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## Development of a recombinant brewing yeast to produce beer from hemp extract (*Cannabis Sativa L.*)

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A thesis submitted in partial fulfillment of the requirements for the Master of Engineering Science degree in Chemical and Biochemical Engineering

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## Abstract

The Cannabis industry is a rapidly-growing market in Canada. With the legalization of edible products in 2019, many cannabis-derived candies, baked goods, beverages appeared on shelves. Cannabis beer can be brewed by replacing barley with pretreated cannabis plant. However, using a traditional brewing yeast to brew cannabis beer will result in incomplete fermentation which will affect the beer's composition and flavour because traditional brewing yeasts are not able to utilize xylose which is an abundant carbohydrate in lignocellulosic extracts. Using a recombinant strain of a brewing yeast and a xylose-fermenting yeast can overcome this issue. The work presented in this thesis compares the fermentation performance of two native xylose-fermenting yeast strains, *Pichia stipitis* and *Spathaspora passalidarum*, and performs the transformation with a brewing yeast via electroporation.

Fermentation performance of the xylose-fermenting yeasts were evaluated in mixed carbohydrate medium, containing cellobiose, glucose and xylose. Under aerobic conditions, carbohydrate consumption rates of both strains were faster than the rates under anaerobic conditions, but aerobic conditions led to ethanol respiration by *P. stipitis* and *S. passalidarum*. Under anaerobic conditions and at high glucose concentrations, *S. passalidarum* sequentially utilized glucose and xylose, while glucose decreased xylose utilization ability of *P. stipitis*. *S. passalidarum* also exhibited higher ethanol tolerance compared to *P. stipitis*. Transformation of brewing yeast strains and *S. passalidarum* were conducted using electroporation-based transformation. Genomic DNA of the donor strain, *S. passalidarum*, was extracted using phenol-chloroform extraction and transferred into host strains, an ale and a lager strain, using an electric pulse. Putative recombinants were selected on plates containing xylose as the sole carbon source, however, obtained recombinants strains were deemed to be unstable due to the aneuploid nature of the host strains.

## Keywords

Xylose-fermenting yeast, *Spathaspora passalidarum*, *Pichia stipitis*, lignocellulosic biomass, hemp extract, cannabis, beer, brewing, genome shuffling, fermentation

## Summary for Lay Audience

The Cannabis industry is a rapidly-growing market in Canada. With the legalization of edible products in 2019, many cannabis-derived candies, baked goods, beverages appeared on shelves. Cannabis beer can be brewed by replacing barley with pretreated cannabis plant. However, using a traditional brewing yeast to brew cannabis beer will result in incomplete fermentation which will affect the beer composition and flavour because traditional brewing yeasts are not able to utilize xylose which is an abundant carbohydrate in plant extracts. Using a recombinant strain of a brewing yeast and a xylose-fermenting yeast can overcome this issue. The work presented in this thesis compares the fermentation performance of two native xylose-fermenting yeast strains and performs the transformation with a brewing yeast shuffling genetic information of both strains.

Fermentation performance of the xylose-fermenting yeasts were evaluated in a mixture of carbohydrates that are found in plant extracts. Under oxygen-limited conditions, *S. passalidarum* had superior fermentation ability than *P. stipitis*. Transformation of a brewing yeast with *S. passalidarum* DNA was conducted. Genetic material of the donor strain was extracted and transferred into the host strain using electrical current. Putative recombinants were selected by growing them in a medium containing xylose as the sole carbon source, however, obtained strains were deemed to be unstable due to the complex genetic nature of the host strains.

## Co-Authorship Statement

### Chapter 3

**Ugur Gulmen:** Designed and conducted experiments, conducted analysis of samples, interpretation of data, and writing

**Dr. Erin Johnson:** Designed and conducted experiments, editing

**Dr. Lars Rehmann:** Supervised the research work, assisted in data analysis, editing

### Chapter 4

**Ugur Gulmen:** Designed and conducted experiments, conducted analysis of samples, interpretation of data, and writing

**Dr. Erin Johnson:** Editing

**Dr. Lars Rehmann:** Supervised the research work, assisted in data analysis, editing

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## Chapter 1

### 1 Introduction

Beer has been a part of human culture dating back thousands of years (Dietrich, Heun et al. 2012). It is the most popular alcoholic beverage in the world, thus, competition in the global beer industry is high. Increasing demand for craft beers lead breweries to seek alternative flavours. Many breweries replaced barley malt with other crops such as sorghum, rice and corn to produce high quality beer for a lower cost and different flavours (Bogdan and Kordialik-Bogacka 2017).

Hemp (*Cannabis Sativa L*) is known to be cultivated for thousands of years and has a long history of applications in the textile, paper, food, pharmaceutical and construction industries (Russo 2007). However, *Cannabis Sativa L* is a strictly controlled crop due to its psychoactive effects of certain strains. Its usage as a recreational drug is illegal in many countries. Hemp is also a type of *Cannabis Sativa L*, however, years of breeding has removed the psychoactive compounds. In 2018, legalization of recreational cannabis use in Canada emerged as a new market which increased the product diversity. Apart from traditional consumption of cannabis, many edible products, which were legalized a year later, such as sweets and beverages have started to take place in regulated cannabis stores. In recent years, hemp has been investigated as a lignocellulosic feedstock for ethanol production and found to be a promising crop for producing bioethanol (Das, Liu et al. 2017).

