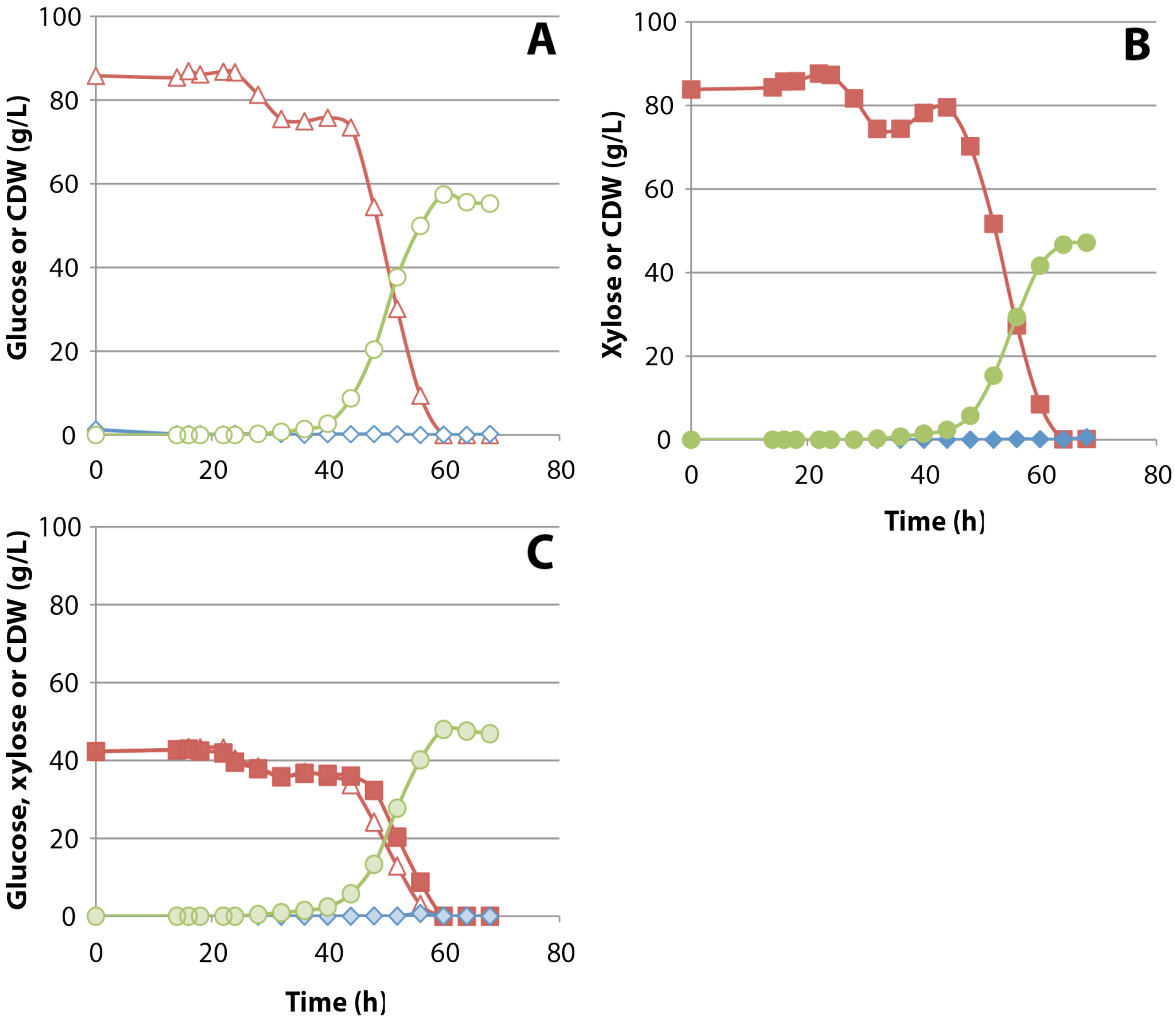

Figure 2-1 *Spathaspora passalidarum* cultivated under fully aerobic conditions. Cells were cultivated at 30°C in 2,000 ml of defined minimal medium (CBS) with glucose (A), xylose (B), or a 50:50 mixture of the two sugars (C). Cultures were aerated with 1 vvm air and an agitation rate of 700 rpm. Symbols: open triangles, glucose; solid squares, xylose; circles, cell mass; diamonds, ethanol.

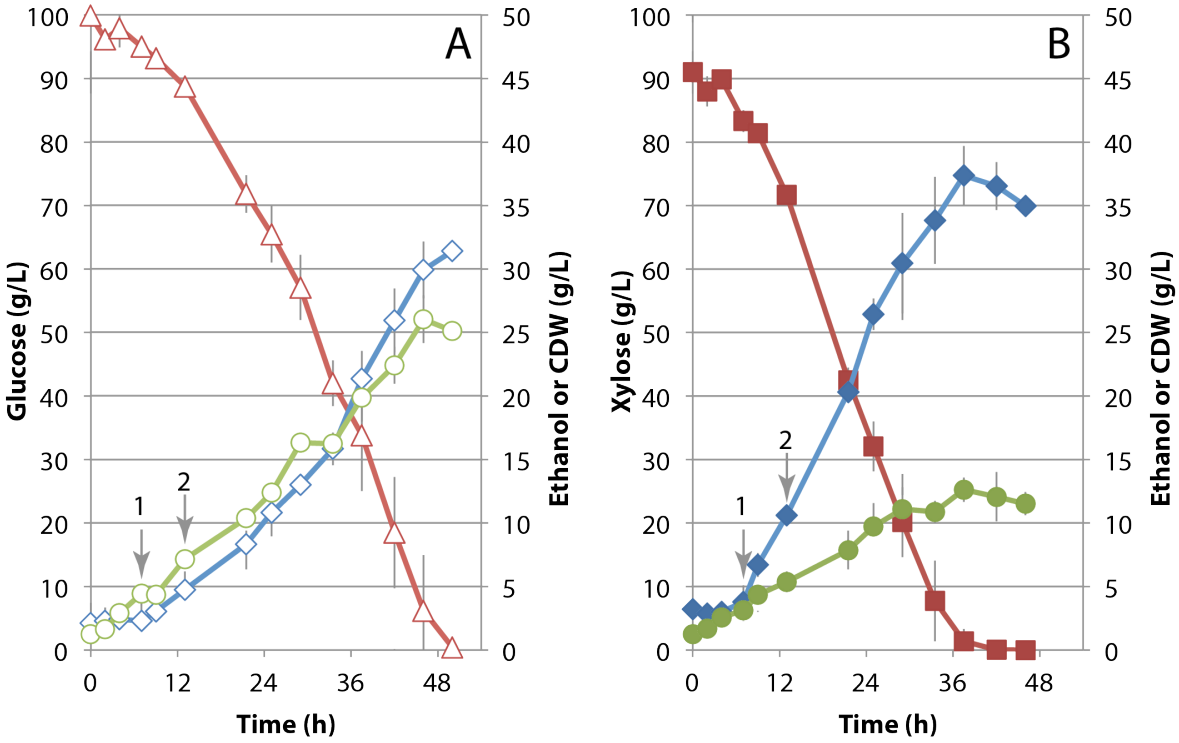

Figure 2-2 *Spathaspora passalidarum* cultivated under oxygen-limited conditions. Cells were cultivated in duplicate 3-L bioreactors at 25°C in 2,000 ml of defined minimal medium (CBS) with either glucose (open symbols) (A) or xylose (closed symbols) (B). Symbols: triangles, glucose; squares, xylose; diamonds, ethanol; circles, cell dry weight; arrow 1, first metabolomics sample and shift to 2.1% O_2; arrow 2, second metabolomics sample. Averages values from duplicate runs are shown. Gray bars depict range of values.

the cell mass was 1.23 mg/ml, and with air sparging, 150 rpm was sufficient to maintain dO_2 at the set point of 2.1% O_2 saturation. Initial growth rates during the air sparging phase were 0.19 and 0.14 h-1 for glucose and xylose, respectively. Specific ethanol production rate were very low (glucose) or undetectable (xylose). After 7 h, cell densities reach 4.45 and 3.15 g/L for glucose and xylose, respectively. At that point, the sparging rate was switch to 90% N_2, 10% air. With agitation capped at 500 rpm and inlet oxygen capped at 2.1%, dO_2 decrease to low or undetectable levels as cell densities increased. Following the switch to oxygen limitation, specific growth rates fell by 2.5- to 5-fold, while specific ethanol production rates increased dramatically (Table 2-2). During the oxygen-limited phase, cell yield on xylose was only about half of that observed with glucose, but the specific ethanol formation rate was three times higher. This resulted in significantly higher ethanol production with xylose, ever though cell yield was notably lower (cf. Figure 2-2 A and B).

Table 2-2 *Spathaspora passalidarum* product yields and specific formation rates on glucose or xylose during the oxygen-limited phase[a].

Sugar	Yield (g/g sugar)		Ethanol productivity (g/L·h)	Q_p (g/g CDW·h)	
	Cells	Ethanol		Cells	Ethanol
Glucose	0.22	0.31	0.72	0.05	0.05
Xylose	0.12	0.41	1.14	0.05	0.17

[a] Ethanol yields, specific growth rates, and ethanol productivities were calculated from time zero to 50 h for glucose and from time zero to 36 h for xylose. Q_p, specific product formation rate.

2.4.3 Cofermentation of glucose, xylose, and cellobiose

During enzymatic saccharification and fermentation of pretreated lignocellulosic, the major sugar present are xylose and glucose, xylan oligosaccharides (from hydrolysis of hemicelluloses), and cellobiose (from enzymatic saccharification of pretreated cellulosic solids). We therefore examined the cofermentation of cellobiose and xylose. In duplicate shake flasks, the xylose consumption rate was much greater than that for cellobiose, but after cell attained a density of ≤ 5 g/L, oxygen limitation induced ethanol production. Cellobiose and xylose were coutilized at 0.49 and 0.94 g/L·h, respectively, and the volumetric ethanol production rate was 0.56 g/L·h (Figure 2-3). In bioreactors, cellobiose and xylose were cofermented from the outset at very similar rates in the presence (Figure 2-4 A) or absence (Figure 2-4 B) of 20 g/L glucose. Prior to inoculation, dO_2 occasionally rose slowly to 0.2 to 0.5% without an apparent effect on ethanol production pr growth. In the absence of glucose, xylose and cellobiose were metabolized at essentially similar rates until xylose was depleted at 48 h (Figure 2.4 A). The rate of ethanol production declined significantly thereafter, but cell growth continued. In the presence of glucose, coutilization of cellobiose and xylose was delayed slightly in the first 15 h, but all three sugar were coutilized at very similar rate from 15 to 68 h, at which point all sugars were consumed and ethanol production ceased (Figure 2.4 B). The maximum rate of ethanol production from a mixture of xyloe and cellobiose was 1.07 g/L·h, and from all three sugar it was 0.73 g/L·h. Ethanol yields during the phase of maximum production rate were 0.43 g/g and 0.42 g/g for xylose-cellobiose and glucose-xlose-cellobiose mixtures, respectively. Overall volumetric productivities and rates (0 to 72 h) were 0.53 g/L·h for the glucose, xylose, and cellobiose mixture and 0.58 g/L·h for the xylose and cellobiose mixture.

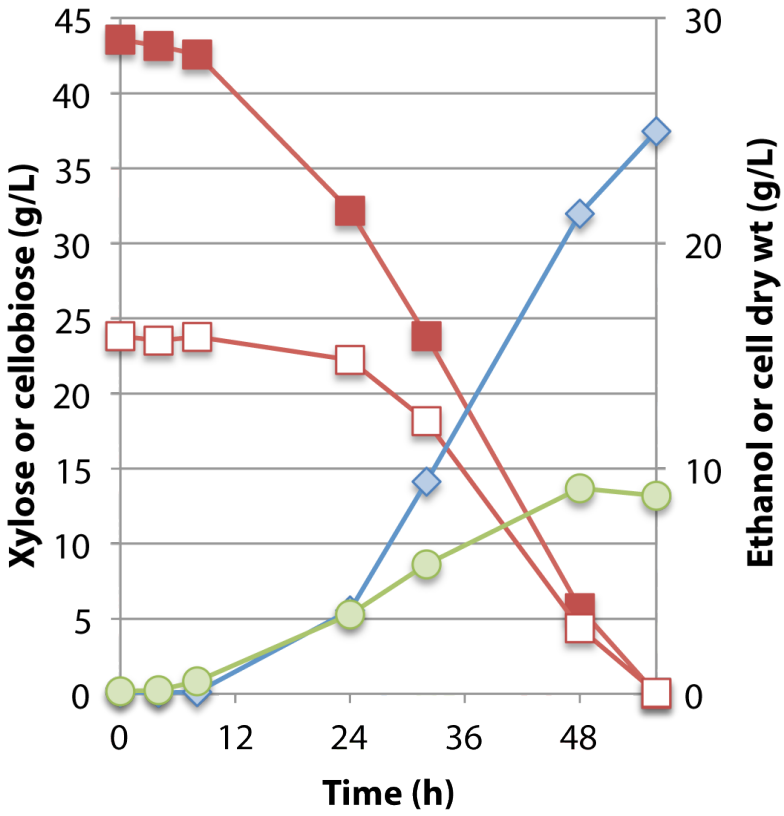

Figure 2-3 Cofermentation of cellobiose and xylose under oxygen limitation in triplicate shake flasks. Symbols: solid squares, xylose; open squares, cellobiose; diamonds, ethanol; circles, cell mass. Minimal defined medium (CBS) was employed. Flasks were incubated at 30°C and shaken at 200 rpm.

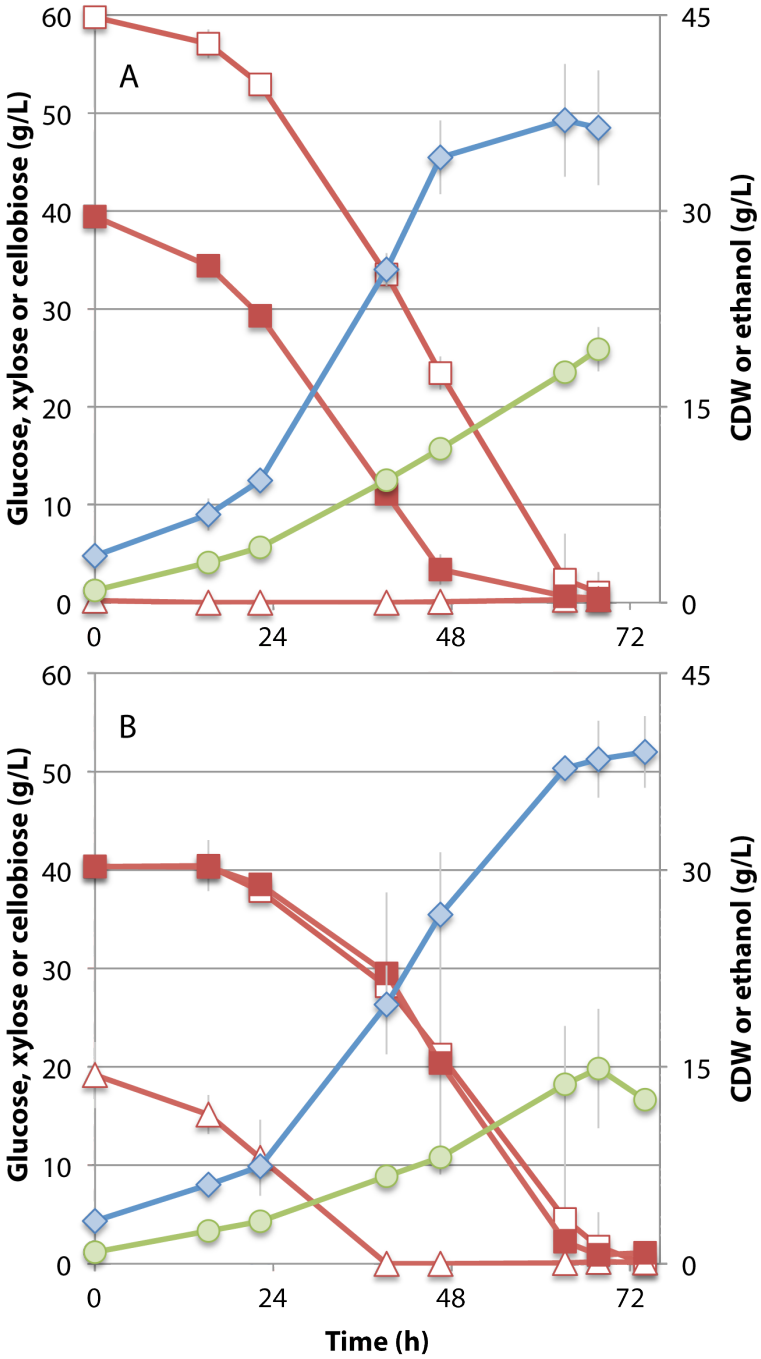

Figure 2-4 Coutilization of glucose, xylose, and cellobiose by *Spathaspora passalidarum*. Cells were cultivated at 25°C. Symbols: open triangles, glucose; closed squares, xylose; open squares, cellobiose; diamonds, ethanol; circles, cell mass.

2.4.4 Hydrolysate fermentation

MHH contained 1.8 times more xylose than glucose, and the AFEX hydrolysate contained ~2.3 times more glucose than xylose. Unlike the AFEX-treated enzymatic hydrolysate of corn stover, MHH did not contrain significant amount of acetic acid or cellobiose, and the glucose/xylose ratio was 65:35 at the start of the fermentation. Fermentation of MHH (Figure 2-5 A) was compared to that of a sugar mixture (SM) designed to mimic the hydrolysate (Figure 2-5 B). Almost from the outset, glucose and xylose were cometabolized in the MHH, but utilization of xylose was delayed by ~12 h in the SM. During the bulk of the fermentation, glucose and xylose were coutilized at similar rates in both the MHH and SM media. Fermentation of the SM proceeded more rapidly to attain a peak of 40 g/L ethanol in 38 h, whereas fermentation of the MHH required 59 h to attain 38 g/L ethanol. During the fermentation phase (19.5 to 49 h), cell and ethanol yield were 0.19 and 0.34 g/g sugar consumed from the MHH, respectively, and 0.25 and 0.37 g/g consumed from the SM, respectively. The fermentation time with AFEX was relatively long (Figure 2-6 A). Its initial acetic acid concentration was about 1.4 g/L. Cells grew under these conditions but did not exhibit significant ethanol production until acetic acid was depleted after 3 days (Figure 2-6 B). Xylose utilization was largely delayed while glucose was consumed, but after 6 days, strains adapted to grow in hydrolysate rapidly converted 84% of xylose into 23 g/L ethanol with a yield of 0.45 and 16% into xylitol.

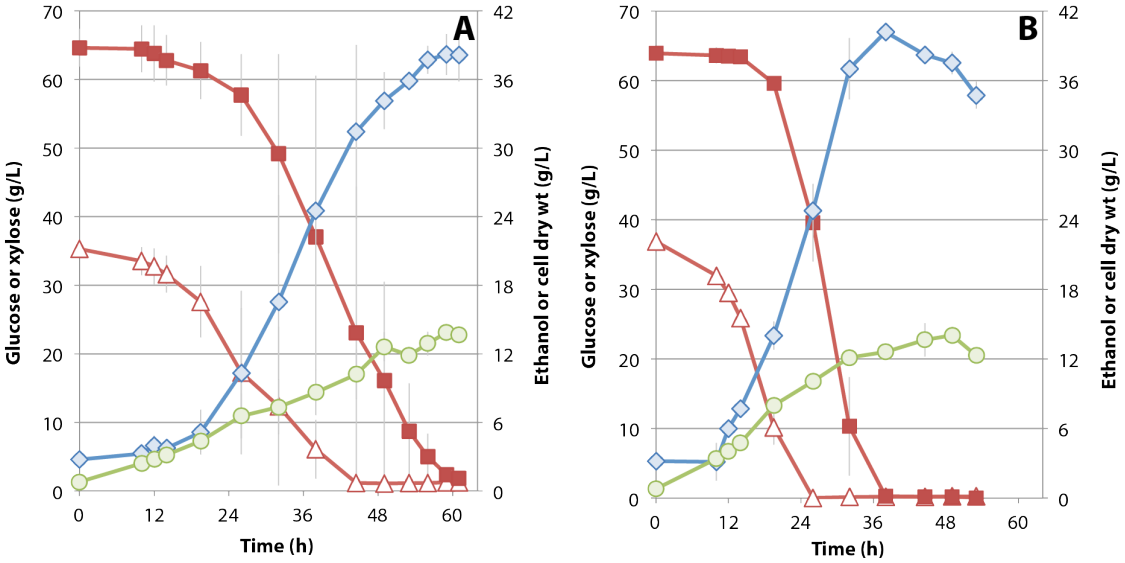

Figure 2-5 Fermentation of a maple hemicellulose hydrolysate (A) and a synthetic sugar mixture (B) by *Spathaspora passalidarum* E7 in defined minimal medium (CBS). Symbols: open triangles, glucose; closed squares, xylose; open squares, cellobiose; diamonds, ethanol; circles, cell mass.. Averages of two bioreactors are shown with range of values (gray bars).

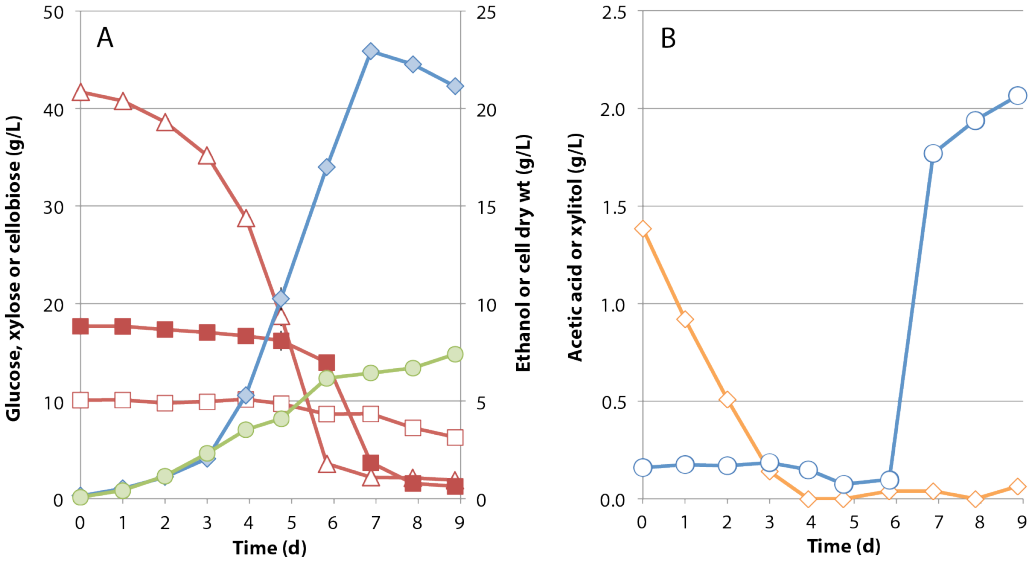

Figure 2-6 Fermentation of an AFEX hydrolysate by *Spathaspora passalidarum* AF2 in defined Slininger medium (Slininger et al. 2006). Fermentation was conducted in duplicate shake flasks (50 ml/125 shaken at 100 rpm). AF2 was cultured in a 125-ml Erlenmeyer flask containing 50 ml of AFEX hydrolysate medium at 100 rpm and 30°C for 5 days (optical density, above 40) and then transferred directly from the adapted flask into another flask containing the same AFEX hydrolysate medium. The initial targeted optical density at 600 nm was 0.5. Duplicate flask fermentations were conducted at 30°C with rotary shaking at 100 rpm. The starting cell density was 0.1 mg cdw/ml. Symbols: (A) open triangles, glucose; closed squares, xylose; open squares, cellobiose; gray diamonds, ethanol; gray circles, cell mass; (B) open diamonds, acetic acid; gray circles, xylitol. Averages of two shake flasks are shown.

2.4.5 Metabolome analysis

To better understand the regulatory and metabolic basis for *S. passalidarum* 's preferential utilization and coutilization of xylose, we analyzed samples for relative metabolite abundance in cell cultivated on glucose or xylose at the ends of the aerobic growth phase and during the ethanol production phase (arrow 1 and 2, Figure 2-2). At the end of initial growth phase (arrow 1), levels of glucose 1-phose (G1P), glucose 6-phose (G6P), fructose 6-phosphate (F6P), fructose 1,6-bisphosphate (F16P), 2 phosphoglycerate (2PG), 3-phosphoglycerate (3PG), phosphoenolpyruvate (PEP) and pyruvate were all significantly higher when cells had been cultivated on glucose than on xylose (Figure 2-7). Likewise, 6-phosphogluconate (6PG), which is at the entry to the oxidative pentose phosphate pathway (PPP), and α-ketoglutarate (αKG), which is derived from the tricarboxylic acid cycle (TCA), were higher on glucose at that time. Amino acids derived from these intermediates followed essentially the same patterns as their glycolytic or PPP precursors (Figure 2-8).

In contrast to these metabolites, levels of pentose phosphate (PP), sedoheptulose 7-phosphate (S7P), and particularly, erythrose 4-phospphate (E4P) were higher at the end of the growth phase when cells were cultivated on xylose than when they were cultivate on glucose. Interestingly, cellular levels of phenylalanine, tyrosine, and tryptophan, which are formed from E4P and PEP, all followed the profile exhibited by E4P rather than that exhibited by PEP (Figure 2-8). In contrast, concentration of histidine (which is derived from ribose 5-phosphaste) followed the profile of 6PG rather than that of PP. Our analytical procedure was not able to distinguish among ribose 5-phosphate (Ri5P), ribulose 5-phosphate (Ru5P), and xylulose 5- phosphate (Xu5P), but although Ri5P is derived directly from 6PG, Xu5P is derived from phosphorylation of xylulose, so the pool of Ri5P might not be directly affected by growth on

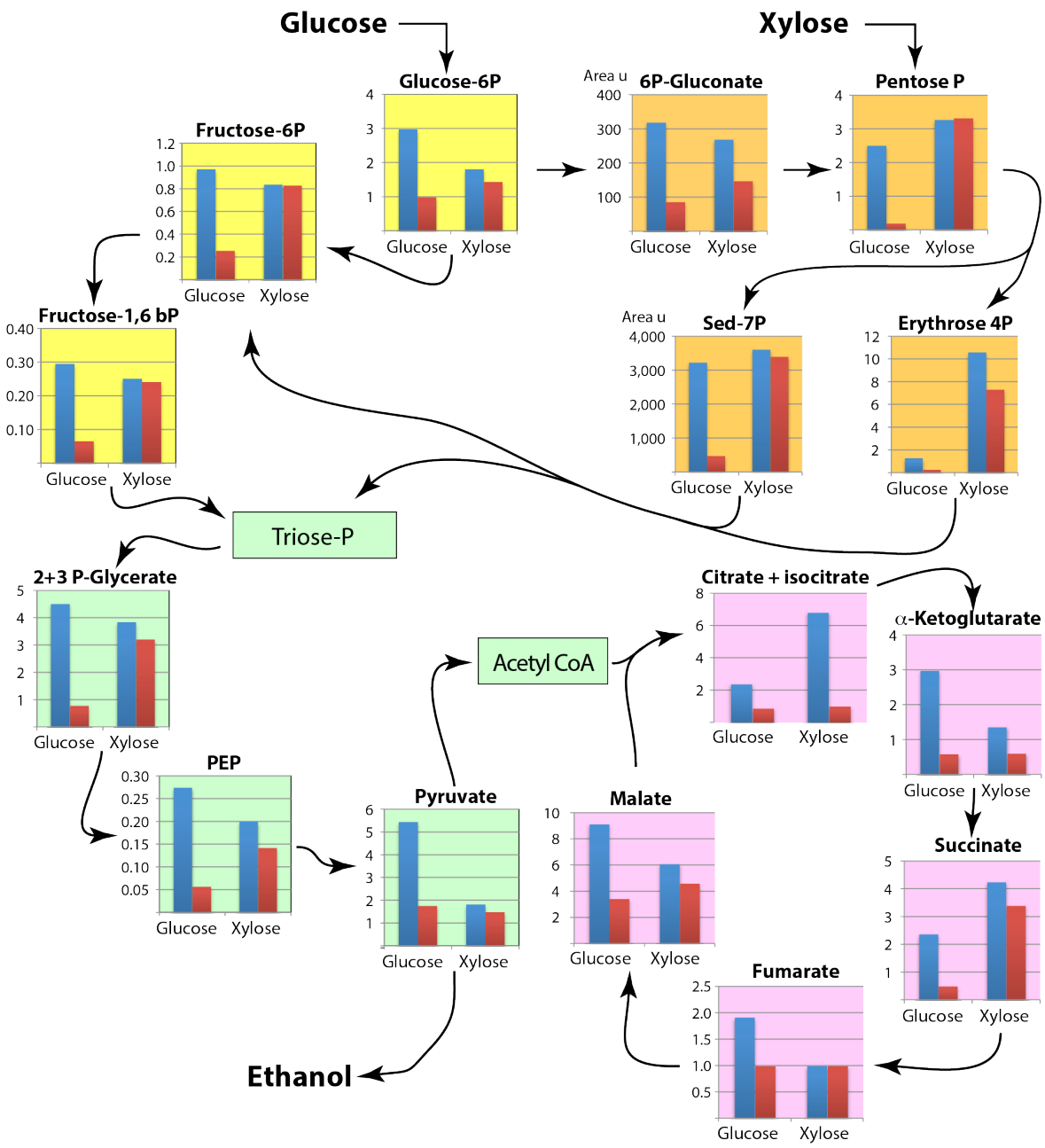

Figure 2-7 The Intracellular levels of glycolytic metabolites and amino acids in *Spathaspora passalidarum* cultivated on glucose or xylose under aerobic (blue bars) and oxygen-limited (red bars) conditions (cf. Figure 2-2 A and B).

Figure 2-7 The Intracellular levels of glycolytic metabolites and amino acids in *Spathaspora passalidarum* cultivated on glucose or xylose under aerobic (blue bars) and oxygen-limited (red bars) conditions (cf. Fig. 2A and B). All units are μmol/g CDW except for 6-phosphogluconate and sedoheptulose 7-phosphate, which are expressed as area units (area u)/g cdw. Fructose-1,6bP, fructose 1,6-bisphosphate Fructose-6P, fructose 6-phosphate; Glucose 6P, glucose-6-phosphate; 6P- Gluconate, 6-phosphogluconate; Pentose P, pentose phosphate; Sed-7P, sedoheptulose 7-phosphate; Erythrose 4P, erythrose 4-phosphate; 2+3 P-Glycerate, 2-phosphoglycerate plus 3-phosphoglycerate; Triose-P, triose phosphate; Acetyl CoA, acetyl coenzyme A.

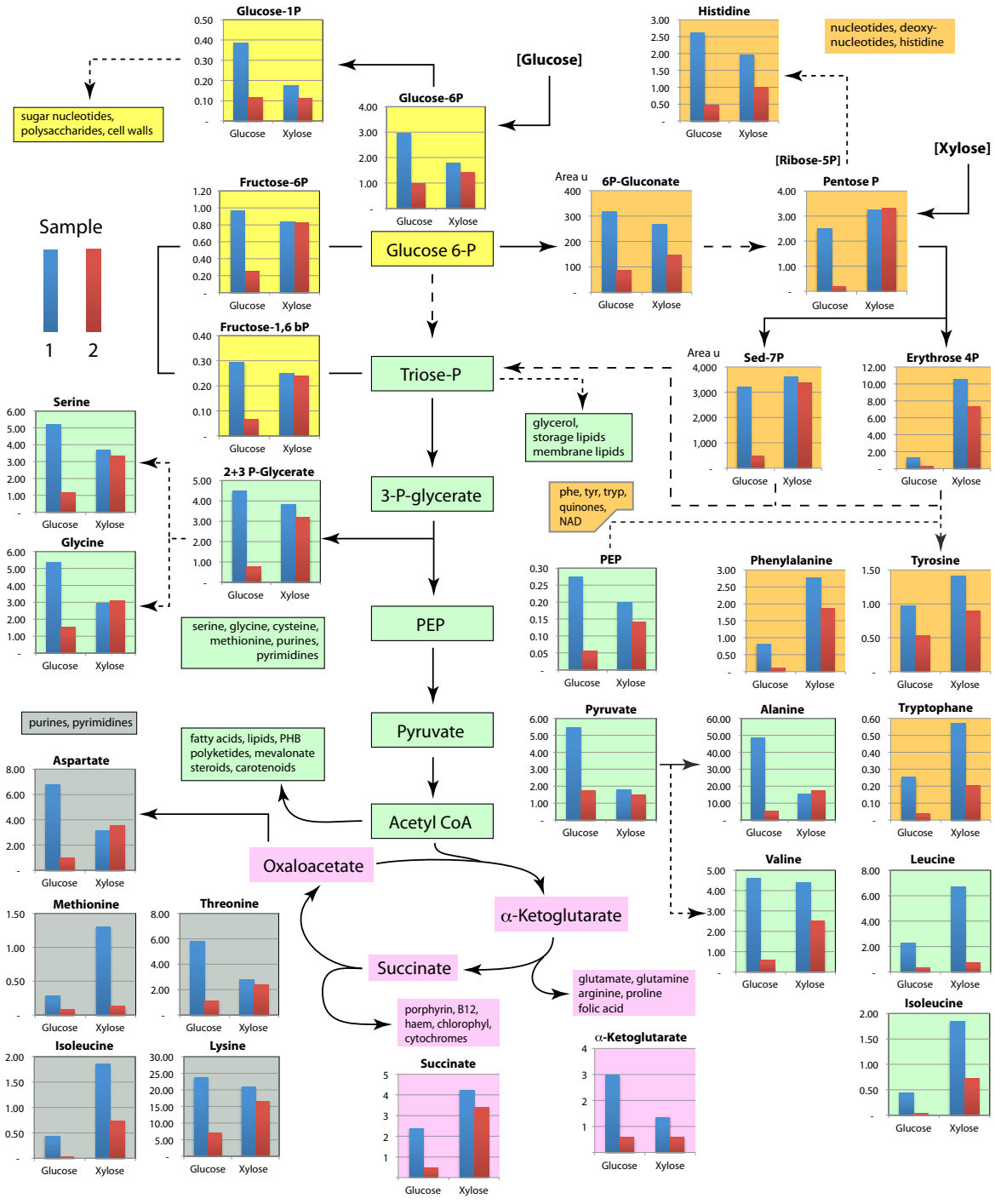
Glucose-1P
sugar nucleotides, polysaccharides, cell walls
[Glucose]
Glucose-6P
Histidine
nucleotides, deoxy-nucleotides, histidine
[Ribose-5P]
[Xylose]
Sample
1
2
Fructose-6P
Glucose 6-P
6P-Gluconate
Pentose P
Fructose-1,6 bP
Triose-P
glycerol, storage lipids membrane lipids
Sed-7P
Erythrose 4P
Serine
2+3 P-Glycerate
3-P-glycerate
phe, tyr, tryp, quinones, NAD
Glycine
serine, glycine, cysteine, methionine, purines, pyrimidines
PEP
Phenylalanine
Tyrosine
Pyruvate
Alanine
Tryptophane
purines, pyrimidines
fatty acids, lipids, PHB polyketides, mevalonate steroids, carotenoids
Aspartate
Acetyl CoA
Oxaloacetate
Valine
Leucine
Methionine
Threonine
α-Ketoglutarate
Succinate
glutamate, glutamine arginine, proline folic acid
porphyrin, B12, haem, chlorophyl, cytochromes
Isoleucine
Lysine
Succinate
α-Ketoglutarate
Isoleucine
Glucose
Xylose
Area u

Figure 2-8 Intracelluar levels of glycolytic metabolites and amino acids in *Spathaspora passalidarum* cultivated on glucose or xylose under aerobic (blue) and oxygen-limited (red) conditions (cf. Figure 2-2 A and B). All units are μmol/g dcw except for 6P-gluconate and sedoheptulose- 7P, which are expressed as area units/g CDW.

xylose like the concentration of S7P and E4P are. Alanine also attained relatively high levels (~50 μmol/g CDW) that were of the same order of magnitude as those of glutamate, glutamine, and arginine (Figure 2-9). The greatest observed difference in metabolite level occurred during the oxygen-limited fermentative phase (arrow 2, Fig 2-2). With the exception of G1P, which is tied closely to growth through synthesis of cell wall glucans, levels of virtually all glycolytic, pentose phosphate, and trichloroacetic acid intermediates (except pyruvate) and the amino acids derived from them were higher on glucose than on xylose. ATP and ADP level were higher on glucose than on xylose (Figure 2-10). Ratios of reduced glutathione to oxidized glutathione stayed almost constant for each of the four conditions, but intracellular concentrations increased about 3.5-fold on xylose relative to those on glucose during the oxygen-limited fermentative phase.

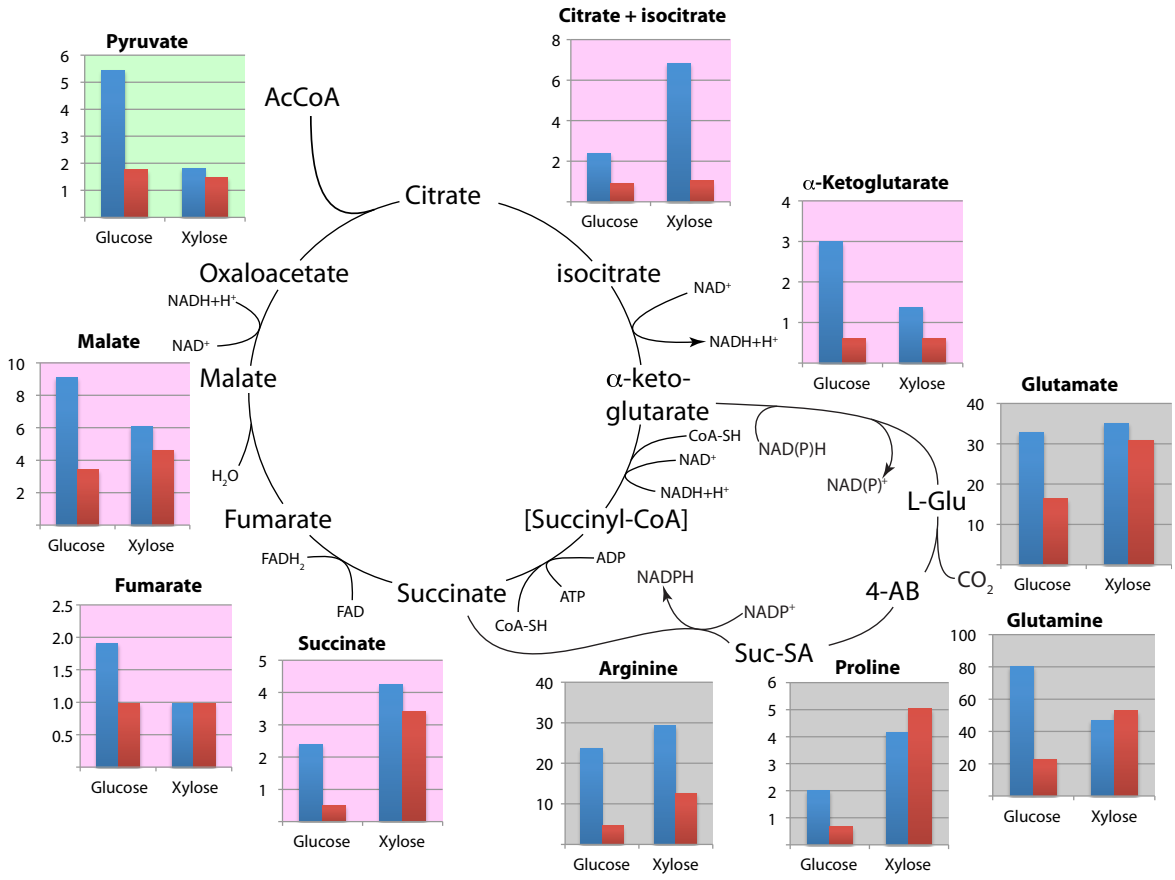

Figure 2-9 Intracellular levels of TCA metabolites and amino acids in *Spathaspora passalidarum* cultivated on glucose or xylose under aerobic (1) and oxygen-limited (2) conditions (cf. Figure 2-2 A and B)

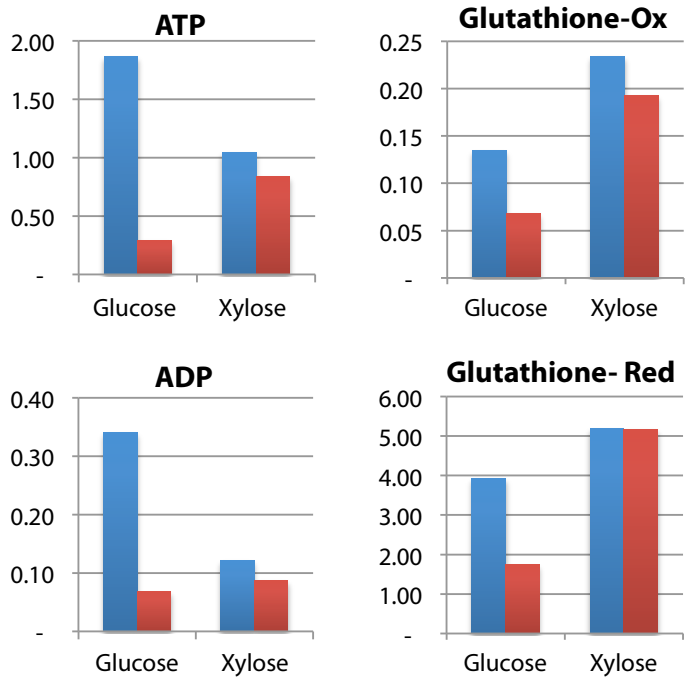

Figure 2-10 Relative levels of ATP, ADP, and glutathione on glucose and xylose under aerobic (1) and oxygen-limited (2) conditions (cf. Figure 2-2 A and B)

2.5 Discussion

S. passalidarum was originally isolated from the midgut of a passalid beetle that preferentially inhabits white-rotted hardwoods (Nguyen et al. 2006). The midgut of wood-boring beetles is hypothesized to be oxygen limited, so evolution for growth under oxygen-limited conditions on mixtures of cellulosic and hemicellulosic sugars might be expected. *S. passalidarum* NN245 is the sole known teleomorphic isolate with the species, but other species in the *Spathaspora* genus and related anamorphic species usch as *Candida jeffriesii* have been isolated and characterized. *Spathaspora arborariae*, which was isolated from rotting wood in Brazilian Atlantic Rain Forest, is also capable of fermenting xylose (Cadete et al. 2009, da Cunha-Pereira et al. 2011), but other members of the *Spathaspora* clade are not (Barbosa et al. 2009, Boonmak et al. 2011). The yeast clade to which *Spathaspora* belongs is related to but distinct from that of *SScheffersomyces* (Kurtzman and Suzuki 2010), which also includes a number of xylose –fermenting yeasts, including *S. stipitis* (Jeffries and Van Vleet 2009). The capacity for producing ethanol from cellobiose is infrequent but occurs over a wide taxonomic range within the yeast. Even so, the traits for xylose and cellobiose fermentation are variable within genera and even species. Although the capacity to ferment both xylose and cellobiose is rare among yeast, the ability to ferment xylose faster than glucose and to simultaneously cometabolize and ferment glucose, xylose, and cellobiose are believed to be unprecedented. Fermentation rates and yields for xylose and cellobiose fermentations by native strains of *Spathaspora* species exceed by those by *Saccharomyces cerevisiae* strains engineering for these traits (Table 2-3).

Table 2-3 Comparison of fermentation kinetics for engineered *Saccharomyces cerevisiae* and native *Spathaspora passalidarum*

Species and strain	Strain background or genetic modification	Cultivation conditions	Medium	Carbon source	EtOH (g/L)	EtOH yield (g/g)	Overall EtOH productivity (g/L·h)	Reference
S. cerevisiae BP10001	*XYL1* with K274R-N276D double mutant	Bioreactor, 200 rpm, pH 5, N_2-sparged	Defined minimal medium based on Jeppsson (2006)	5% Xylose	12.4[a]	0.37	0.003	Krahulec et al. (2010); Petschacher & Nidetzky (2008)
S. cerevisiae OC2-ABGL4Xyl2	Linear integrated 4 copies of BGL genes and 2 copies of xylose-assimilating gene	Shake flask	YPCX medium containing 1% yeast extract and 2% peptone	9% Cellobiose+ 6% Xylose	57.4	0.39	1.196	Saitoh et al. (2010)
S. cerevisiae DA24-16BT3	Mutated *XYL1* preferring NADH (R276H), integrated *cdt1* with multi-copy plasmids containing *ghl-1*	Bioreactor, oxygen limited, 200 rpm	YP medium containing 1% yeast extract and 2% peptone	4% Xylose	12 [a]	0.31	0.263	Ha et al. (2011)
				4% Cellobiose	12 [a]	0.29	0.262	
				10% Cellobiose + 6% xylose	60 [a]	0.38	0.99	
S. arborariae UFMG-HM19.1A	WT	Shaken flasks, 10 g/l pre-grown cells	YP medium containing 1% yeast extract and 2% peptone	2% Glucose	10.5	0.35	5.250	Cadete RM et al. (2009)
				2% Xylose	10	0.37	2.500	
S. passalidarum ATCC MYA-4345	WT	Sealed vials	YP medium containing 1% yeast extract and 2% peptone	3% Glucose	13.5	0.43	0.188	Hou (2011)
				3% Xylose	13.75	0.44	0.191	
S. passalidarum NN245	WT	Bioreactor, low aeration	Defined minimal medium	10% Glucose	31.41	0.31	0.628	This study

[a] Estimated from Figures WT = wild type strain

Under fully aerobic conditions, *S. passalidarum* grew poorly at first on glucose or xylose. Once growth started, however, sugar utilization and cell growth rate were similar, and aerobic coutilization of glucose and xylose was essentially simultaneous. We inferred that the cells underwent physiological adaptation to achieve full aerobic growth. Under typical low-aeration conditions, cells would grow and ferment rapidly, so the long lag under fully aerobic conditions was atypical. Cell yields on xylose, however, were only about 84% of those on glucose under fully aerobic conditions. The higher efficiency of cell growth on glucose was even more apparent under O_2 limitation (Figure 2-2 A). Cell yield on xylose was only 55% of that on glucose (Figure 2-2 B); however, ethanol yield was 32% higher on xylose than on glucose, and the specific fermentation rate on xylose was 3.4-fold higher. This was in contrast to the nearly simultaneous coutilization of both sugars in a mixture under aerobic conditions, where no ethanol production was observed.

A lower net ATP yield for cells grown on xylose could account for the higher glycolytic flux under O_2 limitation. Hou reported a lower cell yield with *S. passalidarum* growing on xylose than on glucose (Hou 2012) and suggested that *S. passalidarum* might use low-affinity, low-capacity facilitate diffusion for growth under O_2 limitation and the high capacity, high-affinity system during aerobic growth (Leandro et al. 2006). Previous studies have shown that *Scheffersomyces stipitis* induced respire-fermentative growth and pyruvate decarboxylase under oxygen-limiting conditions (Klinner et al. 2005, Passoth et al. 1996). It seems likely that similar mechanisms for oxygen-sensing could regulate transport as well. We observed maximal volumetric fermentation rate of 1.14 and 0.72 g/L·h for xylose and glucose, respectively, and our maximal rates for xylose and cellobiose consumption in the presence of glucose were 0.55 and 0.57 g/L·h, respectively, in minimal medium with urea as the nitrogen source. The specific rate

of xylose fermentation was more than 3 times greater than the rate of glucose fermentation under conditions that allowed the induction of transcripts and enzymatic activities important for anaerobic or oxygen-limited metabolism.

Metabolites levels measured during oxygen-limitation support the observation that glycolytic flux is greater in cells cultivated on xylose than those cultivated on glucose (Figure 2-7). Levels of glucose-derived metabolites were higher during the initial aerobic growth phase, but they dropped dramatically as cells shifted to fermentative conditions. By comparison, xylose-derived intermediates were significantly higher on xylose under either aerobic or O_2-limited conditions. Cells cultivated under O_2-limited conditions showed higher levels of ATP on xylose than on glucose. There are several possible reason: (i) glycolytic flux is greater on xylose, (ii) ATP demand is lower, (iii) net ATP yield is higher, or (iv) regulation of ATP biosynthesis is different on glucose and xylose. Because cell yield was significant lower on xylose than on glucose, a decreased ATP demand is probably the dominant effector.

The xylitol reductase (XR) of *Scheffersomyces stipitis* has about 70% as much activity with NADH as it does with NADPH (Verduyn et al. 1985). The capacity to recycle NADH to NAD+ under anaerobic or oxygen-limited conditions can reduce xylitol production and increase the capacity for xylose assimilation (Bengtsson et al. 2009, Jeppsson et al. 2006, Khattab et al. 2011, Klimacek et al. 2010, Kotter and Ciriacy 1993, Matsushika et al. 2008, Petschacher and Nidetzky 2008). Recently, Hou (Hou 2012) reported that the crude XR activity of *S. passalidarum* has a 1.8-fold higher affinity for NADH than for NADPH. By comparison, the XR of *S. stipitis* shows a 1.6-fold higher affinity for NADPH. Moreover, the affinity of the crude XR activity for xylose is 3-fold higher with NADG than with NADPH. The xylitol dehydrogenase (XDH) activity of *S. passalidarum* also has a much higher affinity for xylitol than the corresponding enzyme from *Scheffersomyces stipitis* does(Hou 2012). These kinetic characteristics are much more favorable than those achieved with the best aldose reductase genes previously engineered for high selectivity and activity with NADH (Khattab et al. 2011, Klimacek et al. 2010, Matsushika et al. 2008), and they could account in large part for the high PPP, F6P, and F16P metabolite levels on xylose. The *S. passalidarum* genomes contains two orthologs each for *XYL1* and *XYL2* (Wohlbach et al. 2011), so it is not possible to determine from these data which of these contributes more to the cofactor affinities measured in the crude homogenates.

Most glycolytic regulation occurs metabolically, via allosteric mechanism to determine the overall metabolic flux, rather than hierarchically, through changes in gene expression (Daran-Lapujade et al. 2007). Thus, the relative affinities of enzymes for cofactors and substrates along with enzyme activities at modulated steps determine the overall flow of metabolites. Pyruvate kinase, which catalyzes the final step in glycolysis, is allosterically activated by F16P and is

important for controlling levels of ATP, GTP, and glycolytic intermediates (Jurica et al. 1998). The affinities of NADH-coupled XR and NAD+-coupled XDH would tend to drive both the oxidoreductase pathway for xylose assimilation and the reduction of acetaldehyde to ethanol. The strong xylose assimilation would likewise increase levels of F16P, thereby activating glycolysis via pyruvate kinase. This does not fully explain the higher cell yield and lower glycolytic activity observed with glucose-grown cells, but it does offer a model for further experimentation, and it begins to offer insight into how we might go about engineering and rapid coutilization of these sugar.

We describe what we believe to be the first report of an organism capable of cofermentation of xylose and cellobiose in the presence of glucose. This ability makes S. passalidarum a potentially useful organism for SSF and an interesting organism for unraveling the regulatory mechanisms enabling bioconversion of ligno- cellulosic materials by yeasts.

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T.W.J. conceived the project, directed the work, and wrote the manu- script. Y.-K.S., T.M.L., and J.H. performed the fermentation experiments, contributed to experimental designs, and analyzed results. L.B.W. con- tributed to conceptualization and experimental designs and performed fermentation experiments, data analysis, and editing. A.H. developed and conducted analytical techniques. All coauthors contributed to writing the manuscript.

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Chapter III

Effects of Aeration on growth, ethanol and polyol accumulation by *Spathaspora passalidarum* NRRL Y-27907 and *Scheffersomyces stipitis* NRRL Y-7124

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3.1 Abstract

Spathaspora passalidarum NN245 (NRRL-Y27907) is an ascomycetous yeast that displays a higher specific fermentation rate with xylose than with glucose. Previous studies have shown that its capacity for xylose fermentation increases while cell yield decreases with decreasing aeration. Aeration optimization plays a crucial role in maximizing bioethanol production from lignocellulosic hydrolysates. Here, we compared the kinetics of *S. passalidarum* NN245 and *Scheffersomyces* (*Pichia*) *stipitis* NRRL Y-7124 fermenting 15% glucose, 15% xylose, or 12% xylose plus 3% glucose under 4 different aeration conditions. The maximum specific fermentation rate for *S. passalidarum* was 0.153 g ethanol/g CDW·h with a yield of 0.448 g/g from 150 g/L xylose at an oxygen transfer rate of 2.47 mmol O_2/L·h. Increasing the OTR to 4.27 mmol O_2/L·h. decreased the ethanol yield from 0.46 to 0.42 g/g xylose while increasing volumetric ethanol productivity from 0.52 to 0.8 g/L·h. Both yeasts had lower cells yields and higher ethanol yields when growing on xylose than when growing on glucose. Acetic acid accretions of both strains correlated positively with increasing aeration. *S. passalidarum* secreted lower amounts of polyols compared to *S. stipitis* under most circumstances. In addition, the composition of polyols differed: *S. passalidarum* accumulated mostly xylitol and *R,R*-2,3-butanediol (BD) whereas *S. stipitis* accumulated mostly xylitol and ribitol when cultivated in xylose or a mixture of 12% xylose and 3% glucose. *R,R*-2,3-BD accumulation by *S. passalidarum* during xylose fermentation can be as much as four times of that by *S. stipitis*, and *R,R*-2,3-BD is also the most abundant byproduct after xylitol. The ratios of polyols accumulated by the two species under different aeration conditions and the implications of these observations for strain and process engineering are discussed.

Keywords: *Spathaspora passalidarum*, xylose, fermentation kinetics, oxygen transfer rate, fermentation byproducts, polyol dehydrogenase

3.2 Introduction

Converting lignocellulosic feedstocks into biofuels such as ethanol can temper the demand for gasoline and decrease CO_2 accumulation when properly integrated with cultivation of diverse native grasses (Tilman et al. 2006), sustainable agriculture (Goldemberg and Guardabassi 2009, Schmer et al. 2008) and silviculture (Dwivedi et al. 2012). Furthermore, when used as a fuel for transportation, ethanol from lignocellulosic feedstocks can reduce greenhouse gas emissions by 88% relative to gasoline, which is much greater than the 18% reduction realized from corn ethanol (Farrell et al. 2006, Schmer et al. 2008). Using cellulosic feedstocks can greatly improve greenhouse gas emissions during biofuel production on marginal lands (Gelfand et al. 2013). Hydrolysis of cellulose and hemicellulose generates a mixture of glucose, xylose, arabinose and other monosaccharides. However, the native *Saccharomyces cerevisiae*, which has been used for centuries in alcohol production and which can readily ferment the glucan portion of lignocellulose, cannot metabolize xylose unless engineered to express functional xylose assimilation pathways (Fujii et al. 2011, Kim et al. 2013, Kuyper et al. 2005, Ma et al. 2012, Sanchez et al. 2010). Another approach has been to use native pentose-fermenting yeasts such as *Scheffersomyces* (*Pichia) stipitis* or *Scheffersomyces* (*Candida*) *shehatae* either in their native forms or following directed evolution or metabolic engineering (Hughes et al. 2012, Jeffries and Jin 2004, Li et al. 2012).

One of major challenges for ethanol production from xylose by native pentose-fermenting yeasts is controlling the oxygen supply to maximize ethanol production. Too much oxygen leads to aerobic growth and low ethanol yield (Rizzi et al. 1989); insufficient oxygen slows the fermentation rate, increases xylitol accumulation and causes poor ethanol productivity.

Under strictly anaerobic conditions, both *S. stipitis* and *S. shehatae* are only able to grow for one doubling before fermentation and cell growth stop (Du Preez 1994, Slininger et al. 1991).

Several studies have tested oxygen transfer rates (OTR) ranging from 0.7 to 8.6 mmol O_2/L·h to understand the correlation between aeration and fermentation performance in native pentose fermenting yeasts (Sreenath 1986; Du Preez 1994). An OTR ranging from 1.75 to 5 mmol/L·h was reported as optimal for *S. stipitis* with 5% sugar (Grootjen et al. 1991, Guebel et al. 1991, Laplace et al. 1991). The optimal dissolved oxygen tension (DOT) of *S. stipitis* and *S. shehatae* less than 1% of saturation (about 0.08 mg/L = 2.5 µmol O_2/L), which is below the detection limit of dissolved oxygen sensors, but even so, the physiological responses of these strains could be different at such low DOT levels (Dellweg et al. 1989, Du Preez et al. 1989). *S. stipitis* does not produce ethanol when the DOT is above 3 µmol O_2/L (Rizzi et al. 1989) and a constant specific oxygen uptake rate (qO_2) of 0.3 mmol O_2/g CDW·h can increase the ethanol yield to 0.44 g/g (Dellweg et al. 1989).

A study on *S. shehatae* showed that a qO_2 of 1.19 mmol O_2 /g per cell weight (CDW) was able to maximize ethanol yield (0.327 g/g) with an average ethanol productivity of 2.2 g ethanol/L·h in a fed-batch fermentor containing 10% xylose (Fromanger et al. 2010). Recently, an oxygen transfer coefficient (K_La) from 2.3 to 4.9 h^{-1} was suggested for *S. stipitis* cultivated on 90 g/L of xylose. The in this case K_La corresponds to an OTR from 0.55 to 1.17 mmol O_2/L·h at 30°C. (Silva et al. 2012). Unrean and Nguyen (2012) used an elementary mode analysis to predict that an OTR of 1.8 mmol/L·h was optimal for *S. stipitis* in a 1-L fermentor containing 15 g/L of glucose and 5 g/L of xylose. Sininger et al. (2014) suggested that *S. stipitis* ethanol production is growth associated and that ethanol production can occur even when the growth rate

is as high as 0.5 h^{-1}; the requisite dissolved oxygen necessary to support that rate of growth would be at least 1 to 3 mg O_2/L (or 31 to 93 μmole O_2 /L) at 25°C.