Hemp can be incorporated into beer brewing, as well. The industrial partner of this study, Province Brands of Canada, is currently working on hemp brewing process. However, using hemp in brewing comes with its challenges. In traditional brewing, barley malt is used as the carbohydrates and nitrogen source for yeast to produce ethanol and flavor compounds. Brewing yeasts are able to ferment carbohydrates in barley malt which are hexose carbohydrates such as glucose, fructose and most abundantly maltose which is a disaccharide. Hemp, on the other hand, contains cellulose and hemicellulose in polymeric

form, which when pretreated, can be hydrolyzed to glucose and significant amount of xylose, a pentose carbohydrate. Brewing yeast cannot utilize pentose carbohydrates as it cannot produce necessary enzymes to metabolize them. Using a brewing yeast will leave the fermentation incomplete. Xylose-fermenting microorganisms such as *P. stipitis*, *C. shehatae* and *S. passalidarum* were studied for conversion of lignocellulosic biomass to ethanol (Zaldivar, Nielsen et al. 2001, Nguyen, Suh et al. 2006).

Another major challenge is creating a desirable flavor. Aside from wort composition and brewing conditions, flavor is determined by the flavor-active secondary metabolites that brewing yeasts produce during fermentation, which are strain specific. Recombinant strains can be developed for efficient fermentation of pentose carbohydrates while producing flavor-active compounds. Classical strain improvements methods such as mutagenesis, laboratory evolution and cross-mating are used to develop improved microorganisms but they are time-consuming and labor intensive. Genetic engineering is also another approach but products made using genetically modified organisms (GMOs) are still not widely accepted due to consumer concerns.

Genome shuffling can be used to engineer a recombinant brewing yeast strain. Genome shuffling offers a rapid production of improved microorganisms through recursive genomic recombination with higher gene recombination efficiency (Zhang, Perry et al. 2002). To develop a non-GMO microorganism, strains used in genome shuffling must be in the same taxonomic family (Government of Canada 2016). Many applications have shown that transformation is less time-consuming and easy to operate (Magocha, Zabed et al. 2018). Electroporation-based transformation is the latest method in this field of strain improvement, which is a fast and easy method compared to protoplast fusion, hybridization and mating-based genome shuffling (Zhang and Geng 2012).

In this study, two xylose-fermenting yeast *P. stipitis* and *S. passalidarum* were evaluated for their fermentation properties. Then, an Ale yeast, *S. cerevisiae*, and a Lager yeast, *S. pastorianus* was transformed with the best performing xylose-fermenting yeast.

## 1.1 Research Objectives

The main objectives of this research was to compare the fermentation performance of two xylose-fermenting yeast and develop a recombinant yeast through transformation. The specific objectives are listed below:

- Assess the effect of aeration, temperature, and ethanol tolerance of two xylose-fermenting yeast
- Develop a recombinant yeast strain using a brewing yeast and a xylose-fermenting yeast that had the best fermentation performance in terms of carbohydrate conversion
- Assess the fermentation performance of the recombinant yeast strain

## 1.2 Thesis Organization

This thesis covers five chapters and complies to the “integrated article” format as dictated by the Thesis Regulation Guide by the School of Graduate and Postdoctoral Studies (SGPS) of the University of Western Ontario. The chapters covered are as follows:

<b>Chapter</b>	<b>Scope</b>
1	General introduction and objectives of the research
2	A review of the literature on hemp, brewing and yeast strain development through genome shuffling
3	Comparison of xylose-fermenting yeast based on their fermentation ability of lignocellulosic carbohydrates under aerobic and anaerobic conditions
4	Electroporation-based transformation to develop xylose-fermenting <i>S. cerevisiae</i> and <i>S. pastorianus</i>
5	Summary of the major findings of this research and recommendations for future studies



## Chapter 2

### 2 Literature Review

#### 2.1 Hemp as the feedstock

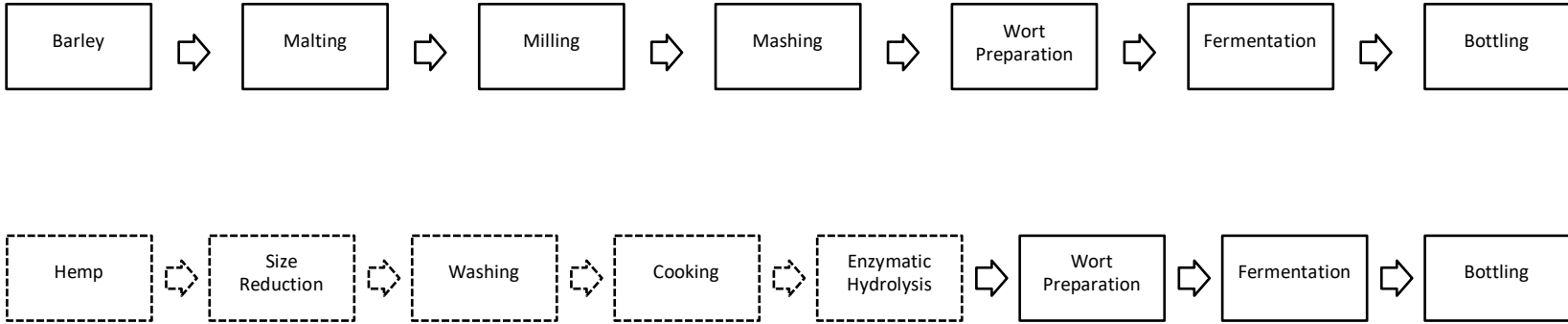
Hemp (*Cannabis Sativa L.*) has many applications in various industries such as textile, paper, construction, food, pharmaceutical and recently the biofuel industry. Hemp fibers are used for textile, paper and as building materials (Nature 1996). Hemp fibers are alternative reinforcement fibers to carbon and glass in composite materials due to its low density, high specific strength and renewability (Dhakal and Zhang 2015). Hempseed oil is used for lightning oil, soap, paint and varnishes as well as for food and dietary supplement for humans and animals (Cherney and Small 2016). It is a good source of essential fatty acids and contains antioxidants which offers cardiovascular and anti-inflammatory benefits (Huang 2000, Mölleken, Mothes et al. 2000). The distinction between marijuana and hemp is based on the psychoactive compounds produced. Marijuana is a type of cannabis which produces high amounts of intoxicating type of cannabinoids, tetrahydrocannabinol (THC), whereas hemp can produce high amounts of cannabidiol (CBD), which has anti-anxiety, anti-psychotic and anti-depressant effects, depending on the species and strain (Pertwee 2008).

In recent years, hemp biomass has been studied for renewable fuel production. Hemp can provide up to 25 metric ton biomass per hectare, can grow in various environmental conditions and has low pesticide requirement as it can overgrow weeds (Struik, P. C., et al. 2000, Prade 2011). Processing hemp oil generates by-products and residues, such as leaves, stalk, stem and roots, which remain unused. This biomass waste can be utilized to produce bio-based chemicals. Hemp has been reported be feasible as a lignocellulosic biomass feedstock for biogas and bioethanol production (Rehman, Rashid et al. 2013, Kuglarz, Gunnarsson et al. 2014) and, as a solid fuel for combustion (Rice 2008). Recently, utilizing hemp in a biorefinery has been shown to produce 149 kg of ethanol and 115 kg succinic acid per 1 ton of dry hemp (Kuglarz, Alvarado-Morales et al. 2016). Hemp contains more

cellulose (44% dry matter) than other crops such as corn stover (37%) and wheat straw (37%) (Sipos, Kreuger et al. 2010). High cellulose content and high biomass yield are properties that make hemp a good potential crop for second generation bioethanol production. Therefore, hemp is a promising candidate for sustainable production of bio-based chemicals. To the author's knowledge, unfortunately, there are no studies on brewing beer from hemp in the literature. However, its high cellulose content may allow producing beer with sufficient alcohol content.

### 2.1.1 Industrial Hemp in Brewing

The main ingredients of beer are hops, water, yeast and barley malt. Barley malt is the ingredient from which the fermentable compounds, carbohydrates and nitrogen sources, are obtained. Barley undergoes malting, milling and mashing processes to extract carbohydrates and nitrogen sources. The malting step involves germination of the barley to produce enzymes to break down starch into fermentable carbohydrates. In the milling step, barley grains are crushed to release enzymes and to increase surface area. The mashing process extracts fermentable compounds through enzymatic hydrolysis of starch and proteins in the presence of hot water. Then, the wort is filtered to remove insoluble material and retain the desired fermentable compounds (carbohydrates and nitrogen sources, flavor compounds), followed by hop addition to boiling wort to extract hop components. Finally, yeast is added to cooled wort for fermentation. Wort most abundantly contains glucose, maltose and maltotriose which are hexose carbohydrates and can be utilized by brewing yeast (Willaert 2006). Non-fermentable carbohydrates such as, dextrins, arabinose, xylose, ribose, isomaltose, panose and isopanose, make a small fraction of wort (Kunz, Seewald et al. 2013).