Whereas *S. cerevisiae* can convert glucose to ethanol under anaerobic conditions, in most cases, engineered *S. cerevisiae* strains have a relatively high xylitol production compared to native xylose-fermenting yeasts and have been reported to require some oxygen during xylose fermentation (Jin et al. 2004). The reason engineered *S. cerevisiae* and native-xylose fermenting yeast need oxygen to ferment xylose is not clear, but it could result from an oxygen requirement for certain key biosynthetic reactions under strict anaerobic conditions (Andreasen and Stier 1953, Fornairon-Bonnefond et al. 2002, Jahnke and Klein 1983, Rosenfeld et al. 2003) or from a need to maintain cofactor balance during xylose assimilation (Fornairon-Bonnefond et al. 2002, Jahnke and Klein 1983, Rosenfeld et al. 2003). During xylose assimilation, *S. stipitis*' xylose reductase (XR) can use either NADH or NADPH as a cofactor, but it has a higher affinity for NADPH. The next enzyme in this pathway, xylitol dehydrogenase (XDH), which converts xylitol into xylulose, depends on NAD^+ almost exclusively (Liang et al. 2014). The different reactivities of the two enzymes with their respective cofactors can deplete NAD^+ and accumulate NADH. If this were the sole basis for the inhibition of growth under anaerobic conditions, then *S. stipitis* should grow anaerobically on glucose; however, this has not been demonstrated for more than one cell division. Engineering *S. cerevisiae* for xylose assimilation through heterologous expression of *S. stipitis* XR, XDH, and xylulokinase (XKS) also creates dependence on respiration in *S. cerevisiae* when xylose is the sole carbon source (Jin et al. 2004, Souto-Maior et al. 2009). Global gene expression and metabolic flux analyses on an engineered *S. cerevisiae* growing on xylose anaerobically suggested that the slower ATP formation from xylose as compared to glucose limits the anaerobic growth on xylose by *S. cerevisiae*, which is possibly

due to the higher ATP requirement for xylose transporters coupled to lower ATP yields from xylose fermentation (Sonderegger et al. 2004). In contrast, using a mutated *S. stipitis* XR with increased K_m for NADPH resulted in 70% xylitol reduction from 50 g/L xylose (Jeppsson et al. 2006). In addition, engineering a mutated XR from *Candida tenuis* with a higher affinity for NADH over NADPH into *S. cerevisiae* reduced the cofactor imbalance and resulted in a 42% increase in ethanol yield (Petschacher and Nidetzky 2008).

One consequence of cofactor imbalance is seen in byproduct formation. It has been reported that small amounts of 2,3-butanediol (BD) and acetoin are as fermentation byproducts during ethanol production by *S. cerevisiae* (Neish 1950), and the genes responsible for 2,3-BD formation in *S. cerevisiae* have been characterized (Gonzalez et al. 2000, Gonzalez et al. 2010). Ligthelm et al. (1988) first reported the accumulation of pentitols (arabitol, ribitol, and xylitol) and glycerol from 40 g/L of glucose (or xylose) by native pentose-fermenting yeasts. But native pentose-fermenting yeasts can accumulate much higher levels of ~~pentitols~~ byproducts compared to glucose fermentation by *S. cerevisiae.* The ethanol yield from glucose by *S. cerevisiae* is about 0.48-0.49 in lab scale but the ethanol yield from xylose by native pentose-fermenting yeast range from 0.4-0.45. This lower yield has been attributed mainly to oxygen requirements for growth and higher cell yields (Slininger et al. 2014). However, no studies have investigated the accumulation of 2,3-butanediol and acetoin in native pentose-fermenting yeast.

A recently isolated native pentose-fermenting yeast, *Spathaspora passalidarum* NN245, is capable of fermenting xylose faster than glucose and can co-utilize glucose, xylose and cellobiose as carbon sources (Hou 2012, Long et al. 2012, Nguyen et al. 2006). *S. passalidarum* and *S. stipitis* are closely related from both taxonomic and environmental perspectives. Both are found in the intestines of beetle larvae and adults, and both belong to the “CUG” clade of yeasts

that substitute serine for leucine when encountering a CUG in their messenger RNA (Wohlbach et al. 2011). Their likely evolution in association with beetles living inside of rotting wood probably accounts for their capacities to metabolize a wide range of lignocellulosic sugars and their capacities to grow at low oxygen tensions (Garcia-Ochoa and Gomez 2009, Nguyen et al. 2006). However, subsequent to the isolation of *S. passalidarum*, few publications have described its cellular physiology with respect to the oxygen requirements for ethanol production and the correlation between carbon source and polyol accumulations. In the present study, we not only investigated the fermentation performance of *S. passalidarum* and *S. stipitis* on glucose, xylose and sugar mixtures as functions of aeration rates but also measured polyol accumulation including 2,3-BDs and pentitols during fermentation with glucose, xylose or a mixture of glucose and xylose.

3.3 Materials and Methods

3.3.1 Yeast inoculum preparation, media

Spathaspora passalidarum NN245 (NRRL Y-27907) and *Scheffersomyces stipitis* NRRL Y-7124 were used in this study. Cultures were first streaked from -80°C stocks and transferred on either YPX or YPD agar plate containing (g/L): agar, 20; yeast extract, 10; peptone, 20; and either xylose, 20 or glucose, 20 and then cultivated for 24 h at 30°C. Cells from fresh plates were transferred into a 1-L Erlenmeyer flask containing 200 ml liquid broth composed of the following nutrients (g/L): xylose (or glucose), 40; peptone, 20; yeast extract, 10. The 1-L culture was grown at 30°C and 200 rpm overnight to reach an OD_{600} of 40, or about 7 g/L cell dry weight (CDW), prior to inoculation. Once the pre-cultures reached this concentration, cells were harvested by centrifugation at 5000 rpm for 3 min and suspended in distilled, deionized H_2O.

The inoculation volume was 4% of the final total volume (the volume of cell suspension plus the volume of the medium); thus, 2 ml of cell suspension was used for 50-ml shake flask fermentation and 3 ml of cell suspension was used for 75-ml shake flask fermentation with a target initial cell concentration of about 1.5 g CDW/L ($OD_{600} \approx 15$). Modified Slininger (MS) medium is based on Slininger's optimal medium (Slininger et al. 2006) except that MS medium contained 3.25 g/L of urea, and no purine, pyrimidine, or thioctic acid were used.

3.3.2 Effects of aeration on fermentation

To determine the maximum ethanol production from xylose by *S. passalidarum*, various aeration rates were applied to evaluate its fermentative ability at 25°C. Tests of aeration rates were performed in 125-ml polycarbonate Erlenmeyer flasks (GeneMate) fitted with 0.22 μm pore membrane filters for oxygen transfer. Flasks containing 50 or 75 ml of MS medium were shaken at 110 or 150 rpm to achieve four different aeration conditions (Table 3-1). Aeration conditions 2 and 3 have been used to evaluate or screen the performance of pentose-fermenting yeasts. (Slininger et al. 2006, Sreenath et al. 1986). The carbon sources consisted of 150 g/L (15%) glucose, 150 g/L (15%) xylose, or a mixture of 120 g/L (12%) xylose and 30 g/L (3%) glucose. Duplicate flasks in each aeration condition were used and a control experiment was performed at the same time with *S. stipitis* in each aeration condition. Xylose fermentations used inocula grown on xylose as the carbon source while glucose and mixed sugar fermentations used inocula grown on glucose. Samples (0.8 ml) were withdrawn daily from each flask for HPLC and cell density determinations. Fermentation rates and yields were based on calculations over the first 48-h to avoid effects of product inhibition or substrate limitation that could interfere with the kinetic values. The sulfite-oxidation method (Garcia-Ochoa and Gomez 2009) was used to determine the oxygen transfer rate (OTR) in each fermentation condition.

Table 3-1 Aeration conditions used in these experiments

Aeration Condition	Liquid volume in a 125-ml flask (ml)	Agitation (rpm)	OTR (mmol O_2/L·h)
1	75	110	2.47
2	75	150	3.65
3	50	110	4.27
4	50	150	4.73

3.3.3 Culture sampling and analyses of sugar, ethanol and major fermentation byproducts

Cell concentration was determined by measuring the optical density at 600 nm (OD_{600}) with an Agilent 8453 spectrophotometer. Yeast CDW was estimated by correlating OD_{600} (5 to 100 OD_{600}) with gravimetric measurements of dried cells from the same cultures: about 2 to 10 ml of cell suspension was filtered through 0.2 μm hydrophilic polyethersulfonate membrane (PALL, New York) and the cell mass was dried as described by Postma *et al.*(1989). Under the conditions used, 1 OD_{600} equals 0.1101 (±0.01) g/l of CDW for *S. passalidarum* NRRL Y-27907and 0.133 (± 0.007) g/L of CDW for *S. stipitis* NRRL Y-7124. All sugars and fermentation metabolites including D-arabitol, xylitol, ribitol, and glycerol were determined by high performance liquid chromatography (HPLC) using a Hitachi auto sampler, pump and refractive index detector and a Bio-Rad Aminex HPX-87H column (300 × 7.8 mm) at 65°C (Fromanger et al. 2010). The mobile phase was 0.005 M H_2SO_4 at flow rate of 0.6 ml/min. External standardization was used with frequent calibration of response factors.

3.3.4 Analyses of acetoin and 2,3-butanediol during fermentation

S. cerevisiae normally produces 2,3-butanediol (BD) consisting of ~67% of *R,R*-2,3-BD, 33% *meso*-2,3-BD and less than 1% *S,S*-2,3-BD (Gonzalez et al. 2000, Romano et al. 2003). We assume less than 1% of *S,S*-2,3BD in the total 2,3-BD production for the native pentose-fermenting and the major composition in 2,3-BD will be *R,R*-2,3-BD and *meso*-2,3-BD. Acetoin, *meso*-2,3-BD, and *R,R*-2,3-BD were confirmed by HPLC-MS and the quantitative analyses of these products, along with polyols, were determined by HPLC (Jiang et al. 2012) using the same methods mentioned in the previous section.

3.4 Results

3.4.1 Fermentation performance with various OTRs in modified Slininger (MS) medium

To determine the potential ethanol productivity of *S. passalidarum* NN245 at various aeration rates, fermentations were carried out at 25°C using 15% glucose, 15% xylose, or a sugar mixture of 12% xylose and 3% glucose under 4 different aeration levels (Figure 3-1). For glucose fermentations, cell growth rate, sugar utilization, ethanol productivity decreased after 48-h and ethanol accumulation on glucose exceeded accumulation on xylose only at the lowest aeration level. On the other hand, for xylose and mixed sugar fermentations, the cell growth rate and ethanol productivity did not slow down until 72 h. On glucose, CDW rose quickly and then dropped after 48 h. With xylose or mixed sugar fermentations, cell yield was much lower from the outset, and cell concentration stayed steady throughout the fermentation. Peak ethanol concentrations occurred between 72 and 120 h, depending on aeration conditions. The highest ethanol concentration was 58.3 g/L from mixed sugar fermentation (12% xylose, 3% glucose) at the lowest aeration level (2.47 mmol O_2/L·h). Similarly, the lowest aeration also produced the highest ethanol yield, 0.45 g/g from 150 g/L xylose This was also the condition in which *S. passalidarum* exhibited the highest specific ethanol productivity, 0.153 g ethanol/g CDW·h (Table 3-2).

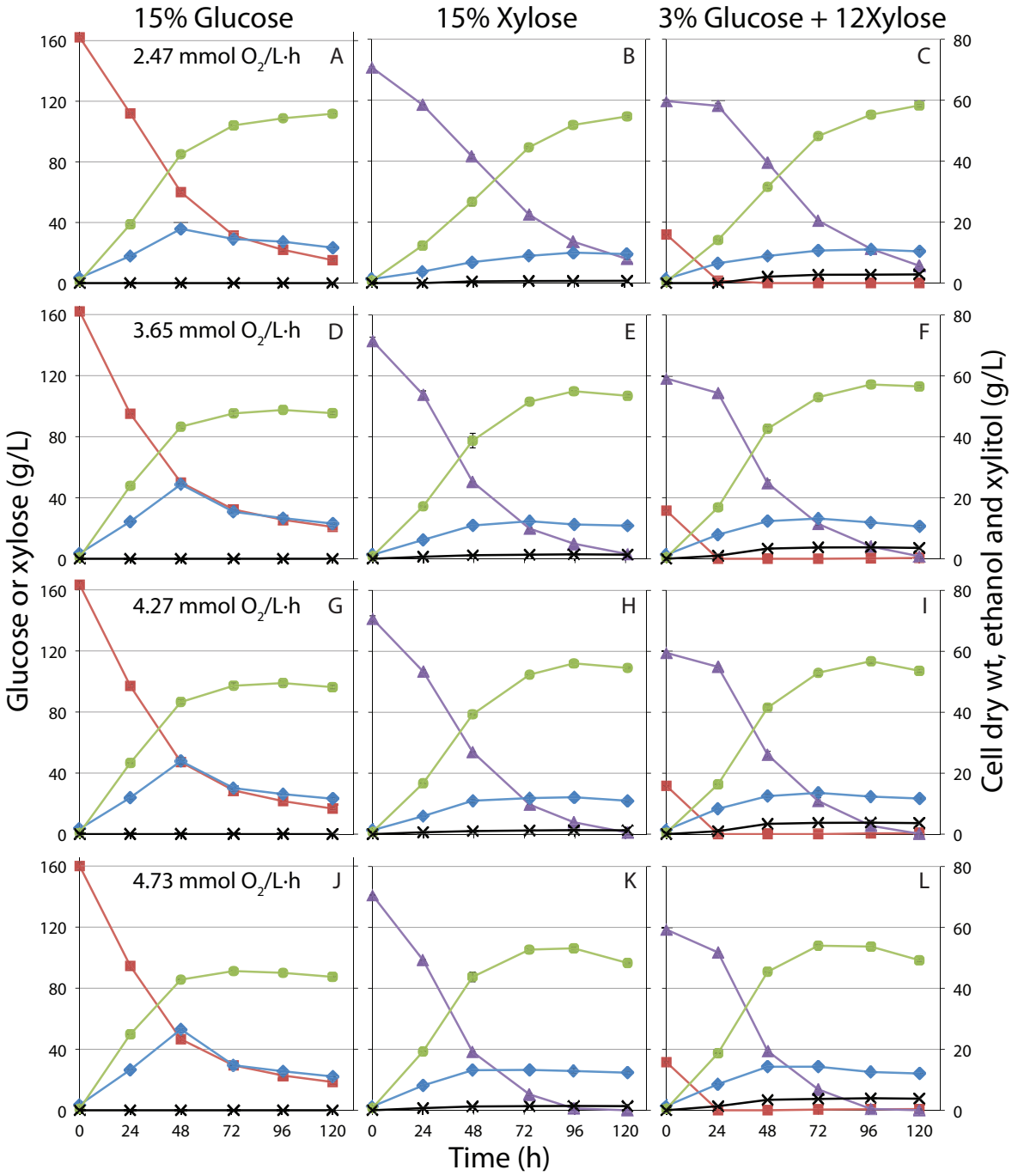

Figure 3-1 Ethanol production from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *Spathaspora passalidarum* NN245 in duplicate shake flasks under different aerations (2.47, 3.65, 4,27, and 4.73 mmol O_2/L·h). Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol.

Table 3-2 Fermentation kinetics in first 48 h from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% glucose by *S. passalidarum* NN245 and *S. stipitis* NRRL Y-7124.

	Spathaspora passalidarum					*Scheffersomyces stipitis*				
15% Glucose										
OTR	μ	$Y_{X/S}$	$Y_{E/S}$	Q_E	q_E	μ	$Y_{X/S}$	$Y_{E/S}$	Q_E	q_E
2.47	0.042	0.158	0.411	0.875	0.110	0.042	0.168	0.391	0.798	0.114
3.65	0.045	0.202	0.382	0.892	0.092	0.044	0.179	0.370	0.891	0.116
4.27	0.045	0.194	0.375	0.892	0.093	0.044	0.176	0.380	0.860	0.116
4.73	0.046	0.218	0.373	0.882	0.086	0.045	0.189	0.383	0.861	0.114
15% Xylose										
2.47	0.033	0.076	0.448	0.547	0.153	0.031	0.101	0.432	0.529	0.122
3.65	0.039	0.075	0.407	0.799	0.144	0.037	0.102	0.395	0.730	0.137
4.27	0.039	0.081	0.415	0.813	0.149	0.037	0.114	0.414	0.719	0.136
4.73	0.041	0.083	0.375	0.907	0.131	0.039	0.119	0.369	0.743	0.130
12% Xylose, 3% Glucose										
2.47	0.033	0.075	0.425	0.649	0.119	0.035	0.109	0.421	0.588	0.129
3.65	0.038	0.075	0.401	0.880	0.127	0.040	0.107	0.380	0.738	0.126
4.27	0.038	0.076	0.389	0.855	0.118	0.039	0.119	0.381	0.699	0.127
4.73	0.040	0.075	0.358	0.938	0.124	0.042	0.125	0.357	0.753	0.126

OTR, oxygen transfer rate, mmol O_2/L·h; μ, specific growth rate, 1/h; $Y_{X/S}$, cell yield, g/g; $Y_{E/S}$, ethanol yield, g/g; Q_E, volumetric ethanol productivity, g/L·h; q_E, specific ethanol productivity, g/g CDW·h

To better understand our findings with *S. passalidarum* at various aeration conditions, we conducted a comparable experiment under the same conditions with *S. stipitis* NRRL-Y7124 (Figure 3-2). The peak ethanol concentration attained with *S. stipitis* was 53.3±0.3 g/L at 2.47 mmol O_2 /L·h from 15% xylose. On glucose, we observed a similar rapid but incomplete sugar consumption accompanied with rapid initial cell growth followed by a decline in CDW after 48 h. Under most circumstances, *S. passalidarum* produced more ethanol compared to *S. stipitis*, except when glucose was the sole carbon source at an OTR of 3.65 or 4.73 mmol O_2 /L·h. The relatively lower ethanol yield on xylose was mirrored by a relatively higher cell yield in comparison to that observed with *S. passalidarum.* The fermentation rates and yields of the two yeasts were similar, but overall *S. passalidarum* performed better than *S. stipitis* under the lower aeration conditions, especially when xylose was the sole carbon source.

We compared the fermentation kinetics of *S. passalidarum* NN245 and *S. stipitis* NRRL Y-7124 at 48-h as functions of the oxygen transfer rates (Figure 3-3). The specific growth rates and cell yields of *S. passalidarum* on glucose were clearly higher than observed with *S. stipitis*, especially for the cell yield at OTR of 4.73 mmol O_2/L·h*,* and with both yeasts, these values increased as aeration increased (Figure 3-3 A, B). However, when the mixed sugar was used, *S. stipitis* showed higher specific growth rates compared to those of *S. passalidarum*. During xylose or mixed sugar fermentations, ethanol yields of both strains dropped by 14 to 16% as the aeration rate increased from 2.47 to 4.73 mmol O_2/L·h. *S. passalidarum's* specific ethanol productivity (q_E) was roughly 50% higher on xylose than on glucose over the entire range of aeration rates examined. By comparison, its volumetric rate of ethanol production from xylose increased with aeration while the volumetric rate with glucose was essentially constant. On the

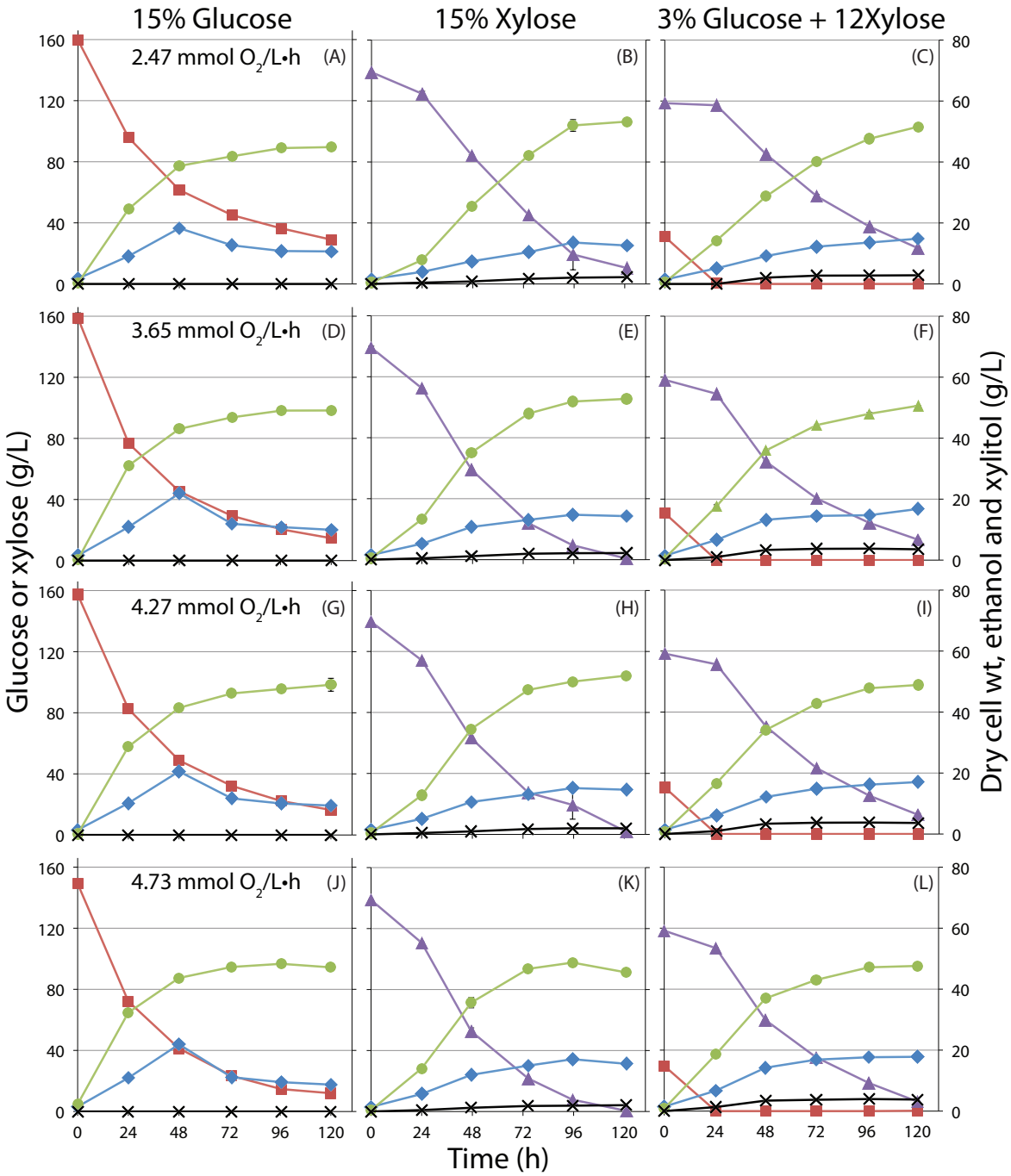

Figure 3- 2 Ethanol production from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% glucose by *Scheffersomyces stipitis* NRRL Y-7124 in duplicated shake flask under different aerations (2.47, 3.65, 4,27, and 4.73 mmole O_2/L·h). Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol.

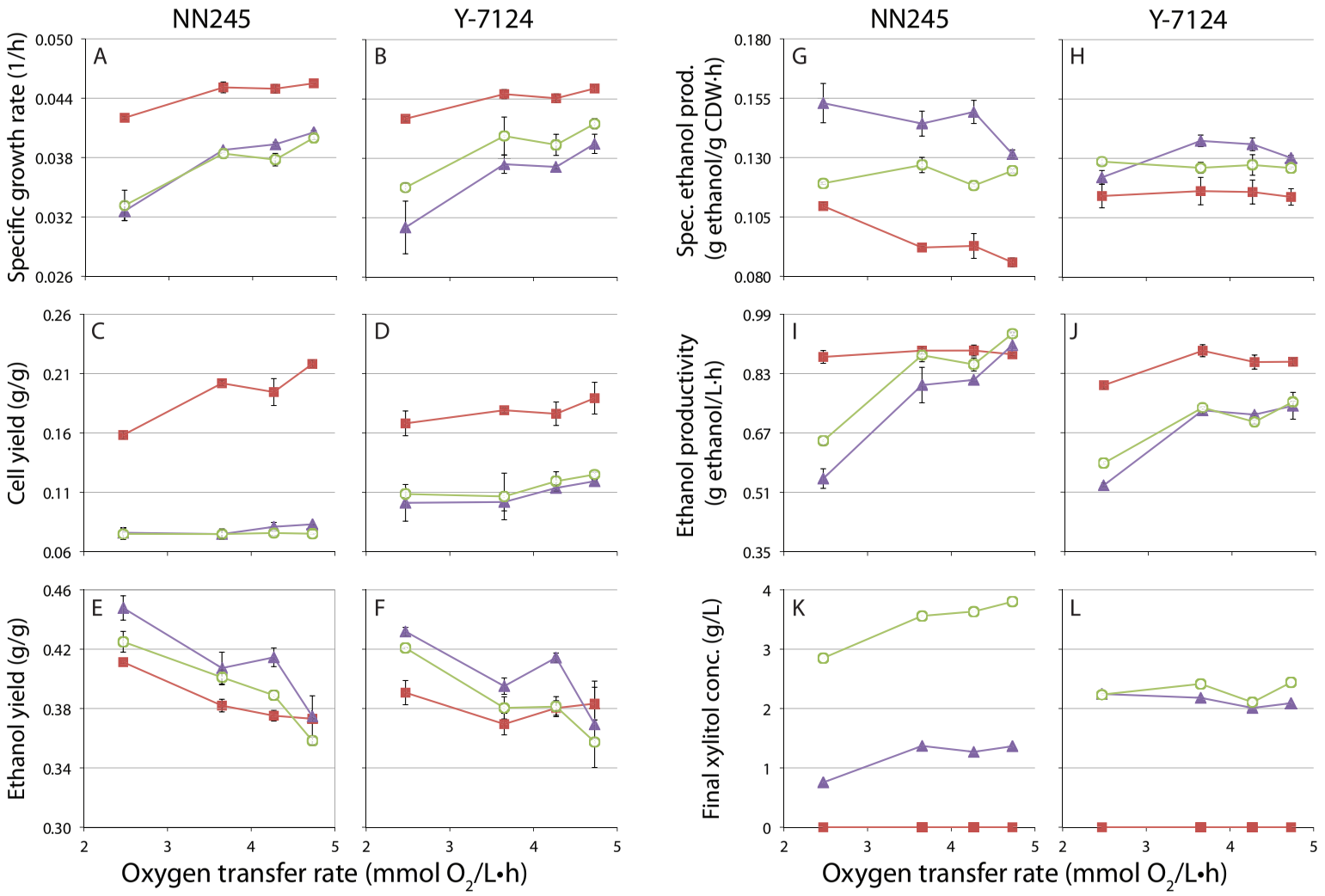

Figure 3-3 Fermentation kinetics at first 48 h and final xylitol concentration from 15% glucose (■), 15% xylose (▲), and a mixture of 12% xylose and 3% glucose (○) by *S. passalidarum* NN245 and *S. stipitis* NRRL-Y7124 under various aerations in duplicate shake flasks. Error bars depict the range of values

other hand, for *S. stipitis*, its q_E responded to the increasing aeration with an optimized OTR at 3.65 mmol O_2/L·h with xylose as the carbon source. For q_E on glucose, xylose or a mixture of the two sugars, *S. stipitis* exhibited a range between 0.114 to 0.137 g ethanol/g CDW·h while *S. passalidarum* showed a wider range of 0.089 to 0.153 g ethanol/ g CDW·h (Table 3-2). The ethanol productivities of *S. stipitis* exhibited similar trends compared to those from *S. passalidarum*, but the rates from xylose and mixed sugar were about 15-20% lower than those from *S. passalidarum* (Figure 3-3).