**Figure 2.1.** Comparison of barley brewing and hemp brewing processes

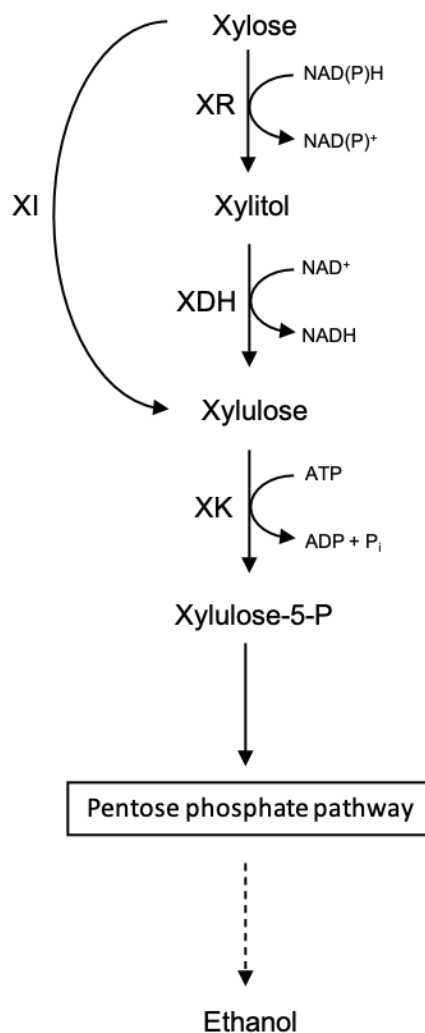
Pretreatment of hemp requires chopping and cutting to reduce the size of lignocellulosic biomass, washing of sand and dirt, cooking and, hydrolysis with much more complex cellulose and hemicellulose enzyme complexes. Cooking disrupts the cellulose-hemicellulose-lignin complex to make it accessible to hydrolytic enzymes (Wyman, Dale et al. 2005). After releasing cellulose and hemicellulose from the complex, they need to be converted into fermentable carbohydrates which are obtained through enzymatic hydrolysis. Carbohydrates are obtained from cellulosic and hemicellulosic part of hemp. Lignin is a heterogeneous aromatic polymer, part of lignocellulosic biomass that provides structural integrity in plants and cannot be fermented. Hemp is rich in cellulose, therefore, hemp extracts mostly contain glucose, however, there is substantial amount of xylose from hemicellulose which cannot be utilized by brewing yeast.

There are several methods for pretreating lignocellulosic biomass, which can be categorized as physical, chemical, physicochemical and biological pretreatment (Haldar and Purkait 2020). The efficacy of pretreatment and hydrolysis depends on the selection of lignocellulosic biomass and the process conditions (Zabed, Sahu et al. 2016). Pretreating lignocellulosic biomass is more expensive and complex than processing barley for brewing.

### 2.1.2 Potential challenges in hemp brewing

The major challenge in hemp brewing is converting xylose into ethanol. Brewing yeast are not able to metabolize xylose due to lack of metabolic pathways (Tian, Zang et al. 2008). Xylose can be naturally metabolized by some bacteria and yeast. Natural xylose-fermenting yeasts utilize xylose through a xylose reductase-xylitol dehydrogenase (XR-XDH) pathway whereas bacteria use a xylose isomerase (XI) pathway (Figure 2.2) (Bruinenberg, de Bot et al. 1984). Xylose is first converted into xylitol by XR with the help of cofactor NADH or NADPH. Then, xylitol is oxidized to xylulose by XDH which is an NADH-dependent enzyme. Finally, xylulose is phosphorylated by xylulose kinase (XK) to xylulose-5-phosphate (X5P) before entering to the pentose phosphate pathway for further

catabolism to ethanol. On the other hand, bacteria directly convert xylose into X5P using XI without requiring any cofactors.



**Figure 2.2.** Xylose metabolism pathways in bacteria and yeast.

XDH: xylose dehydrogenase; XI: xylose isomerase; XK: xylulose kinase; XR: xylose reductase. Yeast use XR-XDH pathway whereas bacteria use XI pathway.

Many yeast and bacteria have been investigated and recombinant strains have been engineered to integrate the xylose metabolic pathway. Recombinant *S. cerevisiae* strains with the XI pathway have been developed but showed slower xylose utilization and lower ethanol yields than recombinant strains with the XR-XDH pathway (Kwak and Jin 2017).

Natural xylose-fermenting yeasts, *P. stipitis* and *C. shehatae*, have been studied extensively for fermenting xylose into ethanol (Zabed, Sahu et al. 2016). One of the major challenges of using natural xylose-fermenting yeast for xylose fermentation is that they have poor growth and fermentation performance in anaerobic conditions. Studies showed that poor performance may be related to cofactor imbalance during xylose assimilation. For example, *Pichia stipitis* and some recombinant *Saccharomyces cerevisiae* use the XR-XDH pathway which has a strong affinity to the cofactor, NADPH, when converting xylose into xylitol using XR. However, the next reaction requires XDH which depends on, nicotinamide adenine dinucleotide, NAD<sup>+</sup>. Different cofactor dependencies lead to NADP, NADH and byproduct (xylitol) accumulation which decreases ethanol yield from xylose (Kwak, Jo et al. 2019). Therefore, oxygen or transhydrogenase is required to regenerate NAD<sup>+</sup> from NADH during fermentation (Harner, Wen et al. 2015). Unlike other xylose-fermenting yeast, cofactor imbalance does not occur when *Spathaspora passalidarum* and *Candida parapsilosis* utilize xylose due to the preference of NADH to NADPH (Lee, Koo et al. 2003, Hou 2012). *S. passalidarum* has also exhibited efficient xylose fermentation performance under oxygen-limited conditions, which is a desired trait for the brewing processes (Hou 2012).

The co-utilization of xylose and glucose is another challenge in lignocellulosic bioethanol fermentation. The presence of glucose inhibits xylose uptake of xylose-fermenting yeasts due to repression of enzymes and transporters in xylose metabolism (Slininger, Thompson et al. 2011). Xylose is not significantly utilized until glucose concentration decreases below 5% (w/v) for *C. shehatae* and 2% (w/v) for *P. stipitis* (Panchal, Bast et al. 1988). Xylose fermentation after glucose depletion may also be an issue as xylose-fermenting yeast have low ethanol tolerance (Harner, Wen et al. 2015). A strain of *S. passalidarum* has been shown to simultaneously ferment xylose, glucose and cellobiose under aerobic conditions (Long, Su et al. 2012). Under anaerobic conditions, however, *S. passalidarum* has been shown to sequentially ferment xylose, glucose and cellobiose and have higher efficiency than *P. stipitis* under anaerobic conditions (Hou 2012). Therefore, *S. passalidarum* may be a good candidate for brewing industrial hemp extract as well as for developing recombinant brewing yeast strains.

### 2.1.3 Beer Flavor

Flavor is the most important outcome of the brewing process. Flavor is determined by secondary metabolites, wort composition, fermentation conditions and yeast type. Secondary metabolites are intermediate compounds and by-products which are formed during fermentation. Higher alcohols, esters, vicinal diketones (VKDs) and carbonyl and sulphur compounds are the major flavor contributors produced by yeast at very low concentrations (Steensels, Snoek et al. 2014). Production of some flavor-active compounds, such as diacetyl, must be kept below a threshold, otherwise, they give beer an undesirable flavor (Olaniran, Hiralal et al. 2017).

The type of yeast used for brewing is a major factor that influences beer flavor. Ale and lager are the two main groups of brewing yeast. Ale strains mostly belong to *S. cerevisiae* species, whereas lager yeast is classified as *S. pastorianus* which is known to be an interspecific hybrid of *S. cerevisiae* and cold-tolerant *S. eubayanus* (Dunn and Sherlock 2008). These yeast strains enable the production of numerous types of beer with different flavor. Lager type beer is brewed at low temperatures which enable lager yeast to slowly ferment carbohydrates and result in very few off-flavor by-products, therefore, lager type beer has a crisp clean flavor (Mosher and Trantham 2017). On the other hand, ale type beer is brewed at warmer temperatures, thus, ale yeasts produce more secondary metabolites. A broader variety of ale type beer exists and ale type beers usually have fruity and floral aroma (Mosher and Trantham 2017).

Brewing process variables such as temperature, pitching rate, specific gravity and wort composition affect yeast metabolism and therefore, the flavor-active compound content in beer. For example, acid and higher alcohol concentrations were found to have increased with higher temperature and decreased with dissolved oxygen level (Webersinke, Klein et al. 2018). Increasing temperature up to 25 °C also improved ester production and supplementing wort with essential nutrients required for yeast growth was reported to be an effective way to optimize ester content in Ale beer (Hiralal, Olaniran et al. 2014). High-gravity brewing (specific gravity > 1.075) is a brewing method that uses wort at higher than normal concentrations. This method was reported to increase ester production in lager

beer, as well (Verstrepen, Derdelinckx et al. 2003). Increasing pitching rate, the amount of yeast added to wort, has been shown to decrease isoamyl acetate production and promote the production of some higher alcohols such as 2- and 3-methyl-1-butanol (Erten, Tangüler et al. 2007).

Strains of brewing yeast have been engineered for enhanced production of flavor-active compounds (i.e. esters) while decreasing production of off-flavor compounds (i.e. VDKs, sulfur compounds) through evolutionary and genetic engineering methods (Table 2.1). Genetic engineering methods are available for rapid and efficient strain improvement. These methods allow direct modification of genomic material of organisms through insertion or deletion of specific genes. Recently, genetic engineering was applied to *S. cerevisiae* for production of flavor-active hop compounds (Denby, Li et al. 2018, Guo, Shen et al. 2019). Using these recombinant *S. cerevisiae* strains in brewing may decrease the cost of production as hops will not be needed for flavor formation. A recombinant *S. pastorianus* was also developed to improve flavor stability of beer and decrease off-flavor formation during fermentation (Wang, Xu et al. 2014, Mertens, Gallone et al. 2019). However, these recombinant strains are considered as genetically modified organisms (GMOs). Although, genetic engineering methods can be used to obtain beer with alternative flavors, legislations on GMOs and low consumer acceptance of using GMOs in brewing restrict strain improvement of brewing yeasts through these methods (Gibson, Geertman et al. 2017).

Non-GM but enhanced strains can be obtained using methods such as, mutagenesis, laboratory evolution or hybridization (Table 2.1). For example, brewing strains were mutagenized to decrease the production of off-flavor compounds such as acetaldehyde and 4-vinyl guaiacol (Shen, Wang et al. 2014, Diderich, Weening et al. 2018). Similarly, laboratory evolution was recently used for reducing off-flavors caused by diacetyl production (Gibson, Vidgren et al. 2018). Some *Saccharomyces* strains were hybridized to broaden the flavor profile of beer by increasing ester production (Steensels, Meersman et al. 2014, Mertens, Steensels et al. 2015, Krogerus, Arvas et al. 2016). Hybridization studies were particularly conducted to understand the hybrid nature of *S. pastorianus* as well as to develop improved lager strains compared to the traditional lager yeast. These strain



improvement methods take advantage of mechanisms that naturally increase diversity among species. According to Canadian General Standards Board, organisms developed using mutagenesis, hybridization or cell fusion techniques are not considered as GMO, as long as the organisms used are in the same taxonomic family (Government of Canada 2016). Therefore, strains developed through these methods can fulfill the demand for diversity in the beer industry while respecting customer concerns and legislations on GMOs (Gibson, Geertman et al. 2017).