3.4.2 Polyol and acetic acid accumulation under different aeration rates

With *S. passalidarum,* the accumulation of xylitol tripled from about 1 g/L to about 3 to 4 g/L when low amounts of glucose were present. Similar but less pronounced trends were observed with *S. stipitis* (Figure 3-3). We found no clear correlation between aeration and arabitol or ribitol accumulation (Figure 3-4). In contrast, acetic acid increased when aeration increased with both of the yeast species and each of the three sugar substrates (Figure 3-5). More acetic acid accumulated on glucose as compared to xylose or mixed sugar fermentations. When the carbon source was xylose or a mixture of 12% xylose and 3% glucose, *S. passalidarum* produced only 40 to 50% as much acetic acid as from glucose alone. The highest acetic acid production was 2.11 g/L from 15% glucose at 4.73 mmol O_2/L·h. *S. passalidarum* produced much less ribitol compared to *S. stipitis* during xylose or mixed sugar fermentation (Figures. 3-4 and 3-5). *S. stipitis* converted roughly 3.3% of the available xylose into ribitol and accumulated as much as 5 g/L. In contrast, *S. passalidarum* generally produced less than 0.6 g/L of ribitol and always less than 1 g/L under all of the conditions tested. *S. passalidarum* accumulated more arabitol than ribitol, and the arabitol concentration could be twice as much as that of ribitol when

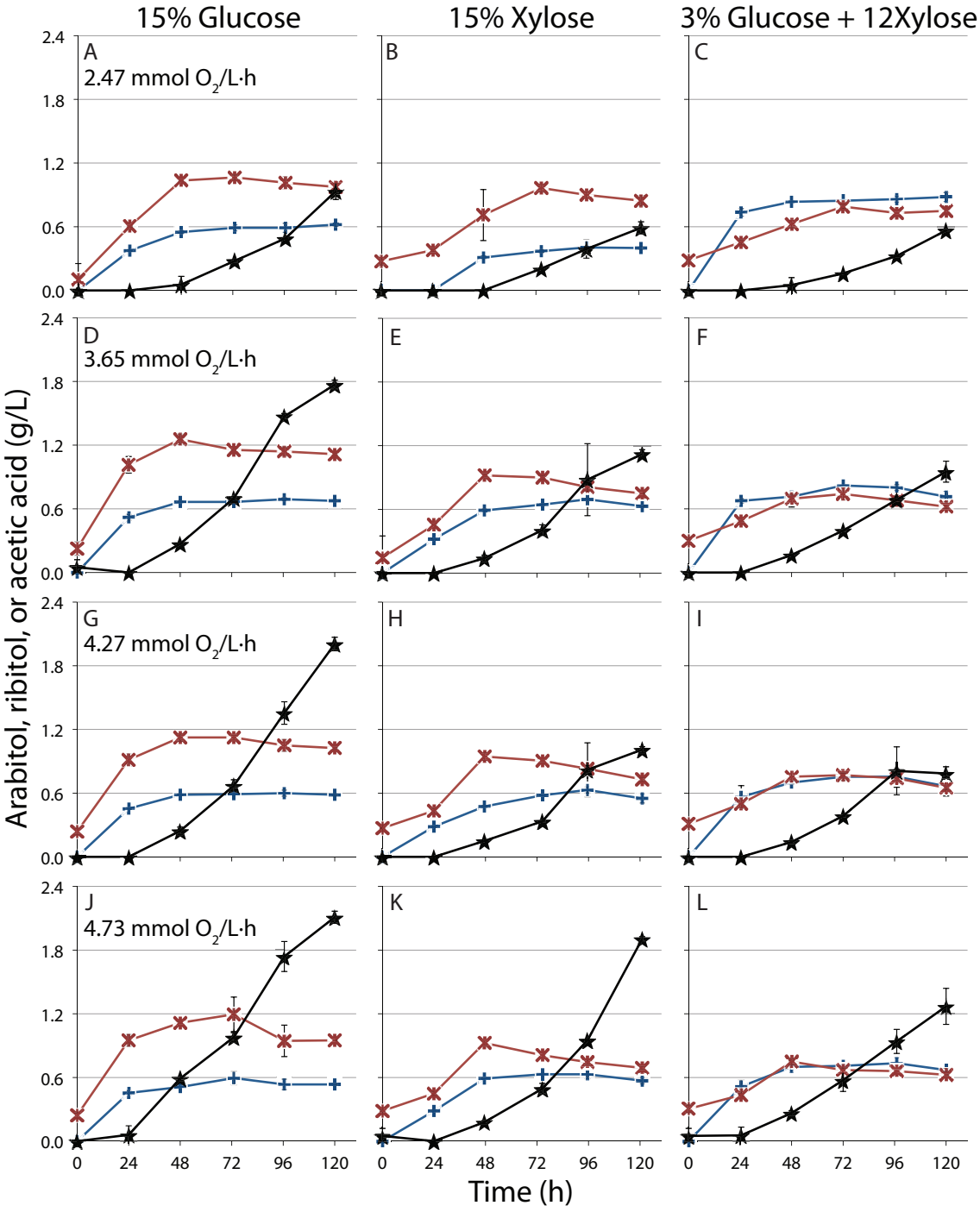

Figure 3-4 The formation of arabitol, ribitol and acetic acid from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *S. passalidarum* NN245 under different aerations (2.47, 3.65, 4,27, and 4.73 mmol O_2/L·h) in duplicate shake flasks. Symbols: ✱, arabitol; ✚, ribitol; ★, acetic acid.

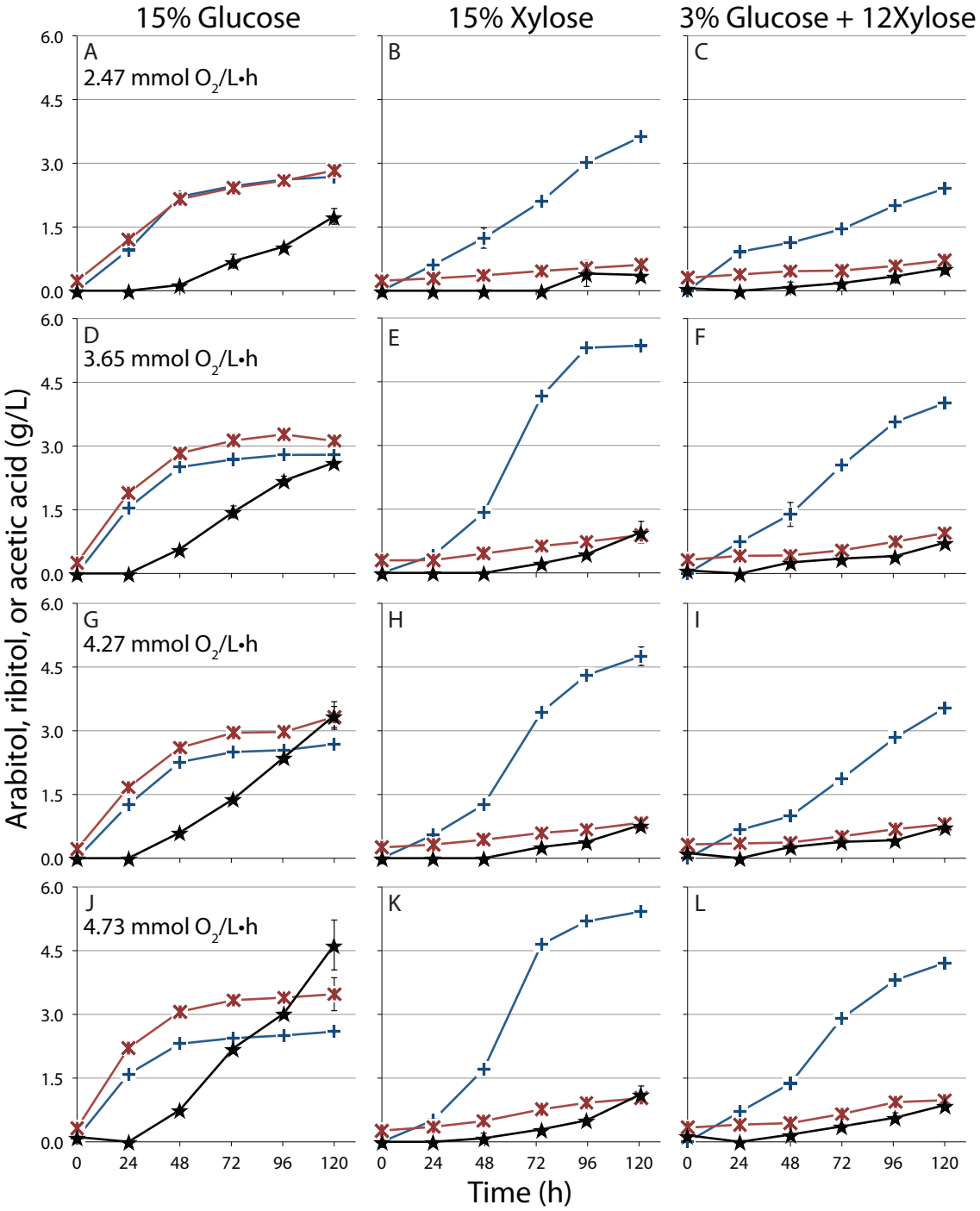

Figure 3-5 The formation of arabitol, ribitol and acetic acid from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% glucose by *Scheffersomyces stipitis* NRRL Y-7124 under different aerations (2.47, 3.65, 4,27, and 4.73 mmole O_2/L·h) in duplicated shake flasks. Symbols: ✖, arabitol; ✚, ribitol; ★, acetic acid

glucose was the sole carbon source (Figure 3-4 A, D, G, J). But when mixed sugars were used, *S. passalidarum* produced arabitol and ribitol in similar quantities. By comparison, *S. stipitis* tended to accumulate much more ribitol than arabitol during xylose or mixed sugar fermentation and the final ribitol concentration could be 5 to 6 times higher than the final arabitol concentration when pure xylose was the sole carbon source (Figure 3-5 B, F, H, K). When glucose was the sole carbon source, *S. stipitis* accumulated similar amounts of arabitol and ribitol at an OTR of 2.47, and higher arabitol accumulation was observed when OTR was higher than 3.65 mmol O_2/L·h.

S. passalidarum and *S. stipitis* both produced glycerol, acetoin, *meso*-2,3-butanediol (BD), and *R,R*-2,3-BD during fermentation (Figures 3-6 and 3-7). With *S. passalidarum*, all of these byproducts accumulated to higher concentrations as aeration increased. Notably, *S. passalidarum* produced four times more *R,R*-2,3-BD than *S. stipitis*, which was the highest byproduct besides xylitol during the xylose and the mixed sugar fermentations. A spike of acetoin production from 0.45 to 0.91 g/L occurred in the first 24-h during glucose and the mixed sugar fermentations but not when xylose was the sole carbon source. *S. passalidarum* accumulated more glycerol when the carbon source was glucose or mixed sugars than when cultivated on xylose. Moreover, during the first 24-h fermentation, a faster accumulation of glycerol was found in the first 24-h from mixed sugar fermentation than from glucose fermentation. For *S. stipitis* (Figure 3-7), during glucose fermentation, the accumulations of glycerol and acetoin correlated with increasing aeration. The accumulation of polyols by *S. passalidarum* appeared to be different than that of *S. stipitis* (Figure 3-8). The ratio of pentitol to total polyol yield (RPPY, mM/mM) can vary from 0.11 to 0.25 for *S. passalidarum*, but the value was around 0.5 for *S. stipitis* when glucose was the carbon source. More striking, the RPPY of *S. passalidarum* ranged 0.28 to 0.42,

but the RPPY of *S. stipitis* was about 0.8 when xylose was the sole carbon source. A summary of acetic acid and polyol accumulation by *S. passalidarum* and *S. stipitis* is shown in Table 3-3.

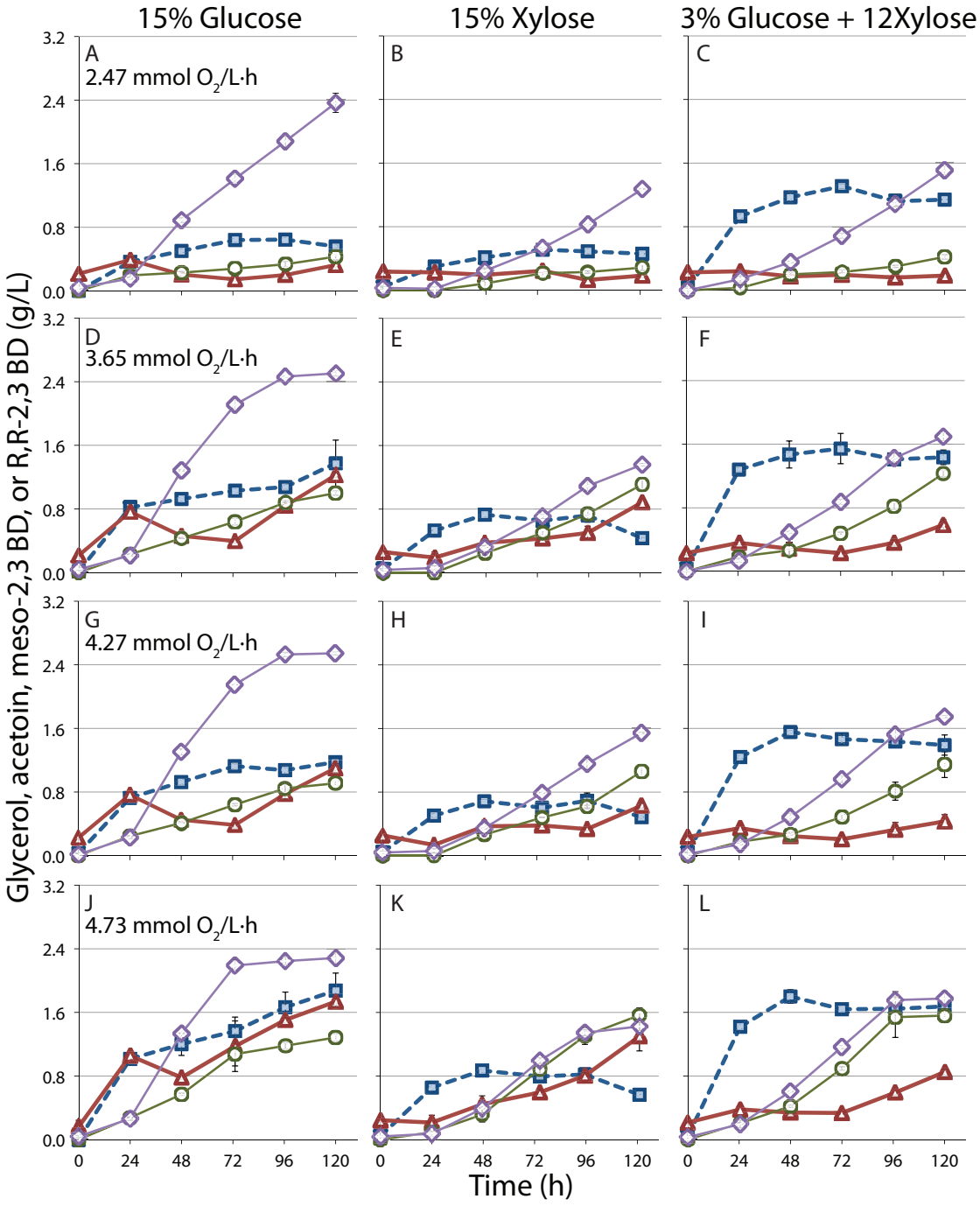

Figure 3-6 The accumulation of glycerol, acetoin, *meso*-2,3BD and *R,R*-2,3 BD from 15% glucose, 15% xylose, and a mixture sugar of 12% xylose and 3% glucose by *S. passalidarum* NN245 under different aerations (2.47, 3.65, 4,27, and 4.73 mmol O_2/L·h) in duplicate shake flasks. Symbols: ■, glycerol; ▲, acetoin; ●, meso-2,3-BD; ◆, R,R-2,3-BD.

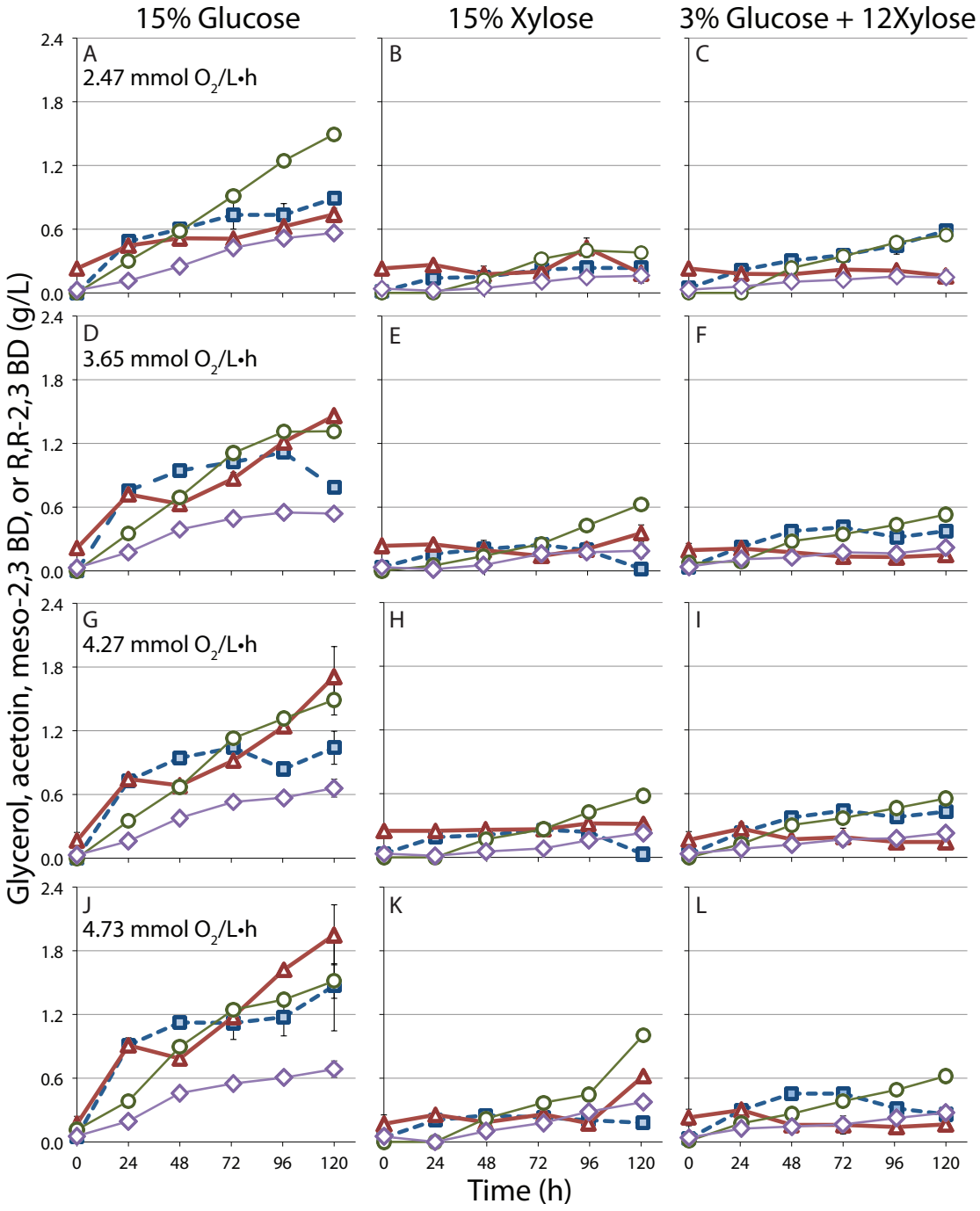

Figure 3-7 The accumulation of glycerol, acetoin, *meso*-2,3BD and *R,R*-2,3 BD production from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% glucose by *Scheffersomyces stipitis* NRRL Y-7124 under different aerations (2.47, 3.65, 4,27, and 4.73 mmole O_2/L·h). Symbols: ■, glycerol; ▲, acetoin; ●, meso-2,3 BD; ◆, R,R-2,3 BD

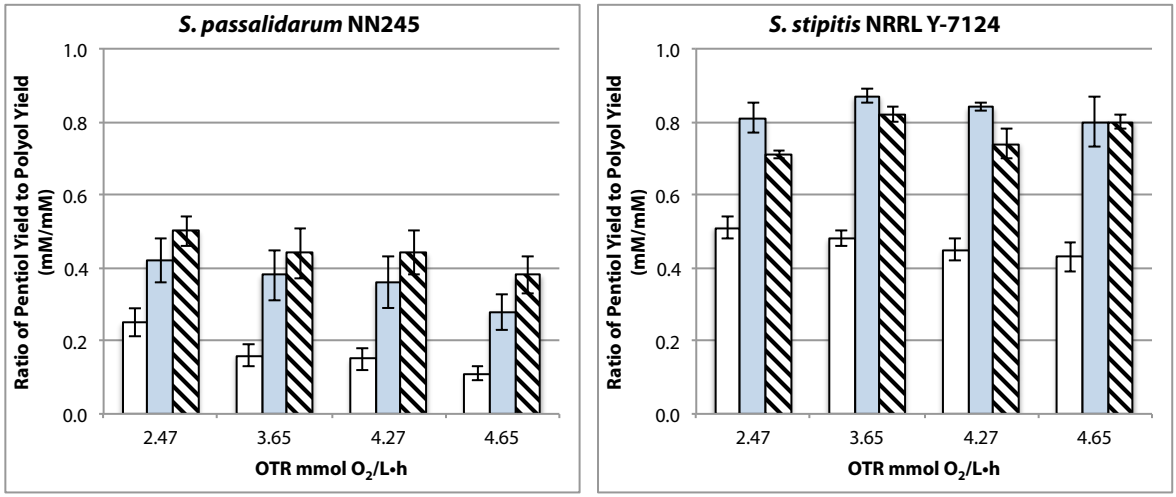

Figure 3-8 Ratio of pentitols yield to polyols yield (mM/mM) from 15% glucose, 15% xylose and a mixture sugar of 12% xylose and 3% glucose by *S. passalidarum* NN245 and *S. stipitis* NRRL Y-7124 under different aerations (2.47, 3.65, 4,27, and 4.73 mmol O_2/L·h) in duplicate shake flasks. Symbols: □, 15% glucose ; ■, 15% xylose; ▨, 12% xylose + 3% glucose. Error bars indicate the range of values. The average ratio of pentitol yield to total polyol yield (RPPY) from 72 to 120 h was calculated by applying following equations (1) to (3)

$$\text{Pentitol Yield} = \frac{\text{Sum of produced pentitols (Arabitol, Ribitol, Xylitol, mM)}}{\text{Sum of consumed sugar (mM)}} \quad (1)$$

$$\text{Polyol Yield} = \frac{\text{Sum of pentitols, acetoin, 2,3 butanediols, and glycerol (mM)}}{\text{Sum of consumed sugar (mM)}} \quad (2)$$

$$\text{RPPY} = \frac{\text{Yield of Pentitols}}{\text{Yield of Polyols}} \quad (3)$$

Table 3-3 Summary of acetic acid and polyols acccumulations[a] , g/L.

Carbon source	Xylitol	Ribitol	Arabitol	Glycerol	Acetoin	*R,R*-2,3 BD	*meso*-2,3 BD	Ratio of 2,3BD[b]	Acetic acid
Spathaspora passalidarum NN245									
15% glucose	nd[c]	0.5-0.6	~ 1	0.6-1.9	0.3-1.7	2.3-2.5	0.4-1.3	1.8-5.6	1-2
15% xylose	0.8-1.4	0.4-0.6	0.7-0.9	0.4-0.6	0.2-1.3	1.3-1.6	0.3-1.6	0.9-4.5	0.6-1.9
12% xylose, 3% glucose	2.9-3.8	0.7-0.9	0.6-0.8	1.1-1.7	0.2-0.9	1.5-1.8	0.4-1.6	1.4-3.6	0.6-1.2
Scheffersomyces stipitis									
15% glucose	nd[c]	~2.7	2.8-3.5	0.8-1.5	0.7-2	0.5-0.7	1.3-1.5	0.4-0.5	1.8-4.7
15% xylose	2-2.3	3.6-5.4	0.6-1	~0.2	0.2-0.6	0.2-0.4	0.4-1	0.3-0.4	0.4-1.1
12% xylose, 3% glucose	2.2-2.4	2.4-4.2	0.7-1	0.3-0.6	~0.2	~0.2	0.5-0.6	0.3-0.4	0.5-0.9

[a] OTR values ranged from 2.47 to 4.73 mmol O_2/L·h after 120-h fermentation from 15% glucose, 15% xylose, and a mixture of 12% xylose and 3% gluocse in duplicate shake flasks by *Spathaspora passalidarum* and *Scheffersomyces stipitis*

[b] The ratio of *R,R*-2,3 BD to *meso*-2,3 BD

[c] nd = Not detectable

3.5 Discussion

3.5.1 Fermentation kinetics and oxygen transfer rates

In the present experiments, the inocula were cultured with yeast extract and peptone (YP), which was the same method described by (Long et al. 2012) because (i) YP medium has been widely use for inoculum preparation, and (ii) only about 4-6 h is needed for *S. stipitis* to adjust the expression level of genes related to glycolytic pathway and PPP during fermentation after transition of cultures based on previous transcription microarray study (Jeffries and Van Vleet 2009). If the inocula had been cultivated in the same MS medium as used for the fermentation trials, it would have reduced the transition, but likely would not have altered the reported rates or yields.

For both yeasts, aeration exhibited a greater effect on cell yields than on specific growth rates during glucose fermentation, but during xylose or mixed sugar fermentation, aeration exerted more influence on specific growth rates than on cell yields (Figure 3-3 A, B, C, D). Also, both yeasts had lower cells yields when growing on xylose than when growing on glucose and higher ethanol yields on xylose than on glucose except at the highest aeration rate examined (Figure. 3-3 E, F). The most likely explanation for the observations among cell yields, specific growth rates, ethanol yield in response to various aerations is that net ATP yields are substantially lower when either yeast is growing on xylose than when growing on glucose, and that the ATP yield from xylose is lower with *S. passalidarum* than with *S. stipitis.* Consequently, cells need to consume more carbon to make the same amount of cell mass. Two metabolic steps could account for the lower ATP yield: sugar uptake by symporters and the xylulose phosphorylation step (Figure 3-9). The energy expended per mole of sugar should be similar

regardless of whether a C5 or C6 sugar is taken up via symport; the same would hold true for phosphorylation, but the C-moles of carbon would be 17% less in each step.

For both strains and for all three carbon sources, our results are consistent with previous studies in *S. stipitis* and *S. shehatae,* which also showed reduced ethanol yield with increasing aeration (Du Preez et al. 1989, Silva et al. 2012). The q_E we observed was similar to what had been reported with *S. stipitis* (Dellweg et al. 1989, Laplace et al. 1991) Interestingly, *S. passalidarum* showed a 1.5 to 2-fold increase in the q_E from xylose compared to glucose due to a 52 to 63% decrease in cell yield. A similar result was also observed by Long *et al.* (2012), who reported a q_E on xylose 3.4-fold higher than on glucose in a 2-L fermenter trial. When xylose or mixed sugar was the carbon source, both yeast improved Q_E when aeration increased, which was also reported previously for *S. stipitis* (Du Preez et al. 1989, Silva et al. 2012).

By comparing the overall fermentation kinetics (Figure 3-2 and Table 3-2), the data suggested that the two yeasts respond differently to the carbon sources and aeration rates. Based on our results, when xylose was the sole carbon source, the ethanol yield of *S. passalidarum* ranged from 0.46 to 0.42 with an OTR corresponding to 2.47 and 4.27 mmol O_2/L·h. These yields compare favorably to ethanol yields from xylose that are obtained with metabolically engineered *Saccharomyces cerevisiae* (Bengtsson et al. 2009, Madhavan et al. 2009, Shen et al. 2012), but fermentations of pure xylose solutions are not generally reported for engineered *S. cerevisiae*, and even more rarely are they reported for sugar concentrations as high as those used here. Increasing aeration dramatically increased ethanol productivity from 0.52 to 0.8 g/L·h. Over this range specific ethanol productivity (q_E) was maintained at ≈ 0.15 g/g CDW·h. If we divide OTR values by the cell mass and assume (i) no change in OTR during fermentation, and (ii) oxygen diffusing into the medium was completely consumed by yeasts (DOT = 0), a qO_2 at

48-h can be calculated around 0.33 to 0.39 mmol O_2/g CDW·h for *S. passalidarum*. With the same method, a qO_2 ranging from 0.34 to 0.4 mmol O_2/g CDW·h can be calculated for *S. stiptis*, which is very close to the value obtained with *S. passalidarum*. Our *S. stipitis's* qO_2 values match some of the studies that were previously reported in the literature, which was about 0.3 mmol O_2/g CDW·h for the xylose fermentation (Dellweg et al. 1989, Grootjen et al. 1991, Guebel et al. 1991, Laplace et al. 1991). Our numbers are higher than some reported from previous research, possibly because of differences in the sugar concentration (cf. Unrean and Nguyen 2012), or medium composition and fermentation configurations (cf. Silva et al. 2012). A summary of the oxygen requirement for xylose fermentation is presented in Table 3-4.