Non-*Saccharomyces* species have also drawn attention due to increasing demand for craft beers and alternative flavors. These species have been primarily considered as spoilage microorganisms. However, studies have shown that these species could also be used for brewing to create unique flavors. For example, *Dekkera bruxellensis*, a yeast used for sour beer brewing, was reported to produce a variety of ester compounds which give fruity or floral aromas (Crauwels, Steensels et al. 2015). The yeast, *D. bruxellensis*, is also able to enzymatically hydrolyze glycosides which can further improve production of flavor-active compounds (Basso, Alcarde et al. 2016). The yeast, *Pichia anomala*, is able to metabolize a wide range of carbohydrates including pentose carbohydrates, xylose and arabinose (Walker 2011). *P. anomala* has been found to be a good producer of ethyl acetate which produces beer with fruity or solvent-like flavors, depending on the concentration (Passoth, Fredlund et al. 2006, White and Zainasheff 2010). *Torulaspota delbrueckii* is a stress tolerant (temperature and pH) yeast species that was shown to be able to produce beer with fruity and floral flavors due to its high ester and amyl alcohol production (Capece, Romaniello et al. 2018). Recently, *Saccharomycopsis fibuligera*, *Schizosaccharomyces pombe* and *Zygosaccharomyces rouxii* have been investigated and found to produce desirable fruity flavors and were able to ferment maltose and maltotriose (Methner, Hutzler et al. 2019).

**Table 2.1.** Recent studies on improving *Saccharomyces* strains through genetic engineering, hybridization, laboratory evolution and mutagenesis.

Application	Parental Strain(s)	Method	Improved Phenotype	Reference
Flavor formation	<i>S. cerevisiae</i>	Genetic engineering	Hop lupulone production	Guo, Shen et al. (2019)
	<i>S. cerevisiae</i>	Genetic engineering	Hop monoterpene production	Denby, Li et al. (2018)
	<i>S. cerevisiae</i>	Genetic engineering	Raspberry ketone production	Lee, Lloyd et al. (2016)
	<i>S. cerevisiae</i> & <i>S. eubayanus</i>	Hybridization	Increased ester production	Krogerus, Arvas et al. (2016)
	Various <i>S. cerevisiae</i> & <i>S. eubayanus</i> strains	Hybridization	Broader flavor profile	Mertens, Steensels et al. (2015)
	<i>S. cerevisiae</i>	Genetic engineering	$\beta$ -ionone production	Beekwilder, van Rossum et al. (2014)
	<i>S. cerevisiae</i> , <i>S. paradoxus</i> , <i>S. pastorianus</i>	Hybridization	Increased isoamyl acetate production	Steensels, Meersman et al. (2014)
	<i>S. cerevisiae</i>	Laboratory evolution	Increased thiol production	Belda, Ruiz et al. (2016)
Flavor stability	<i>S. pastorianus</i>	Genetic engineering	Improved anti-stalling	Wang, Xu et al. (2014)
Off-flavor reduction	<i>S. pastorianus</i>	Genetic engineering	Decreased 4-vinyl guaiacol production	Mertens, Gallone et al. (2019)
	<i>S. pastorianus</i>	Laboratory evolution	Decreased diacetyl production	Gibson, Vidgren et al. (2018)
	<i>S. eubayanus</i>	UV mutagenesis	Decreased 4-vinyl guaiacol production	Diderich, Weening et al. (2018)
	<i>S. pastorianus</i>	UV mutagenesis	Decreased acetaldehyde production	Shen, Wang et al. (2014)

Adapted from Krogerus, Magalhães et al. (2017), Gorter de Vries, Pronk et al. (2019)

## 2.2 Genome Shuffling

Genome shuffling is a combinatorial genetic improvement method which was first introduced by Zhang, Perry et al. (2002). Genome shuffling is recombination between multiple parents of each generation for several rounds to obtain a recombinant that has genetic traits from multiple parents (Leja, Myszka et al. 2011). The main advantage of genome shuffling is that genetic information for the desired phenotypes is not required to develop an improved strain. It is a technique that results in genome-wide changes based on genome plasticity, thus, it does not need the genome sequence data or metabolic network information (Gong, Zheng et al. 2009). However, genome shuffling is random and dependent on the chance of recombination. Therefore, molecular biological techniques are not necessary to extract and implement specific genes for engineering recombinant strains.

In addition, studies, which were reviewed in the following subsections, showed that two rounds of genome shuffling were usually sufficient to obtain an improved strain whereas classical strain improvement techniques such as mutagenesis and laboratory evolution required 20 or more rounds (Zhang, Perry et al. 2002). Thus, genome shuffling is a less time-consuming and less laborious method.

Genome shuffling has three main steps: parental library generation, recursive recombination of parental strains and, screening and selection of recombinants with desired phenotypes. The parental library may be created using chemical or physical mutagenesis to generate more genotypes. Ethyly methanosulfonate (EMS) or nitrosoguanidine (NTG) are the most commonly used chemical mutagens while ultraviolet (UV) radiation is mainly used as physical mutagen (Leja, Myszka et al. 2011). For more efficient mutagenesis, atmospheric and room temperature plasma (ARTP) can be used (Zhang, Lin et al. 2015). On the other hand, parental library generation using mutagenesis may not be always necessary to develop a recombinant strain. There are studies, which were reviewed in the following subsections, in which recombinant strains were developed using genome shuffling but did not use mutagenesis for parental library generation. Instead, initial strains were used as the sole source of genetic material.

The parental strain selection is important for the next step. If the parental strains are not suitable, the desired phenotype will not be obtained (Gong, Zheng et al. 2009). Studies suggested that genome shuffling should be performed either with large initial population diversity or original strains with higher performance to obtain improved hybrid strains (Gong, Zheng et al. (2009), Hida, Yamada et al. (2007)).

Next, parental strains are recombined. The recombinants obtained can be used for the next round of genome shuffling. This step may be repeated several times. Repetition is fundamental for genome shuffling. Repetition of this step can increase the genetic diversity of parental strains since desired phenotypes can be obtain in less rounds compared to the conventional methods, which increases the efficiency of genome shuffling (Gong, Zheng et al. 2009). Genome shuffling can be performed in three different ways. Recursive protoplast fusion and mating-based genome shuffling are the most common methods which shuffles genomes by hybridization of parental strains. The third technique, electroporation-based genome shuffling was recently studied and recombinants strains were successfully developed, as well (Jutakanoke, Tolieng et al. (2017); Ren, Wang et al. (2016); Zhang and Geng (2012)). Unlike hybridization using recursive protoplast fusion or mating, electroporation-based genome shuffling, recombinants strains are developed by transforming a parental strain using an exogenous genomic DNA of a donor strain. The current literature on genome shuffling with these methods that were applied to yeast strains are discussed in the following sections.

Finally, the desired phenotypes are selected through phenotype screening. The type of phenotype screening varies based on the desired phenotype improvement. The growth characteristics of strains are often analyzed for improved substrate utilization or tolerance to a stress factor on agar plates by observing clear zones, hydrolysis zones, and inhibition zones (John, Gangadharan et al. (2008), Magocha, Zabed et al. (2018)). To analyze productivity, color/fluorescence-based assays can be applied to detect target products which are easily pigmented or affected by florescent (Liu and Jiang 2015).

After genome shuffling, genomic DNA of recombinant strains should be analyzed to confirm that recombination was performed successfully. Most studies compared the DNA

content of recombinant strains to their parental strains through Random Amplified Polymorphic DNA (RAPD) analysis or polymerase chain reaction (PCR). For more accurate characterization, whole genome sequencing can be used to effectively identify genetic changes in recombinant strains since it is difficult to determine the amount of inherited genetic information from each parental strain using mentioned analysis methods (Harner, Wen et al. 2015).

### 2.2.1 Recursive Protoplast Fusion (Asexual Hybridization)

Genome shuffling using recursive protoplast fusion differs from conventional protoplast fusion and allows successive rounds of recombination of DNA of multiple parents, whereas protoplast fusion uses only two parents for recombination of DNA in one step (Magocha, Zabed et al. 2018). Thus, more diversity can be achieved and desired phenotypes can be obtained in a shorter period which makes this method more efficient than conventional protoplast fusion (Gong, Zheng et al. 2009). After constructing the parental library through mutagenesis, protoplasts are prepared using cell wall degrading enzymes, such as lysozyme or snailase. Then, protoplasts are fused together using an electric pulse or polyethylene glycol (PEG), following that, cell walls are regenerated. Finally, the hybrids are screened and selected for desired phenotypes which can be used for next round of shuffling.

This method still carries the advantages and disadvantages of protoplast fusion which is the main part of this method. As long as the microorganisms within the same taxonomic family are used as parental strains, hybrid strains developed using recursive protoplast fusion-based genome shuffling are not considered as genetically modified organisms (GMOs) since the process relies on natural homologous recombination and no gene markers are used (Zhang, Perry et al. 2002, Petri and Schmidt-Dannert 2004, Government of Canada 2016). Protoplast fusion is suitable for strains that cannot sporulate, show unstable mating type or cannot mate with each other (Attfield and Bell (2003); Pretorius (2000)). However, the instability of hybrids increases as the genetic background of parents diverges (Giudici, Solieri et al. 2005). Hybrids often lose chromosomes and segregate into parental strains (Pina, Calderón et al. (1986); Attfield and Bell (2003)). High fusion

frequency relies on genetic proximity of the initial strains and applied fusion protocol (Peberdy (1980); Pina, Calderón et al. (1986); Kavanagh and Whittaker (1996); Attfield and Bell (2003)). Therefore, selection of the initial strains and the optimization of protoplast fusion methods plays a critical role for successful genome shuffling through recursive protoplast fusion. Most recent studies on recursive protoplast fusion-based genome shuffling are listed in Table 2.2.