3.5.2 Polyol and acetic acid accumulation

The native yeasts studied here can ferment both glucose and xylose through concerted glycolytic and pentose phosphate pathways. In the process, they can form arabitol and ribitol in addition to xylitol (Figure 3-9). We observed increases in xylitol accumulation by *S. passalidarum* on mixed sugars with a low amount of glucose compared to the results on pure xylose, however cell yields and specific growth rates were very similar under both conditions (Figure 3-2 C, K), suggesting that the increase in xylitol production was not triggered by insufficient DOT. We postulated that the small amount of glucose might have down-regulated glycolytic genes and enzymes in *S. passalidarum*. The difference between final xylitol concentrations produced by *S. passalidarum* and *S. stipitis* during mixed sugar fermentation (Figure. 3-3 K, L) suggested small amounts of glucose had different effects on the two strains. Polyol accumulation during yeast fermentation usually serves as an alternative way to regenerate cofactors such as $NAD(P)^+$ when $NAD(P)H + H^+$ is in excess.[·] Acetic acid accumulation can

occur through the partial oxidation of acetaldehyde when NAD(P)H + H^+ is limited. Polyol production started early in the fermentation

Table 3-4 Fermentation parameters for ethanol production from xylose

Yeast	Type[a]	Xylose (g/L)	Ethanol (g/L)	$Y_{X/S}$ (g/g)	$Y_{E/S}$ (g/g)	Q_E (g/L·h)	q_E	DOT (%)	OTR	qO_2	Reference
S. passalidarum											
NN245	F	140	27-40	~0.08	~0.43	~0.72	~0.15	ns	2.5-4.7	0.33-0.39	This study[b]
S. shehatae											
CBS 2779	FB	129	44	0.07	0.34	0.99	0.34	0.7	ns	ns	du Preez *et al.* 1989
ATCC 22984	FB	100	48.1	~0.13	~0.33	~2.2	~0.22	ns	ns	1.19	Fromanger *et al.* 2012
ATCC 22984	Fr	50	~19.5	0.09	0.39	ns	0.15	~0	3.9	ns	Laplace *et al.* 1991
ATCC 22984	Fr	90	26	0.05	0.28	1.06	*0.12*	ns	8.5	ns	Sreenath *et al.* 1986
S. stipitis											
NRRL Y-7124	Fr	ns	ns	~0.11	0.39	ns	0.12	ns	3.75	0.3	Dellweg *et al.* 1989
NRRL Y-7124	Fr	15b	ns	ns	~0.4	ns	0.24	0	0.7	~0.3	Delgenes *et al.* 1989
NRRL Y-7124	F	50	ns	0.34	0.16	0.5	0.41	ns	5	ns	Guebel *et al.* 1991
NRRL Y-7124	Fr	50	~21.5	0.12	0.43	ns	0.12	0	1.75	ns	Laplace *et al.* 1991
NRRL Y-7124	Fr	50	~18	0.39	ns	ns	0.12	<1	3.75	ns	Rizzi *et al.* 1989
NRRL Y-7124	Fr	90[c]	~27	~0.14	~0.29	~0.3	ns	ns	0.6-1.8	ns	Silva *et al.* 2012
NRRL Y-7124	F	140	~35	~0.11	~0.39	~0.73	~0.13	ns	2.5-4.7	0.34-0.4	This study[b]
CBS 7126	FB	129	~28	~0.15	~0.35-.4	1.6-1.8	0.3-0.4	0.2 - 0.7	Ns	ns	du Preez *et al.* 1989
CBS 6054	C	50	ns	ns	0.48	ns	0.2	0	<1	ns	Skoog *et al.* 1990
BCC15191	Fr	ns	ns	ns	0.4	0.25	ns	ns	1.8[d]	ns	Unrea *et al.*2012

Table 3-4 (Cont.) Fermentation parameters for ethanol production from xylose

$Y_{X/S}$, cell yield; $Y_{E/S,}$ ethanol yield; Q_E, volumetric ethanol productivity; q_E, specific ethanol productivity, g/g DCW·h; DOT, dissolved oxygen tension; OTR, oxygen transfer rate, mmol O_2/L·h; qO_2, specific oxygen uptake rate, mmol O_2/g DCW·h; ns, not stated

[a] F, flask; FB, fed-batch; Fr, fermentor, C, continuous culture.

[b] All the value were calculated at first 48-h

[c] contained 15 g/L of glucose

[d] The result was based on elementary model analysis.

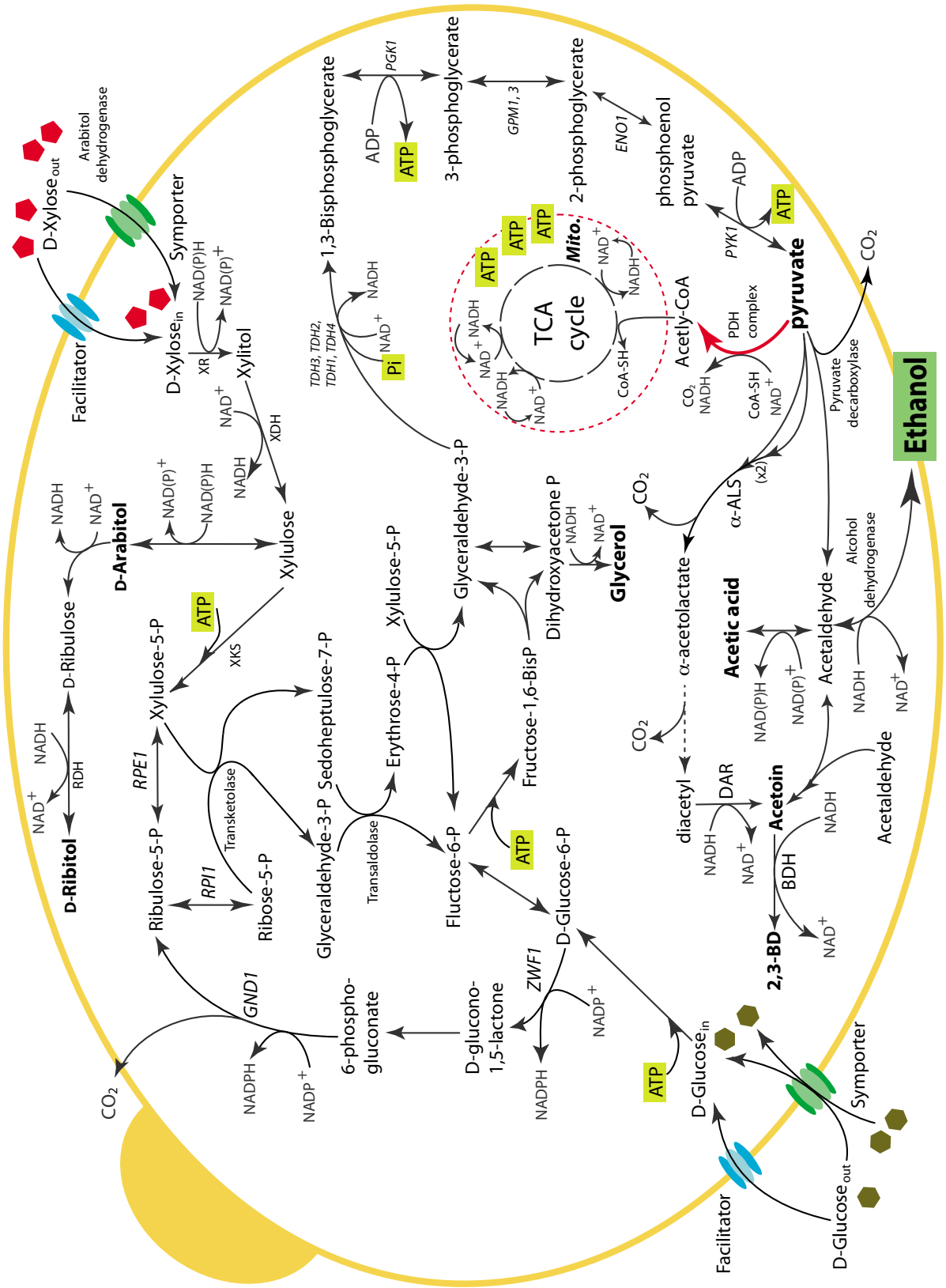
D-Xylose out
Arabitol dehydrogenase
Symporter
Facilitator
D-Xylose in
NAD(P)H
NAD(P)+
XR
Xylitol
NAD+
NADH
XDH
Xylulose
D-Arabitol
NADH
NAD+
D-Ribulose
RDH
D-Ribitol
NAD+
NADH
NAD(P)+
NAD(P)H
RPE1
Xylulose-5-P
ATP
XKS
Ribulose-5-P
RPI1
Ribose-5-P
Transketolase
Glyceraldehyde-3-P
Sedoheptulose-7-P
Transaldolase
Erythrose-4-P
Xylulose-5-P
Glyceraldehyde-3-P
Fructose-1,6-BisP
Dihydroxyacetone P
NADH
NAD+
Glycerol
Fluctose-6-P
ATP
D-Glucose-6-P
ZWF1
NADP+
NADPH
D-glucono-1,5-lactone
6-phospho-gluconate
GND1
NADP+
NADPH
CO2
TDH3, TDH2, TDH1, TDH4
1,3-Bisphosphoglycerate
ADP
ATP
PGK1
3-phosphoglycerate
GPM1, 3
2-phosphoglycerate
ENO1
phosphoenol pyruvate
PYK1
ADP
ATP
pyruvate
Mito.
ATP
ATP
ATP
TCA cycle
NAD+
NADH
NADH
NAD+
NAD+
NADH
CoA-SH
Acetyl-CoA
PDH complex
CO2
NADH
CoA-SH
NAD+
Pyruvate decarboxylase
CO2
α-ALS
(x2)
α-acetolactate
CO2
diacetyl
DAR
NADH
NAD+
Acetoin
BDH
2,3-BD
NAD+
NADH
Acetaldehyde
NAD(P)H
NAD(P)+
Acetic acid
NAD(P)H
NAD(P)+
Acetaldehyde
Alcohol dehydrogenase
NADH
NAD+
Ethanol
Acetaldehyde
ATP
D-Glucose in
Facilitator
D-Glucose out
Symporter

Figure 3-9 The proposed metabolic pathway including glycolysis, pentose phosphate pathway, polyol accumulation, and a simplified tricarbolic cycle in a native pentose-fermenting yesat.

α-ALS, α-acetolactate synthase; ADH, alcohol dehydrogenase; BDH, butanediol dehydrogenase; DAR, diacetyl reductase; XR, xylose reductase; XDH, xylitol dehydrogenase; XKS, xylulose kinase; *ARDH* (arabitol-2-dehydrogenase); *ENO1*, enolase (2-phosphoglycerate dehydratase); *GND1*, 6-phosphogluconate dehydrogenase; *GPM*, phosphoglycerate mutase; *PGK1*, phosphoglycerate kinaes; *PYK1*, pyruvate kinase; *RPE1*, D-ribulose-5-phosphate 3-epimerase; *RPI1*, ribose-5-phophate isomerase ; *TDH*, glceraldehyde-3-phosphate dehydrogenase; *ZWF1*, glucose-6-phosphate dehyrogenase; *Mito.*, mitochodria; the arrow with black dashline indicates a spontaneous reaction.

and once formed, they were not readily re-assimilated under low aeration conditions. By comparison, acetic acid accumulation increased with increasing aeration and cell growth. The highest accumulations by both yeasts were from glucose at an OTR of 4.73 mmol O_2/L·h. Increasing aeration increased cell yield and increased the demand for NADPH, which could be met by making more acetic acid.

Interestingly, *S. passalidarum* accumulated more D-arabitol than ribitol during xylose or glucose fermentation; but *S. stipitis* accumulated much more ribitol than D-arabitol during xylose or mixed sugar (12% xylose, 3% glucose) fermentation. One possible explanation could be a difference in how the two yeasts handle the cofactor imbalance created in the conversion of xylose to xylulose. The xylose reductase activity of *S. passalidarum* shows a higher affinity for NADH than seen with *S. stipitis* (Hou 2012). This could result in a higher flux for xylose assimilation under low aeration conditions. A less efficient glycolytic flux in *S. stipitis* NRRL Y-7124 could lead to greater depletion in NAD^+ in the cytosol (due to lower recycling of NAD^+ by reduction of acetaldehyde to ethanol). This would result in greater conversion of arabitol to ribitol by an oxidoreducatase to regenerate NAD^+. During glucose fermentation, *S. passalidarum* accumulated about 80% of polyols derived from pyruvate while *S. stipitis* only generated about 40% of non-pentitol polyols (Figure 3-9). If the hypothesis of more ATP being generated with glucose compared to xylose is correct, this observation suggests a more active glycolysis with xylose in *S. passalidarum* than in *S. stipitis*. We grew *S. passsalidarum* NN245 in minimal defined medium [medium composition based on (Verduyn et al. 1992)] in an anaerobic chemostat fitted with a copper oxygen scavenger, and observed only xylitol and ribitol (no arabitol) accumulation as major byproducts during fermentation of 5% xylose (data not shown).

This implies that under strict anaerobic conditions, *S. passalidarum* was also forced to further convert arabitol to ribitol.

S. cerevisiae exhibited a ratio of *R,R*-2,3 BD to *meso*-2,3 BD around 2 (Gonzalez et al. 2000) while *S. passalidarum* exhibited a wide range of ratios (0.9 to 5.6) depending on substrates and aeration while the ratio of *R,*R-2,3 BD to *meso*-2,3 BD from *S. stipitis* was only about 0.3 to 0.5. These differences in major byproduct formation during fermentation (Table 3-3) between *S. passalidarum* and *S. stipitis* could also be attributed to the more efficient glycolysis pathway of *S. passalidarum* than that of *S. stipitis*. We examined the literature and the genomes of *S. passalidarum* and *S. stipitis* to identify genes that might encode enzymes that could carry out the conversion of aldo-ketones to polyols. $NAD(P)^+$-dependent cytoplasmic polyol dehydrogenases catalyzing reversible reactions between polyols and aldo-ketones have been intensively investigated in acetic acid bacteria (Cheng et al. 2005) but not in fungi. The first D-arabitol dehydrogenase characterized in fungi is Ard1 (Accession no. P43066), a D-arabitol-2-dehydrogenase (ribulose-forming) from *Candida albicans*, which produces large amounts of D-arabitol from glucose (Wong et al. 1993). A short-chain D-arabitol dehydrogenase in *S. stipitis* (*SsARDH*) with 84% identity to Ard1 of *C. albicans* was reported (Hallborn et al. 1995). Related research indicates these genes reversibly convert D-arabitol to D-ribulose (Murray et al. 1995). A homologue to *Ss*ARDH in *S. passalidarum* (Accession no. EGW30261) with 85.6% similarity can be found through UniProt (http://www.uniprot.org). The ARDH found in *S. passalidarum* could also responsible for the conversion between D-arabitol to D-ribulose. However, the gene or protein responsible for conversion between D-xylulose to D-arabitol (D-arabitol-4-dehydrogenase) has not yet been identified. A ^{13}C nuclear magnetic resonance studies in *C. albicans* suggested the *Ard1* protein may only be responsible for D-arabitol uptake because while

an *ard* null mutant of *C. albicans* is unable to utilize D-arabitol, it can still synthesize D-arabitol from glucose via the same pathway compared to its wild-type parent (Wong et al. 1995).

Recently, a versatile $NADP^+$-dependent D-arabitol dehydrogenase (Ard1p) capable of reversibly catalyzing the reaction from arabitol to either ribulose or xylulose and mannitol into fructose was isolated from a plant pathogenic fungus *Uromyces fabae* (Link et al. 2005). A BLAST analysis of Ard1p on the database finds sorbitol dehydrogenases (SOR) in *S. passalidarum* (24.8% identity) and *S. stipitis* (23.8%) instead of the two short-chain arabitol dehydrogenases from both yeasts. In addition, the two sorbitol dehydrogenases from both *S. passalidarum* and *S. stipitis* along with Ard1p from *U. fabae* belong to the Zn^{2+} alcohol dehydrogenase family with $NAD(P)^+$ binding site. Phylogenic analyses also suggest that Ard1p is more closely related to the *S. passalidarum* and *S. stipitis* sorbitol dehydrogenases than to the D-arabitol-2-dehydrogenases in *S. passalidarum* and *S. stipitis* (Fig 3-10). These finding suggest the possibility of an oxidoreductase in yeast using NAD^+ as a cofactor and converting D-xylulose into D-arabitol, which was also stated in other literature (Cheng et al. 2009, Wong et al. 1995). Two butanediol dehydrogenases from *S. cerevisiae* (Bdh1 and Bdh2) have already been confirmed and characterized (Gonzalez et al. 2000) and possible candidate butanediol dehydrogenase in *S. passalidarum* and *S. stipitis* can also be found by BLAST of their genomes database with *S. cerevisiae* in sequence database (NCBI access number EGW32915 and 34210 for *S. passalidarum*, and XP_001385027 and XP_001386484 for *S. stipitis*). These provide targets for further investigation of polyol accumulation by pentose fermenting yeasts, and may help to elucidate the key differences in hexose and pentose metabolism that lead to differential

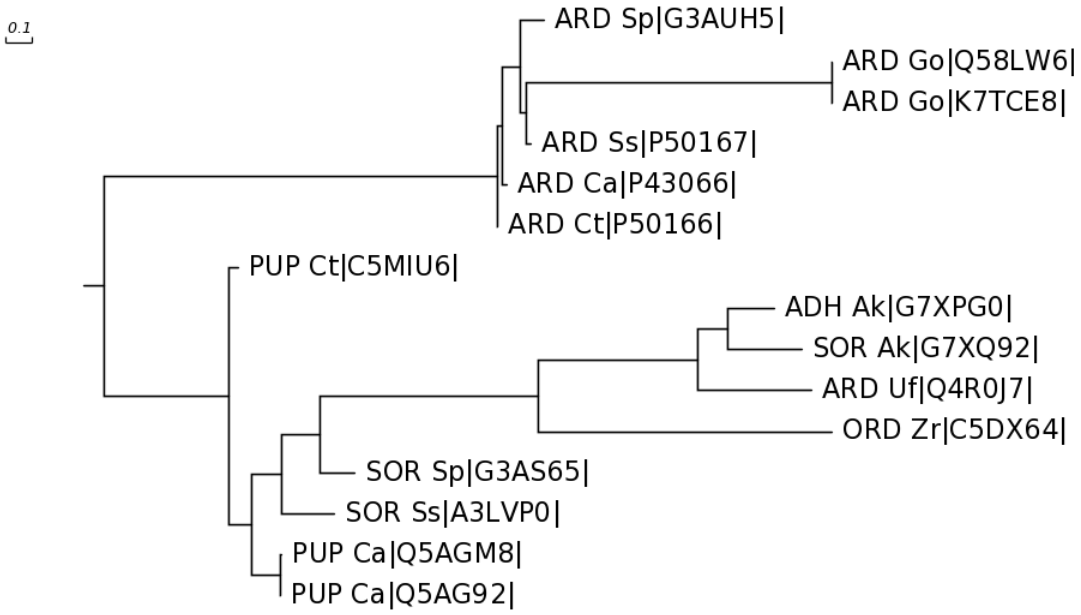

Figure 3-10 Phylogenetic analysis of D-arabitol dehydrogenase and sorbitol dehydrogenase among *Aspergillus kawachii* (Ak), *Candida albicans* (Ca), *C. tropicalis* (Ct), *G. oxydans* (Go), *S. passalidarum* (Sp), *S. stipitis* (Ss), *Uromyces fabae* (Uf), and *Zygosaccharomyces rouxii* (Zr) by using ClustalW2. Nomenclature: protein name species name|6 digit Uniprot access number|; for example, ARD_Ca|P43066| is the arabitol dehydrogenase from *Candida albicans* with Uniport accession no P43066. ADH, alcohol dehydrogenase; ARD, arabitol dehydrogenase; ORD, oxidoreductase; SOR, sorbitol dehydrogenase. PUP = putative uncharacterized protein. Protein phylogeny analysis was carried out by using ProbCons for alignment and PhyML *v3.0 aLRT* with bootstrap number 100 on Phylogency.fr website (http://www.phylogeny.fr/).

byproduct formation in *S. passalidarum* and *S. stipitis* under a range of aeration conditions. A recent publication by Kim et al. (*In press*) successfully introduced a xylose assimilation pathway (*XYL1*, *XYL2*, and *XYL*3) from *S. stipitis* into a pyruvate decarboxylase deficient *S. cerevisiae* to increase the flux into 2,3-BD accumulation. The mutant *S. cerevisiae* produced *R,R*-2,3-BD dominantly (>97%). For *S. passalidarum*, *R,R*-2,3-BD, the second highest byproduct during xylose fermentation and the highest byproduct during glucose fermentation at lower aeration (2.47-3.65 mmol O_2/L·h) suggests *S. passalidarum* could be a promising native pentose-fermenting yeast for metabolic engineering for 2,3-butanediol fermentation.

3.6 Conclusion

Our findings, together with data from our previous studies, confirm the preferential xylose fermentation by *S. passalidarum*, and more importantly, the specific fermentation rate is a least 1.5-fold higher on xylose than on glucose in flask-scale. A q_{OUR} of 0.33 to 0.39 mmol O_2/ g CDW·h for *S. passalidarum* was shown when OTR ranged from 2.5 to 4.7 mmol O_2/L·h with 150 g/L of xylose. Based on the fermentation kinetic data presented above our findings suggest that, under the oxygen transfer rates applied in this study, *S. passalidarum* could be a better native xylose-fermenting yeast than *S. stipitis*. *S. passalidarum* produced a smaller proportion of pentitols (20-50%) in the total polyols accumulated and higher 2,3-BDs compared to *S. stipitis,* suggesting a higher glycolytic flux in *S. passalidarum.*

3.7 Acknowledgements

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3.8 Nomenclature

BD	Butanediol
CDW	Cell dry weight, g/L
OTR	Oxygen transfer rate, mmol O_2/L·h
PPP	Pentose phosphate pathway
Q_E	Ethanol productivity, g ethanol/L
q_E	Specific ethanol productivity, g ethanol/g CDW·h
qO_2	Specific oxygen uptake rate, mmol O_2/g CDW·h
RPPY	Ratio of pentitol yield to polyol yield
$Y_{E/S}$	Ethanol yield, g/g
$Y_{X/S}$	Cell yield, g/g

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Chapter IV

Characterization of selected *Spathaspora passalidarum* adapted to AFEX hydrolysate by repetitive batch transfer

The main content of this chapter will be submitted to peer-reviewed journal.

4.1 Abstract

One of major barriers to commercialize lignocellulosic ethanol production is to overcome the toxicity in pretreated hydrolysates. Degradation products present in hydrolysates inhibit cellular metabolic activity, decrease fermentation rate, and diminish product yield. One strategy to surmount this problem is to develop robust microbes able to thrive in hydrolysates and form products efficiently. Directed evolution and empirical strain development have proven successful in solving these sorts of difficulties. In our study, we used evolutionary engineering to select for resistant strains of the native xylose fermenting yeast *Spathaspora passalidarum* NN245 (NRRL Y-27907). Cells were repeatedly exposed to inhibitors by recycling and mating different adapted strains. We selected promising isolates from these evolved heterogeneous mixtures by direct plating, isolation and high throughput screening. The selected candidate, YK208-E11 showed a 3-fold increase in specific fermentation rate compared to the parental strain and an ethanol yield above 0.45 g per gram substrate by co-utilizing cellobiose, glucose, and xylose. Further characterization of YK208-E11 showed that the strain also makes 20-40% less biomass compared to the parental strain when cultivated in YP medium under fully aerobic conditions. We employed tetrazolium agar overlay in the presence of respiration inhibitors, including rotenone, antimycin A, KCN, and salicylhydroxamic acid (SHAM) to elucidate nature of the mutational events. The results indicated that YK208-E11 has a deficiency in its respiration system that contributes to its low cell yield and higher specific fermentation rate.

Keywords: *Spathaspora passalidarum*, lignocellulosic fermentation, adaptation, evolutionary engineering, tetrazolium chloride, respiration inhibitor.

4.2 Introduction

Spathaspora passalidarum NN245 (Nguyen et al. 2006) is an unusual yeast that shows a high capacity for fermenting xylose, cellobiose and glucose as pure sugars and from hydrolysates of lignocellulose (Hou and Yao 2012, Long et al. 2012). The *Spathaspora* genus (Cadete et al. 2013, Cadete et al. 2009) is closely related to the *Scheffersomyces* clade (Urbina and Blackwell 2012) and shares with it a proclivity for living in the guts of wood-boring beetles of the family *Passalidae*, which is associated with decaying wood (Urbina et al. 2013a, Urbina et al. 2013b). In the longhorn beetle (family *Cerambycidae*), which attack trees and also live in decaying wood, yeasts colonize specialized organs (mycetomes) that are found near the top of the mid-gut (Grunwald et al. 2010, Urbina et al. 2013a). It is not clear that Passalid beetles have similar structures, but yeasts that have evolved in association with wood boring beetles have acquired the capacities for fermenting a wide range of sugars and metabolizing lignin-related compounds found in cellulosic materials.

The physiology of *S. passalidarum* is still poorly understood, even though its genome has been sequenced (Wohlbach et al. 2011). One of its more unusual features is that it appears to be capable of growth with very little oxygen (Hou 2012), and it shows less cell growth (about 50% less) and higher specific rates of ethanol production on xylose than on glucose (Long et al. 2012, Su et al. 2014). Its high capacity for co-fermenting xylose, cellobiose and glucose (Long et al. 2012) makes it a potentially useful for bioconversion; however, its growth is inhibited by weak organic acids (e.g., acetic acid, levulinic acid, and formic acid), and to a lesser extent by 5-hydroxyl methylfurfural (HMF), furfural and lignin degradation moieties found in lignocellulosic hydrolysates (Hou and Yao 2012). Aldehydes such as HMF and furfural are generated by

dehydration of hexose or pentose during pretreatment of cellulosic biomass. The reactive aldehyde functional groups of furfural and HMF are thought to induce the accumulation of reactive oxygen species (ROS) (Allen et al. 2010), and the ROS in turn lead to mutations, inactivate enzymes and disrupt membrane integrity (Yasokawa and Iwahashi 2010). HMF and furfural cause an extended lag phase, and yeasts detoxify HMF and furfural by reducing the aldehyde to an alcohol by aldehyde reductase (Liu et al. 2009).

Acetic acid is released by hydrolyzing the acetyl side chain on the xylan backbone while levulinic acid and formic acid are generated by further dehydrating HMF and furfural (Klinke et al. 2004). Acetic acid can also be formed by acetamide with aqueous acetic acid (Martin 1965). When the pH is low (< 5), protonated weak acids are able to pass through the cell membrane and dissociate in the cytosol, which leads to intracellular anion accumulation and an increase in H^+ concentration in the cytoplasm (Palmqvist and Hahn-Hagerdal 2000). Intracellular acidification decreases metabolic activity, reduces the rate of biosynthesis and diminishes nutrient uptake by reducing the proton gradient across the cytoplasmic membrane. To maintain pH homeostasis in the cytoplasm, the H^+-ATPase hydrolyzes ATP to extrude protons. At the same time, multidrug resistance transporters detoxify weak acids by pumping the anionic form out of the cell (Mira et al. 2010). Because the concentration of protonated acid increases at lower pH, the inhibitory concentration varies with the pH during fermentation. Detoxification of weak acids requires ATP, and weak organic acids generally reduce cell growth and inhibit carbohydrate metabolism.

Studies have shown that acetic acid also hinders xylose uptake in *Saccharomyces cerevisiae* engineered for xylose fermentation and that inhibition is more severe for xylose consumption than for glucose utilization (Casey et al. 2010). A recent study demonstrated that acetic acid inhibits the non-oxidative pentose phosphate pathway, and overexpression of

transaldolase in xylose-fermenting *S. cerevisiae* was able to increase ethanol production from xylose 1.7-fold in the presence of 30 mM acetic acid as compared to a control strain (Hasunuma et al. 2011). While acetic acid is probably the single most important inhibitor in hydrolysates, other lignocellulose degradation products are also of concern.

Phenolic compounds such as 4-hydroxybenzaldehyde, 4-hydroxybezoic acid, vanillin, and *p*-coumaric acid come from partial degradation of lignocellulose (Klinke et al. 2004). Because phenolic compounds are lipophilic, they can easily enter the cell membrane, which increases the membrane permeability and finally causes the loss of membrane integrity. Notably, *p*-coumaric acid and ferulic acid, which are generated by breaking down arabinoxylans, contain an unsaturated side chain, and even a small amount (≈ 5 mM) can severely inhibit *S. cerevisiae* during glucose fermentation (Larsson 2000). These and other toxins in hydrolysates can be removed by over liming (Huang et al. 2009), anion exchange, charcoal adsorption (Stoutenburg et al. 2011) or by oxidation with laccase (Jonsson et al. 1998). These processes, however, are expensive and add greatly to the cost of sugar production. Selection of appropriate pretreatment processes can reduce the need for subsequent hydrolysate detoxification. The ammonia fiber expansion (AFEX) pretreatment process uses aqueous ammonia to deacetylate hemicellulose and to disrupt the ester linkages between hemicellulose and lignin. This increases the hydrophilicity of the substrate, increases porosity, and allows greater enzyme access (Vismeh et al. 2013). In the process organic acids such as *p*-coumaric and ferulic are aminated by converting carboxylic groups are into amides. These modifications reduce the toxicity of the treated material (Chundawat et al. 2010)

Another cost effective means to overcome toxicity is to engineer or adapt the fermentative cells to the pretreated hydrolysate. This can be accomplished by selecting for

resistance to individual components such as acetic acid or furfural (Nilsson et al. 2005) or by serial selection against successively higher concentrations of hydrolysate (Stoutenburg et al. 2011). Even though it is generally easier to select for resistance to individual components, the difficulty in knowing which are the most important or the possibility of synergistic toxicities arising from multiple inhibitors can favor direct selection for resistance to successively higher concentrations of diluted hydrolysate.

The overall aim of our study was to identify yeasts that would resist a wide range of inhibitors from various feedstocks and pretreatment processes. We hypothesized that the major inhibitors such as acetic acid, furfural and HMF would be present in most hydrolysates, albeit at various concentrations, and that selecting for resistance to one hydrolysate would help confer resistance to a hydrolysate with a different origin. We further hypothesized that by combining traits from separately selected and adapted populations, we would be able to identify isolated strains with favorable resistance traits over a wide range of hydrolysates. In this process, we focused on the use of enzymatic hydrolysates from AFEX-pretreated corn stover because when properly prepared, it showed less inhibition than hydrolysates from most other methodologies.

4.3 Materials and Methods

4.3.1 Yeast strains, evolutionary engineering, and batch adaptation

The wild type (WT) strain *Spathaspora passalidarum* NN245 (NRRL Y-27907) was used as the parental strain for the adaptation process. During adaptation, both the parental and evolved strains were stored in 15% sterile glycerol at -80°C. During the adaptation, the parental and evolved strain were recovered from the frozen stocks and streaked on a fresh plate before inoculation to ensure that any differences in performance was the result from adaptation to the applied specific hydrolysate. Figure 4-1 presents the adaptation process used in this study, and a detailed list of evolved strains with description is showed at Table 4-1. Wild type NN245 was first adapted to mixed southern hardwood (MSH) for two passages to obtain YK101.2 (11.6 generation), and YK101.2 was adapted with two different routes to obtain two descendent strains YK102.12 (12 passages, 46.9 generations) and YK103.33 (33 passaged, 139.6 generations). YK105 was obtained by recycling four different adapted strains, which have been adapted to 4 different hydrolysates. These four hydrolysates included MSH hydrolysate, loblolly pine (LP) hydrolysate, maple hydrolysate (MA) with additional acetic acid, and AFEX-pretreated corn stover (AFCS) hydrolysate.

Following batch adaptions in shake flasks, about 40 ml of cell suspensions from YK101.2, YK102.2, YK103.10, and YK103.19 were each transferred into a 500-ml flask containing 150 ml of AFCS hydrolysate. The final volume of mixed culture was around 300 ml. It was maintained at 30°C without shaking for more than 3 months to avoid excess aeration into the system. The stationary incubation was used to select adapted strains capable of growing under very limited oxygen. Plating on ScX agar was used to test cell viability. Plating on YPD agar and microscopic

inspection were used to check for possible contamination during adaption. During the adaption process, the hydrolysate was supplemented with urea, phosphate buffer, trace metal and vitamin solutions as previous described (Long et al. 2012).

The second batch adaptation was carried out with candidate strains selected from high throughput screening. A full loop of selected strains YK206-D11, YK206-F2, and YK208-E11 from high throughput screening was transferred from fresh AFCS plates into 4% HYPX broth in a 125-ml flask and cultured at 30°C, 200 rpm. Appropriate amounts of overnight cultures (OD ≈ 25) then were collected and transferred into 125 ml flasks containing 50 ml of AFCS hydrolysate to reach an OD of 5. The initial pH in the AFCS hydrolysate was 5. Daily sampling (0.8 ml) was withdrawn to monitor cell density, and extracellular metabolites. Cells were directly transferred to subsequent shake flasks when the maximum ethanol concentration was attained, which usually occurred in about 6 to 7 days. 1% YP (100 μL of 5x YP), which gave a final concentration of 0.01 g/L yeast extract and 0.02 g/L peptone, was supplied in the subsequent flasks to provide enough mineral and vitamins for cell division and detoxification of AFCS hydrolysate during adaptation.

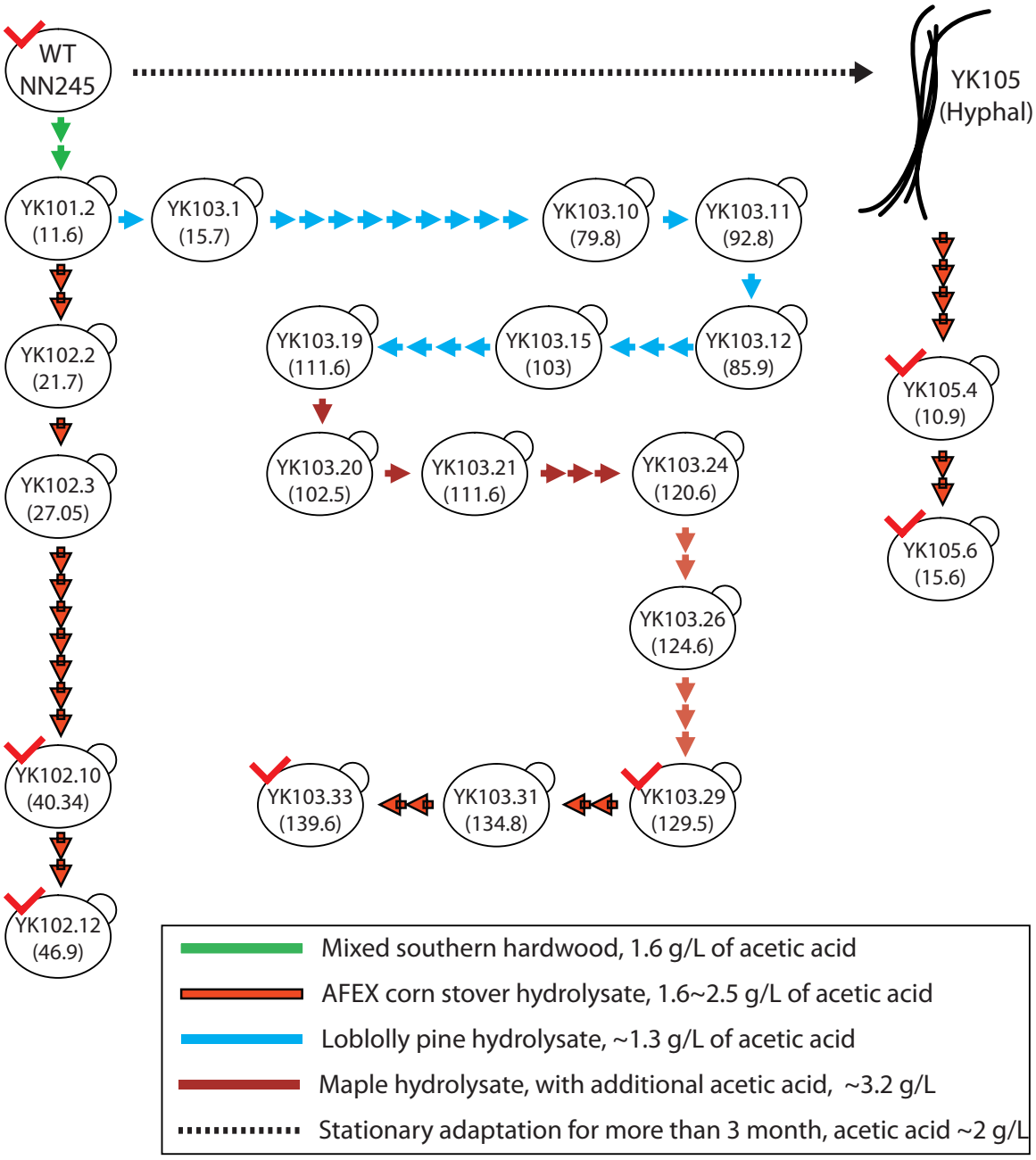

Figure 4-1 A lineage map of adapting *Spathaspora passalidarum* in this study. The number under the strain ID with ( ) indicates the total number of generations. Strains with check mark indicate the stains were selected for shake flask benchmark 1. DP = double phosphate buffer.

Table 4-1 The list of adapted *S. passalidarum* strains used in this study

Strain ID	Hydrolysate Used[a]	Description	Cell Generations[b]	Used for
YK101.2	MSH	Cultivated wild type (WT) parental strain NN245 in MSH hydrolysate for 2 rounds	11.6	Adaptation
YK102.2	MSH → AFCS	From YK101.2, cultivated in AFCS hydrolysate for 2 rounds	21.7	Adaptation
YK102.10	MSH → AFCS	From YK101.2, cultivated in AFCS hydrolysate for 10 passages	40.34	Benchmark 1
YK102.12	MSH → AFCS	From YK101.2, cultivated in AFCS hydrolysate for 12 passages	46.9	Benchmark 1
YK103.10	MSH → LP	From YK101.2, cultivated in LP hydrolysate for 12 passages	79.8	Adaptation
YK103.19	MSH → LP	From YK101.2, cultivated in LP hydrolysate for 19 passages	111.6	Adaptation
YK103.29	MSH → LP → MA	From YK101.2, cultivated in LP hydrolysate for 20 passaged then MA hydrolysate for 10 passages	129.5	Benchmark 1
YK103.33	MSH → LP → MA →AFCS	From YK103.29, cultivated in AFCS hydrolysate for 4 passages	139.6	Benchmark 1
YK105 (Hyphal)	Mixed→ AFCS	Recycling YK101.2, YK102.2, and YK103.10, and YK103.19 and stationarily cultivated in AFCS hydrolysate for more than 3 months	NA[c]	Adaptation
YK105.4	Mixed→ AFCS	From YK105, cultivated in AFCS hydrolysate for 4 passages	10.9	Benchmark 1
YK105.6	Mixed→ AFCS	From YK105, cultivated in AFCS hydrolysate for 6 passages	15.6	Benchmark 1

[a] MSH, mixed southern hardwood; LP, loblolly pine; AFCS, AFEX pretreated corn stover hydrolysate

[b] One generation equals to one doubling

[c] NA, Not applicable

Table 4-1 (cont.) The list of adapted *S. passalidarum* strains used in this study

Strain ID	Hydrolysate Used[a]	Description	Cell Generations[b]	Used for
YK206-D11	AFCS	From HT screening of YK105.6 population and cultivated in AFCS hydrolysate for 3 passages	11.96	Benchmark 2
YK206-F2	AFCS	From HT screening of YK105.6 population and cultivated in AFCS hydrolysate for 3 passages	12.12	Benchmark 2
YK208-E11	AFCS	From HT screening of YK105.6 population and cultivated in AFCS hydrolysate for 3 passages	11.49	Benchmark 2

[a] MSH, mixed southern hardwood; LP, loblolly pine; AFCS, AFEX pretreated corn stover hydrolysate

[b] One generation equals to one doubling

4.3.2 Hydrolysate preparation and hydrolysate plates

MSH, MA and LP hydrolysates were generously provided by SUNY-ESF (Syracuse, NY). MSH and MA hydrolysates were prepared using hot water extraction followed by two-stage membrane filtration and dilute acid hydrolysis (Hu et al. 2010). LP hydrolysate was prepared by pretreating with oxalic acid (Li et al. 2011). AFEX pretreated hydrolysate was generated from corn stover and provided by DOE Great Lakes Bioenergy Research Center (Madison, WI). The solid loading for enzymatic hydrolysis was 15.8%, which is equal to 6% (w/w) solid loading. After 96-h of enzymatic hydrolysis, the hydrolysate was centrifuged and filter sterilized. The final AFCS hydrolysate contains around 60 g/L of glucose and 30 g/L of xylose with a final pH of 4.8. Jin et al. (2013) has published a detailed description describing the preparation of AFEX hydrolysate.

To shorten the time during high throughput screening, the acetic acid in AFCS hydrolysate was removed with the combination of reverse osmosis and diafiltration (AFCS-RO) by using the similar method describe by (Lee et al. 2011). The acetic acid concentration in AFCS-RO was ≈ 0.9 g/L. AFCS hydrolysate plate was used to obtain individual colonies for high throughput screening. AFCS hydrolysate plate contained 50% (v/v) of AFCS hydrolysate with 2% agar.

4.3.3 Medium

YPD or YPX agar contained (g/L): glucose or xylose, 20; agar, 20, yeast extract; 10 and peptone, 20. YPD (4%) and YPX (4%) liquid broths were prepared by adding appropriate amounts of autoclaved 40% of glucose (dextrose) or xylose and sterile water into autoclaved 5x YP stock solution to the final concentration (g/L) of: yeast extract, 10; peptone, 20; glucose or xylose, 40.

Since the inoculation in high throughput screening was performed by directly transferring cultures from a 96-well deep block, to avoid the excess amount of nutrients in the initial growth phase during high throughput screening with AFCS-RO hydrolysate. YPX (4% xylose) medium with half amounts of yeast extract and peptone (YPXH) was used. YPXH was also used to grow the precultures for repetitive shake flask adaptation of YK206-E11, YK206-F2, and YK208-E11. Synthetic (Sc) minimal liquid broth contains a final concentration of 6.7 g/L of yeast nitrogen base with either 20 g/L of glucose (ScD) or 20 g/L of xylose (ScX). For Sc medium agar plateplates, a final concentration of 2% agar was used.

Stock solutions of respiration inhibitors were prepared individually and filter sterilized with following concentration: rotenone (ROT), 50 mM; antimycin A (AA), 5 mM; KCN, 5M, and salicylhydroxamic acid (SHAM), 1.3 M. Prior to plating, respiration inhibitors were diluted to following concentration: 50 mM, 0.5 mM, 0.5 M, and 0.2 M, respectively and 75µL of each respiration inhibitor was applied on the plate. If we assume only 5 ml from each plate was perfused after applying inhibitors, the final concentration (mM) applied in this experimenton each plate would be: ROT, 75 µM; AA, 7.5 µM; KCN, 7.5 mM; SHAM, 3 mM.

For tetrazolium agar, a 50x stock solution of 2,3,5-Triphenyl-2H-tetrazolium chloride (TTC) was prepared by dissolving 2.5 g of TTC in 50 ml double distilled waster and filter sterilized. A 1.5% agar containing 0.1% tetrazolium chloride, 20 g/L of glucose (or xylose) and 50 mM phosphate buffer (pH 7) was use for overlaying (Ogur et al. 1957) .

4.3.4 High throughput screening of adapted population

We used high throughput screening to select possible candidates from the adapted population YK105.6. Cells of YK105.6 from shake flask benchmark 1 plated on five AFCS hydrolysate plates yielded 10 to 20 single isolated colonies on each plate. About 12 isolated

colonies from each AFCS hydrolysate plate were selected yielding a total of 58 isolated colonies, which were transferred into a 96-deep well block (NUNC) containing 500 µL of 4% YPXH from row B to G and row 2 to 11. Wells B6 and G11 were not inoculated as they served checks for contamination and the outer wells (row A and H, column 1 to 12) were filled with 500 µl of sterile H_2O to control the evaporation. The deep well block then was sealed with breathable tape (Axygen) and incubated at 30°C, 225 rpm. After 48 h, 10 µl of cell suspension from well B2 to G11 in the deep well block were directly transferred into a standard 96-well plate (NUNC) with a lid cover containing 190 µL of AFCS-RO in each well from row B to G and column 2 to 11. Wells B6 and G11 were not inoculated and outer wells were filled with sterile water for evaporation control. The inoculated 96-well plates were placed in Tecan F500 or M1000 mutimode plate readers. The plates were shaken for 2 min out of every 10 with an amplitude of 2 mm. Cell density was measured for 24 to 48 h at 595 nm immediately following each 2-min shaking. Seven rounds of screening with AFCS-RO and one control screening with 4 % YPX were carried out to confirm the screening result. Both plotting the data by Excel and analyzing growth rates using an automated analysis (GCAT, https://gcat.glbrc.org) were performed to evaluate the results. Promising candidate strains were streaked on AFCS hydrolysate plates and incubated at 30°C. Glycerol stocks were prepared by transferring 100 µL of cell suspension from 48-h grown 96-well deep plate into 96-well plates containing 100 µL of 2% YPD (yeast extract, 10g/L; peptone, 20g/L; dextrose, 20 g/L) supplemented with 30% v/v glycerol. The glycerol stock plates were stored at -80°C.

4.3.5 Shake flask benchmarking

Two shake flask screenings were performed in this study. The first compared the abilities of adapted strains to ferment AFCS hydrolysate. These strains had been adapted to different

hydrolysates over various durations (Table 1). The second experiment was conducted with adapted strains YK206-D11, YK206-F2, YK208-E1, which were selected from high throughput screening of the YK105.6 population and adapted with AFCS hydrolysate for an additional one month. Cells were grown on 4% YPX overnight (OD ≈ 30), and an adequate amount of cells were collected and spun-down at 3000 rpm for 3 min. The cell pellet was suspended in sterile double distilled H_2O and inoculated into a 125-ml Erlenmeyer containing 50 ml of AFCS hydrolysate.

Nutrients such as nitrogen, mineral solution and vitamins with concentrations mentioned previously were provided in the first benchmark experiment at pH 5 (Long et al. 2012). For the second benchmark experiment, 100 µL of 5x YP, which gave a final concentration of 1% YP (v/v), was provided, and the initial pH was increased to 6. The targeting initial OD for benchmark 1 and 2 were 7 and 3, respectively, which corresponds to ≈ 1 and 0.4 g CDW/L. Samples (0.8 ml) were withdrawn daily to monitor cell density, and extracellular metabolites.

4.3.6 Analysis of metabolites and cell dry weight

Cell optical density was measured with an Agilent 8453 spectrophotometer at 600 nm (OD_{600}). To determine yeast cell dry weight (CDW) during hydrolysate fermentation, cells growing in AFCS hydrolysate (2 to 10 ml) were collected and filtered through a 0.2 µm membrane based on the protocol developed by Postma *et al*. (1989). 1 OD unit = 0.1416 g CDW/L in hydrolysate. For the aerobic growth experiment with YP medium, 1 OD unit = 0.16 g CDW/L. Extracellular metabolites including arabitol, xylitol, ribitol, and glycerol were determined by high performance liquid chromatography (HPLC) using the method described previously (Su et al. 2014).

4.3.7 Drop test and tetrazolium overlay with respiration inhibitors

Drop tests were executed with YK206-D11, YK206-F2, YK208-E11, and parental strain NN245 on ScD, ScX and ScX supplied with lignotoxin (ScX+LT), which was kindly provide by Dr. Yaoping Zhang (DOE GLBRC). Cells were cultured overnight with 2% YPD or 2% YPX at 200 rpm, 30°C. Four ten-fold serial dilutions were carried out. The top row had a cell density (OD_{600}) of 0.2. Cell suspension (3 μL) from each dilution was placed on each plate and incubated at 30°C until sizable colonies could be evaluated visually.

For the tetrazolium overlay, yeast cells grown in ScD broth were plated on ScD agar, and inocula cultured on ScX broth were plated on ScX agar. To insure the cells used for plating were in vigorous aerobic growth phase, a two-stage inoculation preparation was used. A small seed inoculum was cultured at tilted (45 degree) culture tubes containing 3 ml of ScD broth shaking at 225 rpm, 30°C for 6-8 h. Then, adequate amounts of each cell suspension was transferred into 125-ml Erlenmeyer flasks containing 25 ml of ScD to reach initial OD_{600} ~ 0.005 and shaking at 225 rpm, 30°C. Cells were used for plating when culture are in the active log phase (OD_{600} between 0.8~2). Respiration inhibitors (75 μL) were first added onto the surfaces of ScD plates separately, spread out evenly, and after the plates dried, 90 μL of yeast cells with an OD_{600} of 2×10^{-5} was plated onto each ScD plate, which yielded ~200 colonies on a control plate without respiration inhibitors. The ScD plates were incubated at 30°C until colonies had developed (2-6 days). Each plate was then gently overlaid with tetrazolium agar. Prior to overlaying, the tetrazolium agar was maintained at 45°C. Fresh plates (held not more than 14 day at 4°C since plating) were used for the tetrazolium overlay experiment. After overlaying, the plates were incubated at 30°C for 12 h (Trevors 1982). A total five conditions were tested on both strains separately, which included overlaying with each of respiration inhibitors: ROT, AA, KCN, and

the combination of AA plus SHAM (AASH). No inhibitor was added to the control plate. An experiment with ScX plates was also performed with the identical protocol mentioned here. A flowchart of tetrazolium overlay is presented in Figure 4-2. The results were documented by scanning each plate with an EPSON v350 (300dpi) scanner and analyzed by adobe photoshop measuring the sizes of the colonies in the scans using Adobe Photoshop CS5. The colony diameter was measured by using calculating the ratio of the diameter of a Petri dish in mm to the pixels measurement on the computer (85 mm =2025 pixel). Fourty colonies from each plate were measured. The *p*-values were calculated use Microsoft Excel 2011.

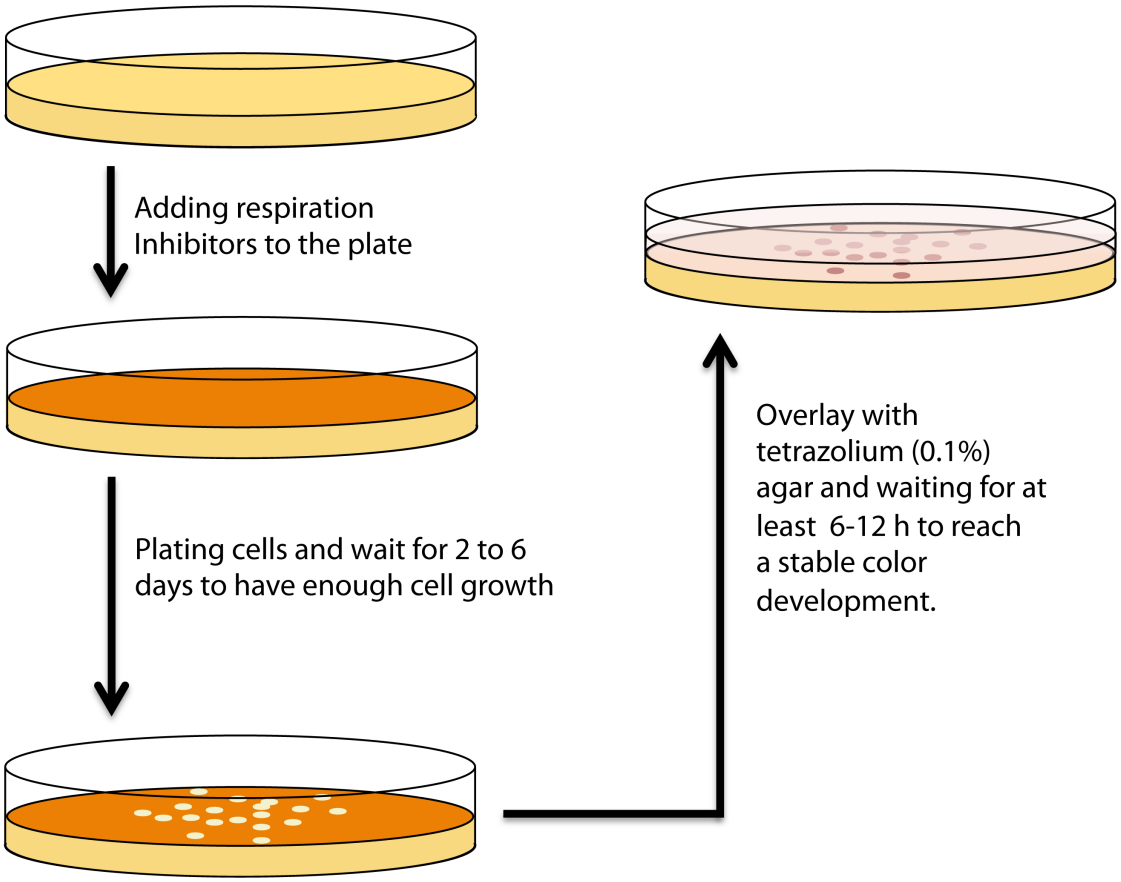

Figure 4-2 The follow chart of performing tetrazolium overlay with the parental *S. passalidarum* strain NN245 and evolved strain YK208-E11

4.4 Results

4.4.1 Adapted strain YK105 and the first benchmark

We hypothesized that growth in AFCS hydrolysate under a highly oxygen limited environment could select for an adapted strain able to ferment it. Since we had already adapted various strains to hydrolysates containing many of the same inhibitors (YK101.2, YK102.2, YK103.10 and YK103.19) by culturing them on different hydrolysates (Table 1), we decided to mix these cultures and transfer them into a stationary flask containing AFCS hydrolysate (6% glucose, 3% xylose). The flask was kept at 30°C for more than 3 months. The limited nutritional and aeration conditions induced mating as evidenced by the microscopic observation of asci (data not shown). After 3 months of adaptation in a stationary flask, the mixed culture was streaked on both YPX and YPD plates. Interestingly, the isolated colonies formed a rough surface on both YPD and YPX plates. Further inspection under microscope showed that the most abundant cell morphologies (~95%) from YPX plates had hyphal structures rather than the yeast or pseudomycelial forms previously observed. Both pseudomycelial cells and hyphal structures were found from cells cultivated on YPD plates. The hyphal cells were thinner and more elongated as compared to the pseudomycelial cells from adapted strain YK102.10. Photographs of yeast colonies from YK105 and its microscopic hyphal structures are presented in Figure 4-3.

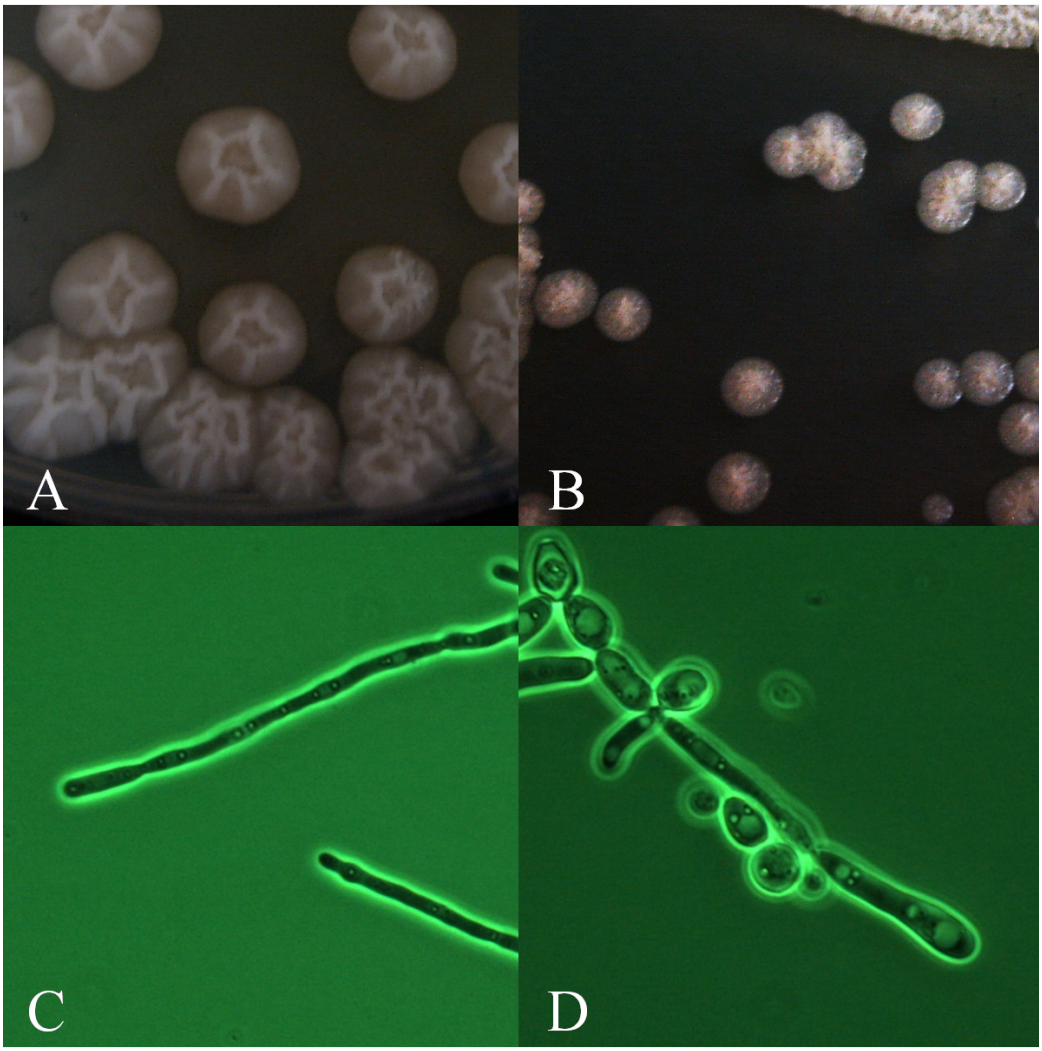

Figure 4-3 Colony morphologies and cell morphologies under microscope of filamentous adapted *Spathaspora passalidarum* strains YK105 and YK102.10 (A) Growth of YK105 on 2% YPD after 5 days. (B) Growth of YK105 on 2% YPX after 5 days. (C) Hyphal cells of strain YK105 cultured with AFCS hydrolysate (D) Pseudomycelia cells of YK102.10 cultured with AFCS hydrolysate.

A single colony from the streaked YPX plated was then streaked three successive times on YPX plates to determine whether its hyphal morphology would revert to a yeast-like phase, but it did not. The hyphal strain was subjected to D1-D2 sequencing (Kurtzman and Robnett 1998), and the product sequences were analyzed in UW-Biotech Center (Madison, WI). This segment was found to be identical to the parental *S. passalidarum*, NN245, and this hyphal strain was named YK105 (Figure 4-1). Later, YK105 was subjected to repetitive batch adaptation in AFCS hydrolysate by sub-culturing six times in shake flasks, and both strains YK105.4 (after 4 rounds of subculture) and YK105.6 (after 6 rounds) were compared with other adapted strains from various rounds of adaptation (YK102.10, YK102.12, YK103.29, YK103.33, and WT) in AFCS hydrolysate to evaluate their fermentation performance. The decimal after each strain number refers to the number of rounds of subculture in that series. The results showed that after long period of fermentation (10 days), only YK105.4, YK105.6, and NN245 (wild-type) were able to consume both glucose and xylose and produce ethanol from the hydrolysate (Figure 4-4). YK105.6 performed slightly better than YK105.4. Ethanol yields from YK105.6 and NN245 were 0.414 and 0.36, respectively. YK105.6 exhibited an ethanol productivity of 0.128 g ethanol/L·h with a peak ethanol of 32 g/L while NN245 had an ethanol productivity of 0.113 g ethanol/L·h with a peak ethanol of 28.4 g/L.

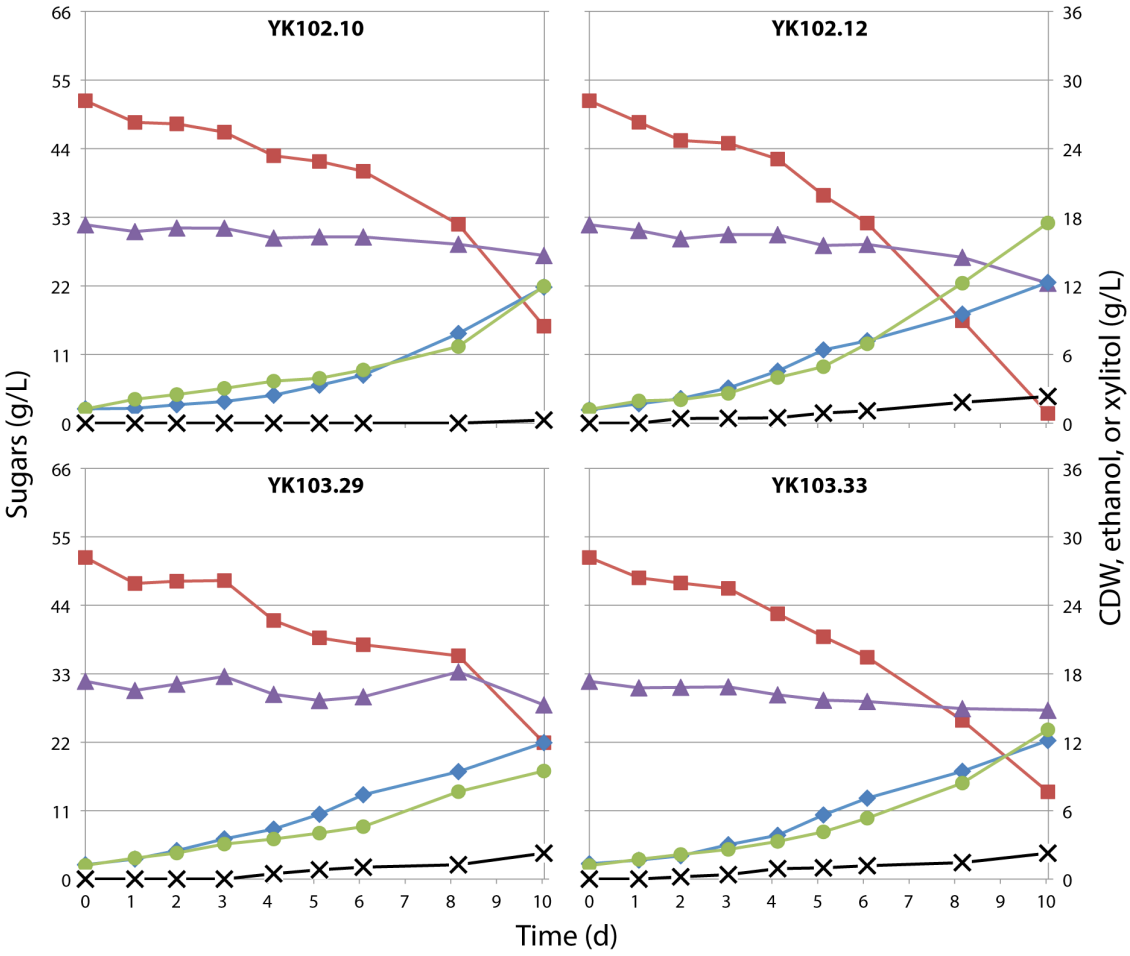

Figure 4-4 Shake flask screening of adapted *Spathaspora passalidarum* strain YK102.10, YK102.12, YK103.29, YK103.33, YK105.4, YK105.6 and wild type strain NN245 with AFCS hydrolysate. Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol.

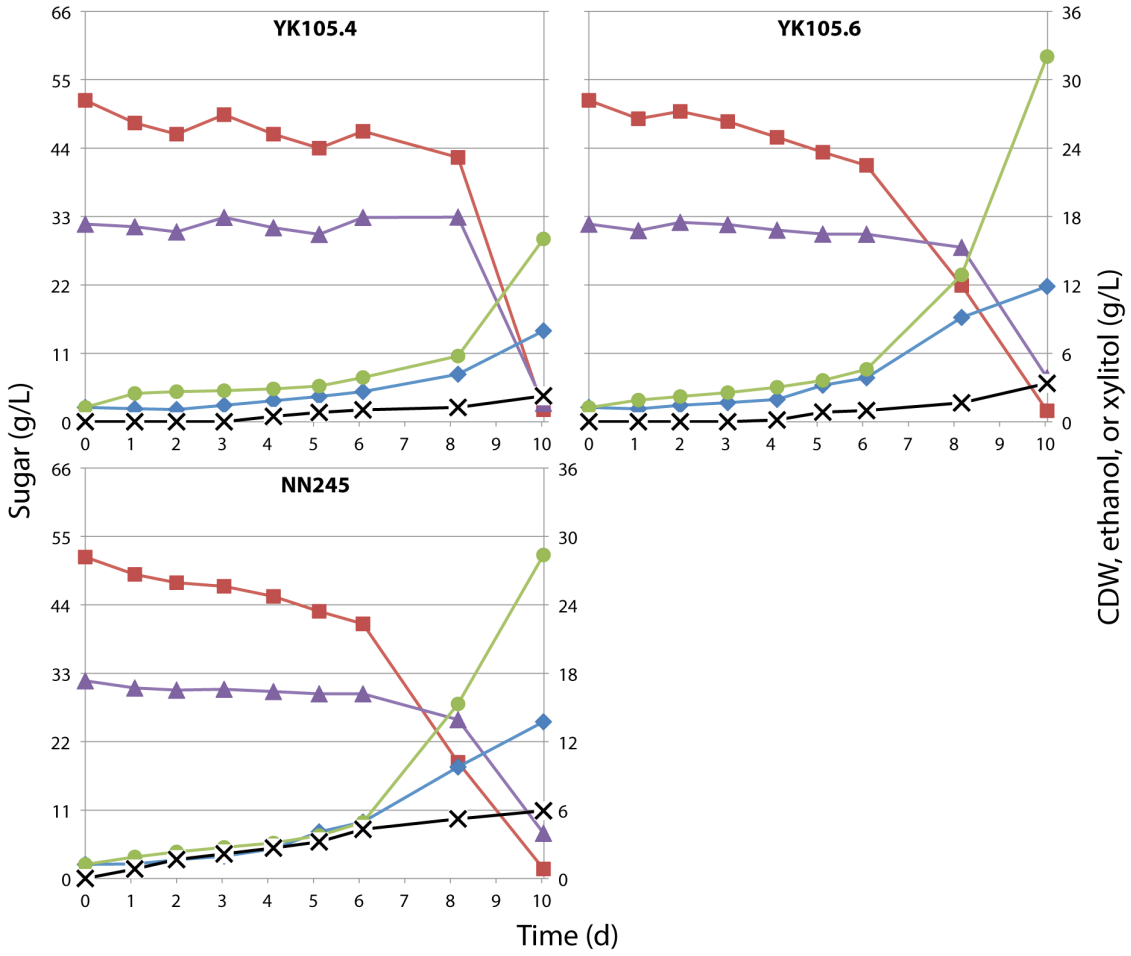

Figure 4-4 (cont.) Single shake flask screening of adapted *Spathaspora passalidarum* strain YK102.10, YK102.12, YK103.29, YK103.33, YK105.4, YK105.6 and wild type strain NN245 with AFCS hydrolysate. Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol

4.4.2 Adaptation and benchmarking of selected strains

Because we adapted YK105.6 by transferring a portion of the population into subsequent flasks without picking single isolated colonies from plates, we hypothesized that it was a mixed population with some cells having substantially better fermentation potential than others. We isolated 58 colonies from the YK105.6 adapted population by cultivation on AFCS hydrolysate plates and subjected them to 7 rounds of high throughput screening in 96 well plates with AFCS-RO hydrolysate as the medium. Three promising candidates (YK206-D11, YK206-F2, and YK208-E11) were identified based on their relatively high growth rates or the HPLC profile in the starter conditions. These strains were adapted to AFCS hydrolysate for one additional month (Figure 4-5). During adaptation, the culture was transferred every 6 to 7 days. Analyses of the supernatant solutions showed an average ethanol yield of 0.443 g/g and cell yield of 0.065 g/g. The average ethanol productivity during adaptation period was of 0.197 g/L·h. A triplicate shake flask experiment was performed to compare YK206-D11, YK206-F2, and YK208-E11 with the parental WT strain NN245 in AFCS hydrolysate (Figure 4-6). Both adapted strains and parental WT exhibited the potential to co-utilize glucose, xylose, and cellobiose for ethanol production. Both YK208-E11 and YK206-F2 consumed most of glucose (>95%) in 4-5 days and utilized about two-thirds of xylose in 6 days while WT strain NN245 converted about 85% glucose but used less than one-third of xylose in 6 days. YK206-D11 behaved unstably and had a slower average sugar utilization and fermentation rate compared to parental WT. Among the three adapted strains, YK208-E11 stood out due to its peak ethanol (38.3 g/L) production at 120 h, and because it has a shorter lag phase for glucose consumption compared to YK206-F2. The ethanol productivity of YK208-E11 was 0.309 g/L•h, which was 1.6 times higher than that of the WT strain (0.186 g/L•h).

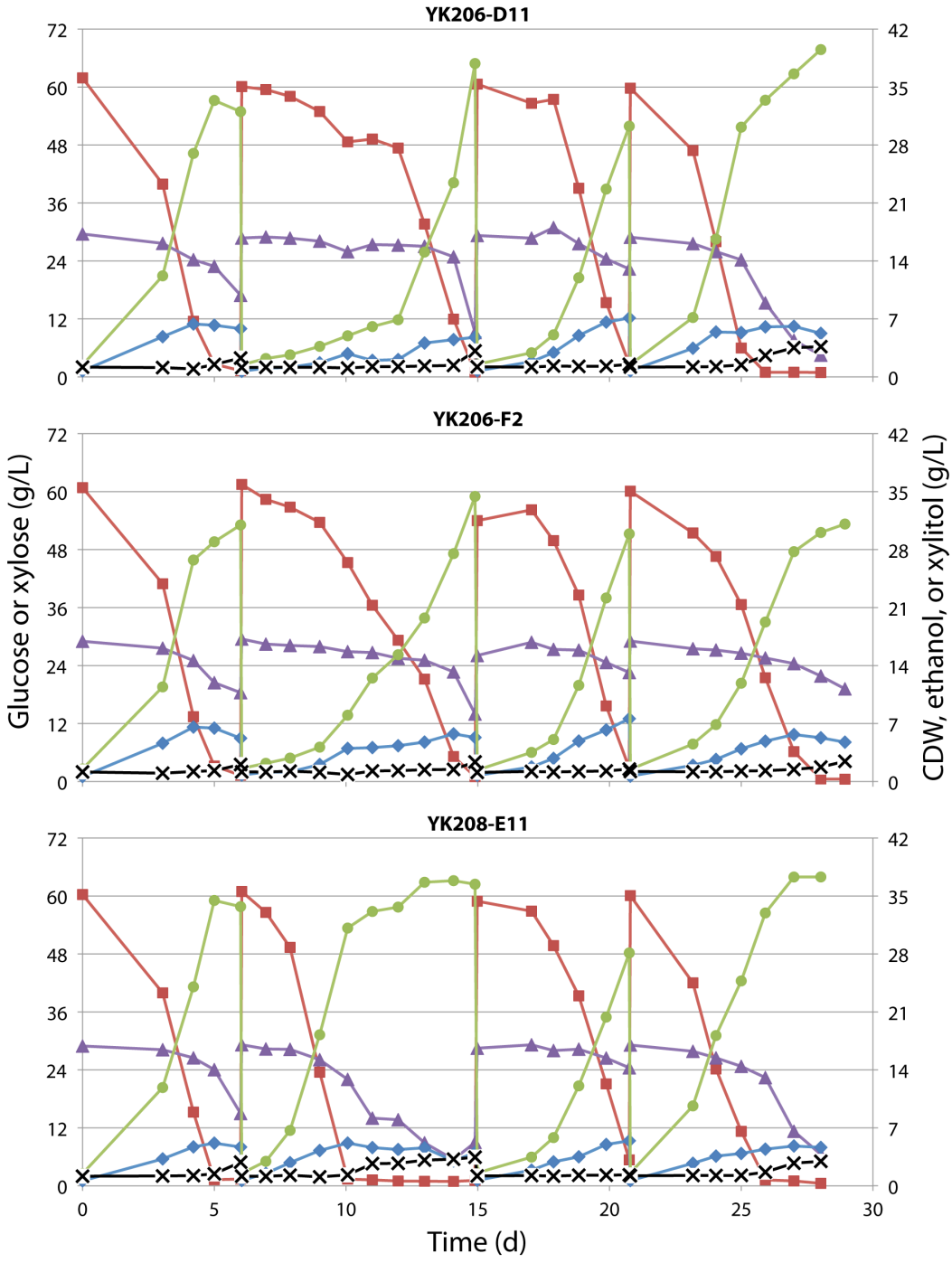

Figure 4- 5 The repetitive batch adaptation of *Spathaspora passalidarum* YK206-D11, YK206-F2, and YK208-E11 in shake flasks. Symbols: ■, glucose; ▲, xylose; ●, ethanol; ◆, cell dry weight; ✕, xylitol.

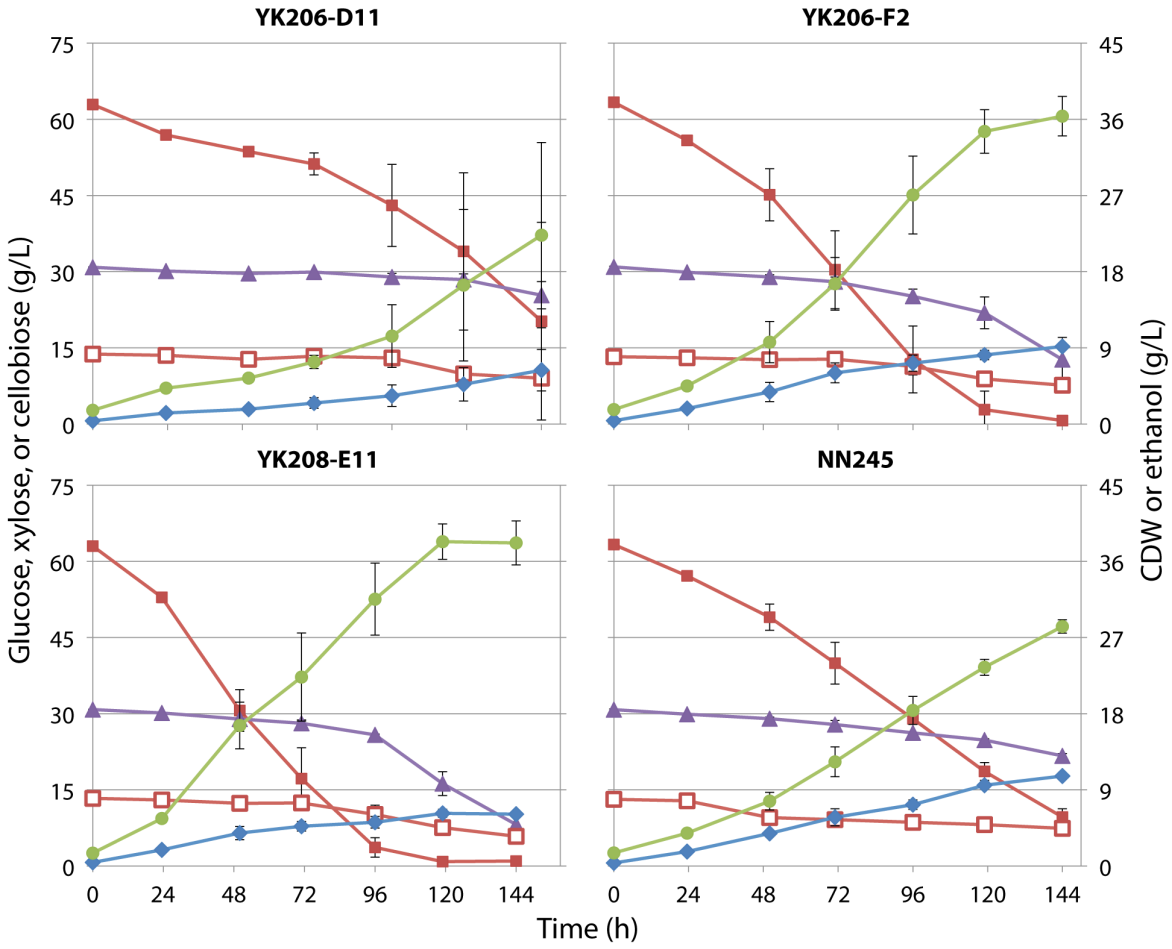

Figure 4- 6 Fermentation of AFCS hydrolysate in shake flasks by adapted *Spathaspora passalidarum* YK206-D11, YK206-F2, YK208-E11, and parental WT strain NN245. Symbols: ■, glucose; ▲, xylose; ▭, cellobiose; ●, ethanol; ◆, cell dry weight. Error bars indicate the standard deviation from triplicate shake flask.

By comparing CDW in the end, we found that YK208-E11 accumulated only about half as much cell mass compared as the WT strain, and the YK208-E11's specific ethanol productivity was 0.05 g/g CDW•h, or about 3 times higher than the WT strain (0.017 g/g CDW•h).

Co-utilization of cellobiose (35-56% of consumption) was also observed during the experiment. The ethanol yield of YK208-E11 was 0.48 g/g when excluding the cellobiose uptake and was 0.445 g/g when including cellobiose consumption. We also compared the acetic acid and fermentation byproducts formations (Figure 4-7). Interestingly, YK206-F2 and parental NN245 showed faster acetic acid degradation compared to YK206-D11 and YK208-E11. The initial xylitol concentration appeared at 1.4 g/L was possibly due to the background interference but YK208-E11 still accumulated more xylitol and glycerol compared to others. The accumulation of arabitol by YK208-E11 was also faster and slightly higher compared to those by other strains.

4.4.3 Characterizing the physiology of YK208-E11

To further confirm that YK208-E11 had a lower cell yield than the WT strain, we first executed a drop test with YK206-D11, YK206-E11, YK208-E11 and parental NN245 on ScD, ScX, and ScX plus lignotoxin (Figure 4-8). Strain YK208-E11 grew slowly on all three media compared to the other strains, as determined by the relative diameters of micro-colonies in the drop test. We then examined both YK208-E11 and NN245 in either 4% YPX or 4% YPD broth under aerobic conditions (200 rpm). The inocula were prepared by culturing yeast cells in either 4% YPX or YPD overnight and directly transferred into medium with corresponding carbon source (glucose-grown starters for glucose flasks and xylose-grown starter for xylose flasks) with cultures having an initial $OD_{600} \approx 0.005$. Under this condition,

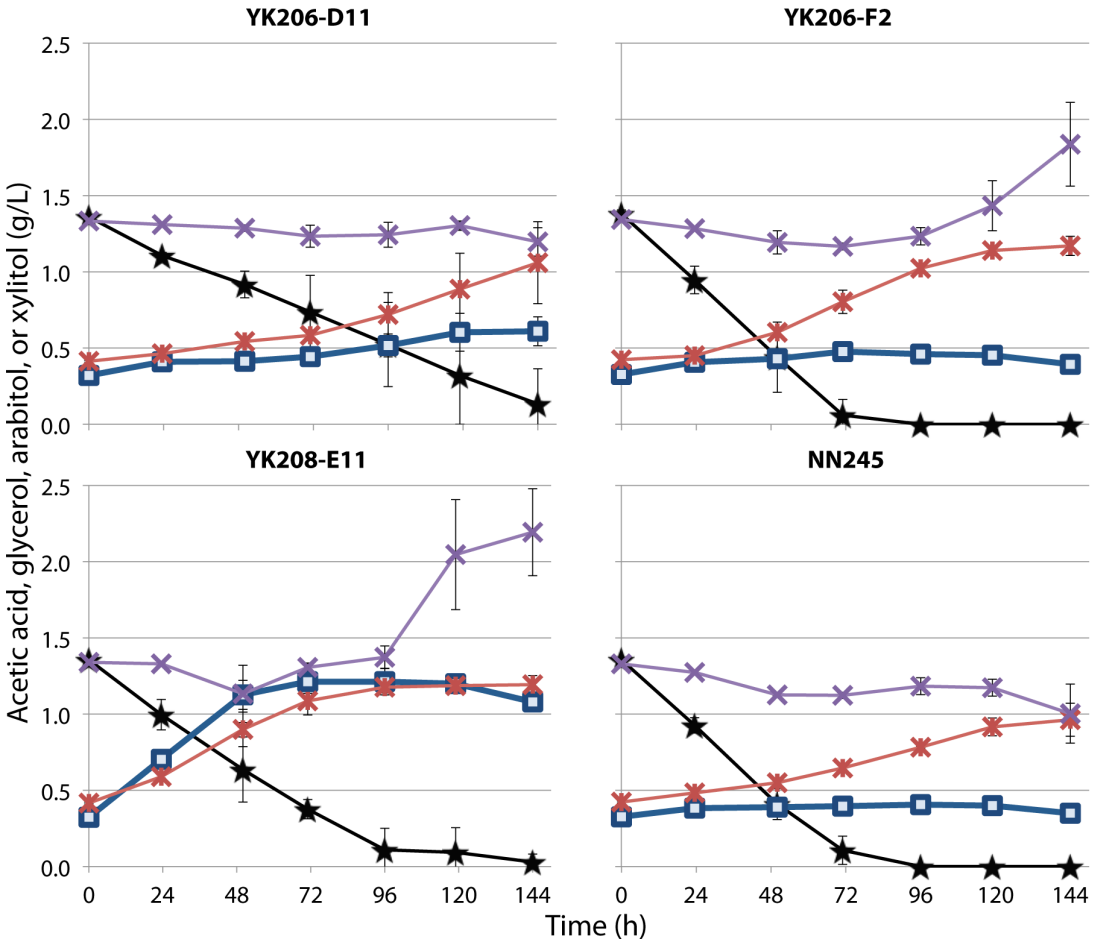

Figure 4-7 Concentrations of acetic acid, glycerol, arabitol, and xylitol by adapted *Spathaspora passalidarum* YK206-D11, YK206-F2, YK208-E11, and parental WT strain NN245 during AFCS hydrolysate benchmark experiment in triplicate shake flasks. Symbols: ★ , acetic acid; ▣ , glycerol; ✱ , arabitol ; ✕ ,xylitol. Error bars indicate the standard deviation from triplicate shake flasks.

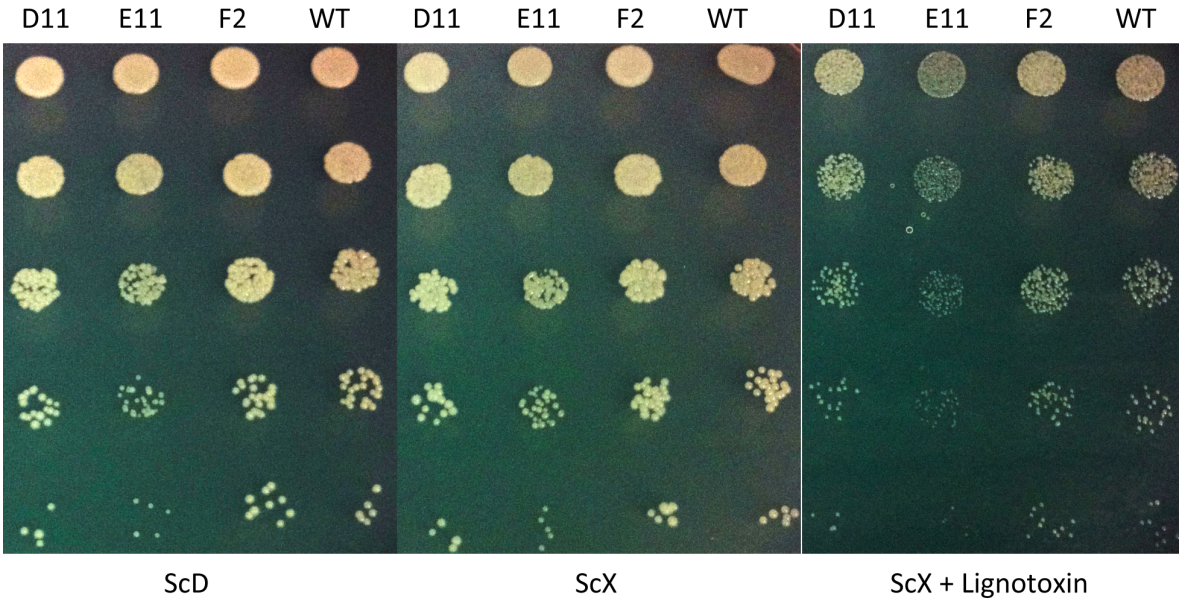

Figure 4-8 Drop tests of *Spathaspora passalidarum* adapted strains YK206-D11, YK206-F2, YK208-E11, and parental wild-type NN245 on ScX, ScD and ScD + Lignotoxin plates. Cell suspension (3 µL) with initial OD of 0.2 was placed in the top row and four ten-fold dilution (3 µL) were carried. Plates were maintained at 30°C 2-3 days.

YK208-E11 still made less biomass compared to WT (Figure 4-9). The difference was especially apparent when glucose was the carbon source. In that case, the cell yield of YK208-E11 was about 66% of the NN245 WT yield. When xylose was the carbon source, the YK208-E11 cell yield was about 77% of the WT.

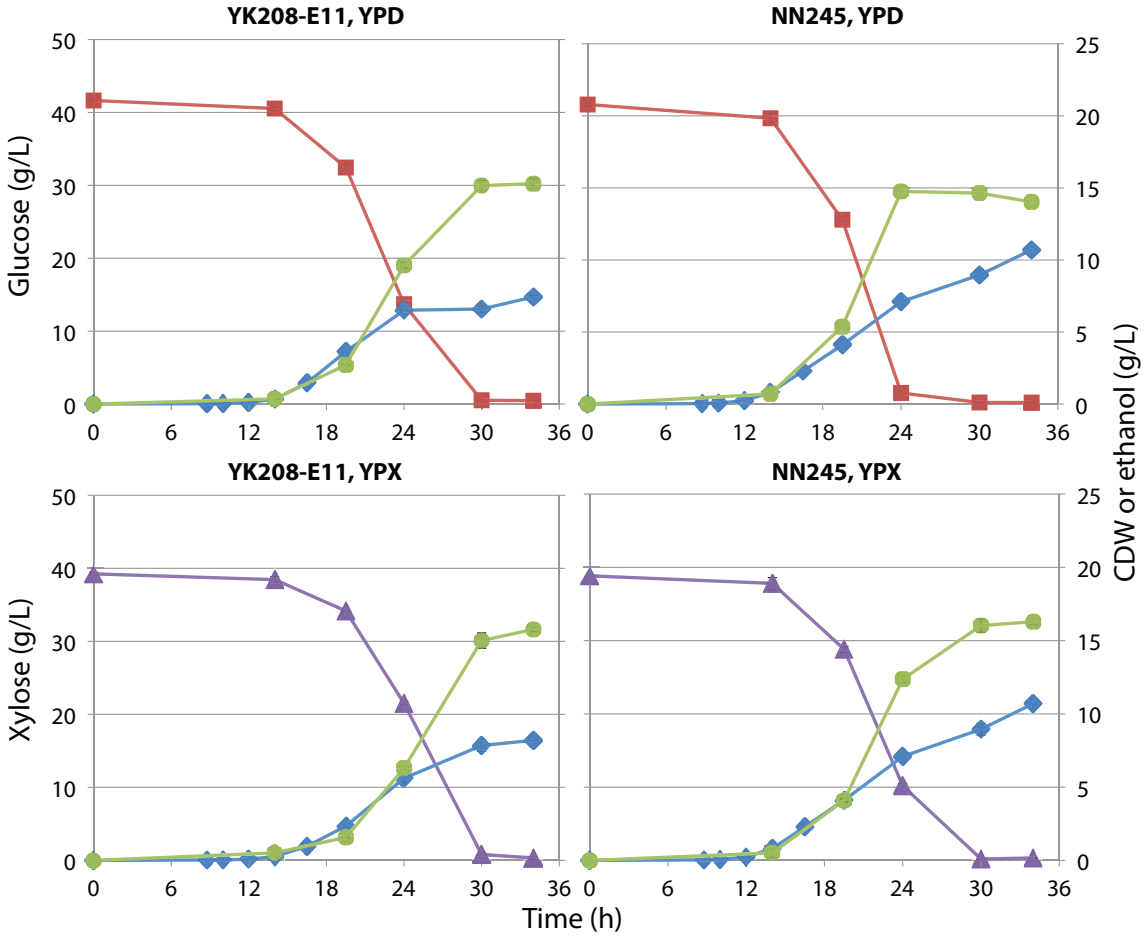

Figure 4-9 Growth of *Spathaspora passalidarum* NN245 and evolved strain YK208-E11 with 4% YPD or 4% YPX in triplicate shake flask under aerobic condition (200 rpm).

The lower cell yields of YK208-E11 implied either (1) possible deficiencies with ATP production in the electron transport chain (ETC) in mitochondria and/or (2) the strain is auxotrophic for one or more essential metabolites. However, since it could grow on the defined minimal Sc medium when we performed the drop test, we ruled out the possibility of it being auxotrophic. To test if any disruption of ETC, we plated cells on ScD and ScX agar containing respiration inhibitors and after colonies had grown out, we overlaid them with tetrazolium agar containing 0.1% of tetrazolium chloride. For control plate and plate overlaid with KCN, incubating for 48 h was sufficient for colony development (Table 4-2). In general, WT strains exhibited less incubation time compared to YK208-E11, except when AA was used. No difference in the incubation time between glucose or xylose, however, a shorter incubation for xylose (72 h) compared to glucose (96 h) was observed

Surprisingly, when glucose was the carbon source, WT colonies exhibited deep red (control) or red under most conditions, except when both AA and SHAM (AASH) were added on the plate. In that condition, the colonies could be pink, dark red, a pink circumference and dark red center. For E11 with ScD, it showed exclusively dark red. However, when xylose was the carbon source, pink, faint pink, or white colonies were observed under most circumstance except for E11 without any inhibitors, which exhibit red colonies, and E11 with both AA and SHAM, in which no colony was observed after 7 days (Figure 4-10). Also, YK208-E11 exhibited darker color compared to WT strain under the same condition. This finding contradicted our hypothesis because a white or faint pink color usually suggests disruption in the mitochondrial ETC, which has been used to select respiration deficiency in *S. cerevisiae* and dark red usually indicates respiration (Barclay et al. 2001, Conconi et al. 2000, Trevors 1982). We compared colony numbers (Table 4-2) from both strains with various conditions. For glucose,

no significant difference (p value ranged between 0.24-0.58) when comparing each condition to the control plate (no inhibitors) except for the condition with AASH ($p = 0.072$). Similar results were also confirmed from ScX, which the p value of the control to the plates of AASH was lower than 0.0005. It was also noticed that when WT strain appeared white when growing on ScX + AA or ScX +AASH plate and on the latter one, the colony only grew on the edge of the Petri dish. Variations in colony size were also observed, which led us to quantitate the colony diameters for each condition (Figure 4-11). For NN245 with glucose, similar diameters were observed except for the plate with AASH, which showed much smaller colonies. For NN245 with xylose, both plates with AA and AASH exhibited significantly much smaller colony sizes ($p<0.005$) compared to the control plate.

These observations suggest the *S. passalidarum* WT strain NN245 is cyanide-resistant and possesses rotenone insensitive NADH dehydrogenases, which do not couple with the formation of a proton-gradient. Interestingly, YK208-E11 exhibited smaller colonies compared to the control plate under all the conditions we tested here, especially for the xylose plates with AASH (no colony formed) and AA, in which the diameter of cells was only about half of that the control plates. For YK208-E11 with glucose, cells grew on the plates with AASH showed only 1/3 of the colony diameter compared to those on the control plates. By comparing the standard deviation, it is clear that introducing AASH increased the variation in the colony size on both WT and YK208-E11. Also, YK208-E11 exhibits more variation compared to WT based on the condition we tested here.

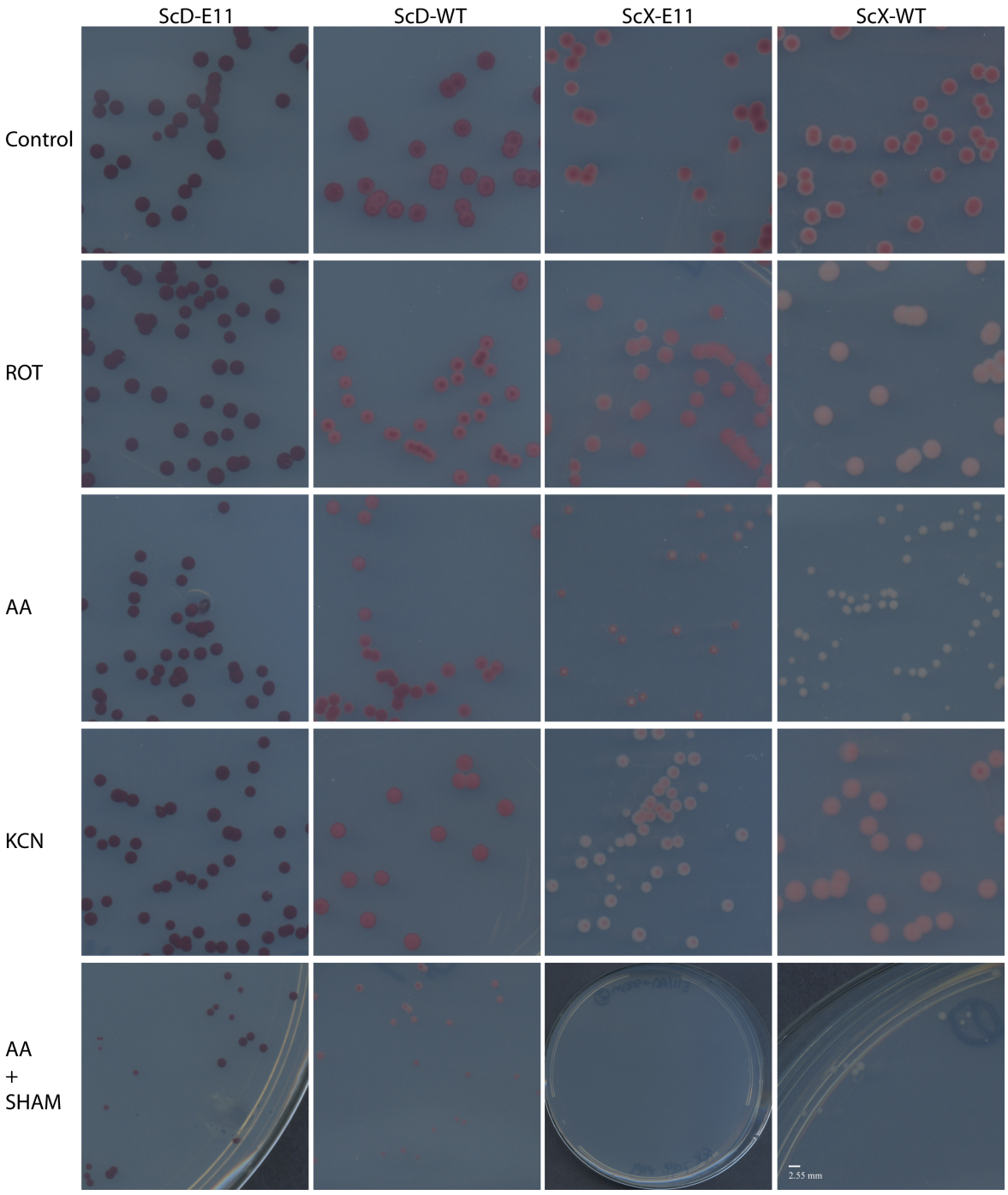
ScD-E11
ScD-WT
ScX-E11
ScX-WT
Control
ROT
AA
KCN
AA
+
SHAM
2.55 mm

Figure 4-10 Comparison of *S. passalidarum* YK208-E11 and parental WT strain NN245 on either ScD or ScX plate overlaying with tetrazolium (0.1%) with respiration inhibitors. Inhibitor concentration used for plating: ROT, 50 mM; AA, 0.5 mM; KCN, 0.5 M; SHAM, 0.2 M. Plating volume: 75 μL for the inhibitor and 90 μL of cell suspension ($OD_{600} = 2\times10^{-5}$).

Table 4-2 The incubation time and numbers of colonies of *S. passalidarum* evolved strain YK208-E11 and parental WT strain NN245 observed in tetrazolium overlay experiment.

Carbon source	Inhibitors	YK208-E11			NN245			*p-value*[a]
		Time (h)	Colony		Time (h)	Colony		
Glucose	control	48	207	191	48	119	244	NA[b]
	ROT	72	258	260	48	157	174	0.5816
	AA	96	164	148	96	157	157	0.2486
	AASH	144	113	118	120	127	111	0.0329
	KCN	48	231	148	48	148	139	0.5106
Xylose	Control	48	209	194	48	118	213	NA
	ROT	72	202	148	48	109	197	0.5559
	AA	72	145	112	72	178	<50	0.2658
	AASH	144	0	0	120	23[c]	11[c]	0.0003
	KCN	48	152	202	48	189	162	0.7821

a The *p-value* was calculated against the control plate.

[b] NA = Not applicable.

[c] The colony grew exclusively on the edge of the Petri dish.

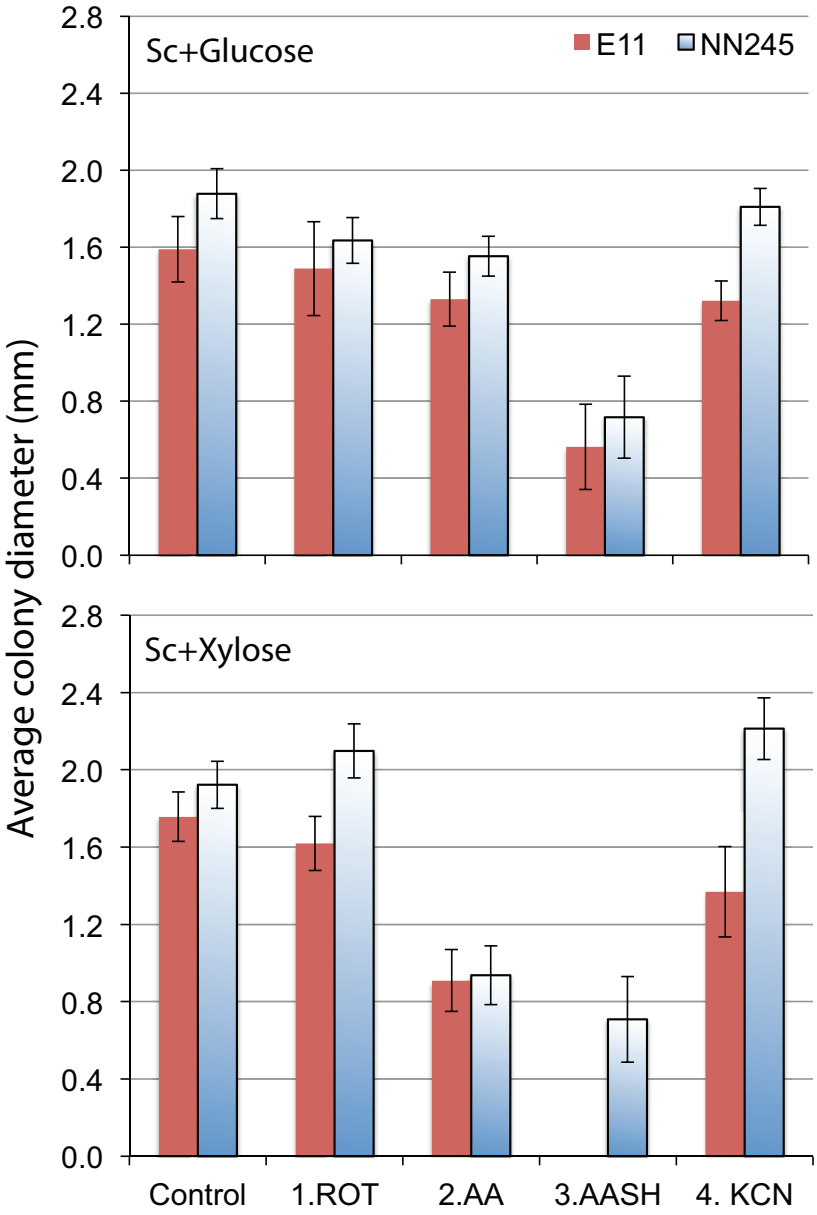

Figure 4-11 Average colony diameters of *S. passalidarum* NN245 and YK208-E11 on ScD (top) or ScX plates (bottom) containing respiration inhibitors and overlaying with tetrazolium (0.1%) agar (n=80). Inhibitor concentration used for plating: ROT, 50 mM; AA, 0.5 mM; KCN, 0.5 M; SHAM, 0.2 M; AASH, AA + SHAM. Plating volume: 75 µL for the inhibitor and 90 µL of cell suspension ($OD_{600} = 2\times10^{-5}$).

4.5 Discussion

4.5.1 Batch adaptation and selection for potential fermentative candidates

Batch and continuous culture based methods have been applied to select for *S. cerevisiae* with improved resistance to acetic acid (Cakar et al. 2012, Demeke et al. 2013, Dragosits and Mattanovich 2013, Kim et al. 2012, Koppram et al. 2012, Oud et al. 2012, Wright et al. 2011). Likewise, numerous strains have been adapted to resist furfural and HMF (Koppram et al. 2012, Lin et al. 2009, Tian et al. 2011). Recursive selection and adaptation – particularly when it is combined with mating between successive rounds of selection has proven particularly effective in obtaining strains of *S. cerevisiae* resistant to hardwood spent sulfite liquor (HW SSL) (den Haan et al. 2013, Kim et al. 2013, Pereira et al. 2013, Pinel et al. 2011). One reason for the efficacy of this approach could be that in developing resistance to complex mixtures of inhibitors, different yeasts acquire different mutational events, and through recursive mating, the various resistance mechanisms are combined into the most resistant strains. Efficient xylose-fermenting diploid strains of *S. cerevisiae* can also be obtained by mating strains that have been engineered independently (Kim et al. 2013). In our experiments, four lines of cells that had been adapted to separate hydrolysates were combined into a single flask and cultivated on AFCS hydrolysate under oxygen limited (stationary) conditions for three months. Microscopic observation of the culture broth indicated that mating occurred during this time.

One of our hypotheses was that increasing the number of passages during batch adaptation could affect the adaptation and using more passages during the batch adaptation in hydrolysates might significantly reduce the number of cells and strains with fermentative ability because most fermentative growth (as opposed to respiration) generates less ATP and

fermentative cells tend to grow slower and produce fewer cells as compared to respiring cells. By comparing the cell densities at day five (Figure 4-1 and table 4-1), we could see that YK102.10 and YK102.12, transferred in AFCS for 10 and 12 passages, respectively, were able to escape from the lag phase faster than YK105.4 and YK105.6. Similar observations were found when comparing YK103.29 and YK103.33 to YK105.4 and YK105.6. These results imply that (1) a population with faster initial growth would be selected during serial transfers in batch adaptation, (2) this selection could benefit cells that can make more cell mass, and (3) this would put fermentative cells at a disadvantage.

We also exercised the same concept for selecting the promising candidate from AFCS plates. In this case, YK208-E11 was selected because of its lesser growth in the 96-well plate and its relative higher ethanol concetration during the inoculum preparation. During the second batch adaptation, our data clearly suggested a better fermentative performance of YK208-E11 compared to YK206-D11 and YK206-F2.

4.5.2 Characterized evolved strain YK208-E11

The consistently lower cell yield compared to other strains was also confirmed by drop test and growth in shake flask under aerobic conditions (200 rpm). To investigate the possibility that YK208-E11 had a disruption or defect in its mitochondrial ETC, we used a tetrazolium agar overlay to detect respiratory activity in the presence or absence of four different respiration inhibitors. It should be noted that the most applications of tetrazolium dyes measure cell proliferation by assuming dye reduction will be proportional to the number of viable cells in the exponential growth phase. In addition, although it is generally assumed the reduction of tetrazolium salts is intercellular and related to energy production, most of reduction appears to be non-mitochondrial (Berridge et al. 2005).

Figure 4-12 shows a typical respiratory chain including the action site of each respiration inhibitor in native xylose-fermenting yeasts such as *S. stipitis* or *S. passaliadrum*. If we assume that the reduction of tetrazolium is associated with energy production and cellular respiration, then cells growing on ScD should exhibit a darker color compared to ScX. The results (Figure 4-10) suggest that cells growing on glucose area more respirative than those on xylose, which implies cells are more fermentative on xylose than on glucose as suggested by our earlier study (Long et al. 2012, Su et al. 2014).

Nevertheless, YK208-E11 showed a darker color as compared to WT under all the conditions tested here (Figure 4-10), which was contrary to our earlier hypothesis about tetrazolium reduction. Based on the mechanism assumed earlier a lighter tetrazolium color reaction should have been observed with YK208-E11 compared to WT. A possible explanation is that tetrazolium chloride serves as an indicator for cellular reducing power, and under most conditions this reaction requires NADH (Berridge et al. 2005), which is also required for yeast cells to detoxify aldehyde inhibitors, such as HMF and furfural (Liu 2011). Since the tetrazolium salts are lipophilic, it can diffuse through cell membrane and be reduced in the cytosol. It is possible YK208-E11 contains higher levels of cytosolic NADH compared to the WT strain, and a darker color from YK208-E11 was shown due to a greater reduction of tetrazolium chloride.

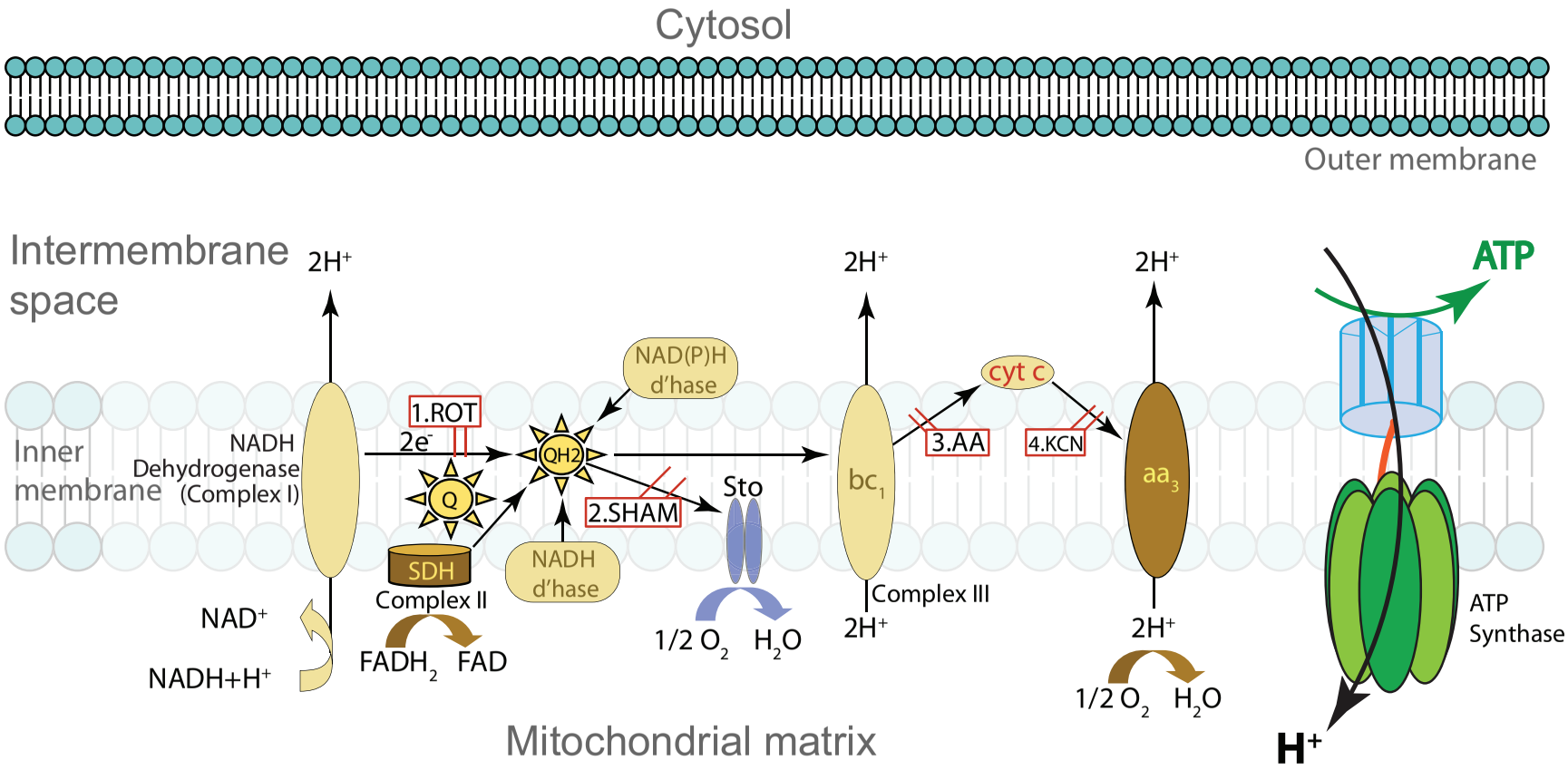

Figure 4-12 A typical diagram of alternative and standard redox components in the electron transport chain (ETC) in *S. passalidarum* or *S. stipitis*. ROT, rotenone; SHAM, salicylhydroxamic acid; AA, antimycin A. NAD(P)H d'hase, retonone-insesitive dehydrogenase

Under most conditions (except ScX plates containing AA), we observed smaller colonies with YK208-E11 compared to the colonies of the WT strain. This suggested a lower cell yield or growth rate and corresponded to our earlier observations in shake flasks and drop tests. Possible explanations for this observation could be (1) genes related to anabolism in YK208-E11 were disrupted and/or (2) less ATP was produced in YK208-E11 under aerobic conditions due to a disruption in the ETC. When YK208-E11 was cultivated on ScD and ScX plates with KCN, it showed colony diameters 31% and 37% smaller respectively as compared to NN245. Additionally, when YK208-E11 was cultivated on ScX with AASH, no colonies were observed. Growth was completely inhibited under this condition. By comparison, WT cells cultivated on ScX with AASH still demonstrated few colonies (~20), despite those are only 10% of the colony compared to the ScX without inhibitors, and those colonies are white with various sizes with growth exclusively on the edge of the Petri dish (Figure 4-10). These observations suggest a possible mutation on the alternative oxidase (AOX) pathway in YK208-E11, which causes the strain is more sensitive to the respiration inhibitor as compared to the WT. *S. stipitis* is known to resist to cyanide (Jeppsson et al. 1995). Studies also suggest that cyanide-resistant AA-resistant yeast possess a SHAM-sensitive AOX pathway, which transfers electrons directly from ubiquinol to molecular oxygen (Rosenfeld and Beauvoit 2003). Since *S. passalidarum* and *S. stipitis* are evolutionarily closely related, it is not surprising that *S. passalidarum* is also cyanide-insensitive and has a SHAM-sensitive AOX pathway. Studies have shown that when the cyanide-resistant AOX pathway is present in yeast or fungi, the complex I is also present, and the presence of cyanide-resistant AOX pathway is very common in yeast and fungi (Joseph-Horne et al. 2001, Veiga et al. 2000, Veiga et al. 2003a, Veiga et al. 2003b).

When plating WT cells on ScD and ScX with AASH, only about 50% and 10% of colonies grew out compared to the control plates, respectively, which suggests that the WT *S. passalidarum* is also SHAM-sensitive. However, the difference in numbers of colonies that grew out on glucose and xylose plates with AASH suggests the carbon source maybe the possible cause for this observation. Besides, the ScX plates containing AA showed no difference in colony size (p=0.25) from both YK208-E11 and parental WT, but the ScD plates overlaid with AA showed a difference in colony size (p<0.005). Also, a shorter incubation time for ScX + AA compared to ScD + AA. Theses findings indicate the xylose-induced fermentative phenomenon could be related to the action site of AA, which is from cytochrome bc_1 complex to cytochrome *c*.

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4.8 Abbreviation

AA	Antimycin A
AFEX	Ammonia fiber expansion
AFCS	AFEX pretreated corn stover hydrolysate
HMF	5-hydroxyl methylfurfural
SHAM	Salicylhydroxamaic acid
TTC	Tetrazolium chloride
ROT	Rotenone

4.9 References

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Chapter VI

Conclusion and Future Directions

5.1 Conclusion

Based on our studies, we conclude the following findings:

1) *Spathaspora passalidarum* unique compared to other native xylose-fermenting yeast such us *Scheffersomyces stipitis* and *Scheffersomyces shehatae* because *S. passalidarum* is able to coferment glucose and xylose when glucose concentration is below 30 g/L, and it is capable of co-utilizing cellobiose, glucose, and xylose for ethanol production.
2) During oxygen-limited condition, fermentation of xylose is more rapid than glucose, and cell yields on xylose are consistently lower than the corresponding values on glucose, which leads to a higher specific ethanol productivity on xylose than glucose.
3) Both kinetic studies and tetrazolium overlay suggest cells are more respirative when glucose is the carbon source, and cells are more fermentative when xylose is the carbon source.
4) Adaptation by serial batch transferred and cell recycling could potentially select for strains with higher hydrolysate tolerance. However, batch adaptation should be carried out cautiously because fermentative cells normally exhibit lower cell density compared to respirative cells and subsequent transferred might lead to strains with higher respiration capacity.
5) The evolved strain YK208-E11 exhibited much higher specific fermentation rate compared to the parental strain due to its remarkable less cell density. Further characterization suggested that YK208-E11 has a deficiency in its alternative oxidase pathway that contributes to its low cell yield and higher specific fermentation rate.

5.2 Future direction

The native xylose-fermenting yeast *S. passalidarum* NN245 exhibited extraordinary characters such as cofermentation of glucose and xylose, higher glycolytic pathway on xylose than glucose, and its xylose-reductase has higher affinity on NADH. However, how xylose induces its fermentative pathway still needs to be elucidated and more efforts is needed to improve its fermentation capacity and hydrolysate inhibitor tolerance and detoxification. These goals can be achieved by:

1) Culturing yeast cells in bioreactors with additional respiration inhibitors and performing real time analysis with tetrazolium dyes. Some tetrazolium dyes used today even can target the location site where the reduction occurs (e.g. on the cell surface, in the cytosol, in the mitochondria, etc.) (Berridge et al. 2005).
2) Performing chemostat studies combined with proteomics or transcriptomics will also provide more insight on the regulation system in *S. passalidarum* during glucose and xylose fermentation and combination with additional respiration inhibitors (antimycin A, rotenone, and etc.)
3) Exercising metabolic modelings based on kinetic data, stoichiometry, elementary mass balance, transcriptomics, proteomic or metabolics studies.
4) Insertion of sugar transporters or genes related to pentose phosphate or glycolytic pathway by metabolic engineering can possibly increase the overall flux for ethanol production and enhance strain's fermentative capacity.
5) Future adaptation with hydrolysate should be executed with continuous culture to maintain the selection pressure. This process should be keep at extremely low-oxygen condition to eliminate the growth of respirative yeast. The possible candidate

should be screening for its fermentative capacity to avoid false-positive fermentative strain.

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