Recently, hybrids from an industrial *S. cerevisiae* (Ethanol Red®) strain have been developed for thermotolerance and inhibitor tolerance to improve ethanol production from pretreated sorghum (Batog and Wawro 2019). Ethanol Red *S. cerevisiae* was developed for bioethanol production which is capable of providing high ethanol yields at high temperatures (Leaf by Lesaffre 2020). After two rounds of protoplast fusion, the hybrid strain exhibited 40% higher ethanol productivity than its parental strains. RAPD analysis also confirmed that the hybrids had genetic material from both parental strains. Hybrids of *S. cerevisiae* YS86, an industrial brewing strain, were developed for reduced acetaldehyde yield (Yin, Liu et al. 2017) and increased glutathione yield (Yin, Ma et al. 2016), which achieved 65% decrease in acetaldehyde production and 3-fold increase in glutathione production, respectively. However, genetic stability of these hybrids was not investigated.

Various other yeast strains have also been developed for use in the biofuel, food and pharmaceutical industries. Genome shuffling was successfully applied to *Candida parapsilosis* DSM 70125 for improved arabinol yield to be used in the food industry for low calorie sweetener production (Kordowska-Wiater, Lisiecka et al. 2018). One stable hybrid had 16% higher arabinol productivity than the parental strain. *Pichia anomala* TIB-x229 was subjected to two rounds of protoplast fusion for increased sugar alcohol production (Zhang, Lin et al. 2015). The improved strain had 32% increased sugar alcohol production than the parental strain. For ethanol production from lignocellulosic hydrolysates, two rounds of protoplast fusion on *Pichia stipitis* ATCC 58376 resulted in a genetically stable hybrid strain that produced 50% more ethanol than the wild-type *P. stipitis* (Shi, Zhang et al. 2014).

In the most recent reports, intergeneric hybridization was successfully performed using recursive protoplast fusion-based genome shuffling, as well. A hybrid yeast strain was developed using *S. cerevisiae* NCIM 3090 and *P. stipitis* NCIM 3497 in order to obtain a strain that had higher carbohydrate utilization, ethanol productivity and ethanol tolerance than the parental strains, which are indispensable traits for bioethanol production (Jetti, Gns et al. 2019). Two rounds of protoplast fusion resulted in a genetically stable hybrid strain which was able to utilize both glucose and xylose in lignocellulosic biomass and achieved 124% and 14% higher ethanol productivity than the parental strains *S. cerevisiae* NCIM 3090 and *P. stipitis* NCIM 3497, respectively. A thermotolerant yeast strain with xylose utilization capacity was developed using a thermotolerant yeast *S. cerevisiae* ScY01 and a natural xylose-fermenting yeast *S. passalidarum* NRRL Y-27907 for improved ethanol productivity and co-utilization of xylose and glucose at high temperature (Lin, Cai et al. 2019). After one round of protoplast fusion a hybrid strain that had 39% increased glucose consumption and 35% increased xylose consumption rate was obtained. Further improvements through adaptive evolution resulted in evolved strains with even higher ethanol productivity from co-utilization of glucose and xylose at 40 °C compared to the hybrid strain, which demonstrates the applicability of evolutionary engineering to hybrid strains developed through this method. It should be noted that genetic diversity of the parental strains was not increased through mutagenesis in any of these applications of intergeneric hybridization.

**Table 2.2.** Recent studies on recursive protoplast fusion-based genome shuffling

Parental Strain(s)	Phenotype of Interest	Source of Diversity	No. of GS rounds	Industry	Reference
<i>S. cerevisiae</i> ScY01 <i>S. passalidarum</i> NRRL Y-27907	Thermotolerance, Xylose utilization	Whole genome transfer	1	Biofuel	Lin, Cai et al. (2019)
<i>S. cerevisiae</i> NCIM 3090 <i>P. stipitis</i> NCIM 3497	Ethanol tolerance, Xylose utilization	Whole genome transfer	2	Biofuel	Jetti, Gns et al. (2019)
<i>S. cerevisiae</i> (Ethanol Red)	Thermotolerance, inhibitor tolerance	EMS mutagenesis	2	Biofuel	Batog and Wawro (2019)
<i>C. parapsilosis</i> DSM 70125	Arabitol yield	UV mutagenesis	2	Food	Kordowska-Wiater, Lisiecka et al. (2018)
<i>S. cerevisiae</i> YS86	Acetaldehyde yield	UV + NTG mutagenesis	2	Beer	Yin, Liu et al. (2017)
<i>S. cerevisiae</i> YS86	Glutathione yield	UV+NTG mutagenesis	2	Beer, Food, Medicine	Yin, Ma et al. (2016)
<i>P. anomala</i> TIB-x229	Sugar alcohol yield	UV + ARTP mutagenesis	2	Food, Medicine	Zhang, Lin et al. (2015)
<i>P. stipitis</i> ATCC 58376	Ethanol yield	UV mutagenesis	2	Biofuel	Shi, Zhang et al. (2014)



### 2.2.2 Mating-based Genome Shuffling (Sexual Hybridization)

Recursive mating-based genome shuffling relies on mating and sporulation cycles of yeast species. In this method, mutants of haploid parental strains are mixed and subjected to mass mating to produce diploid generations. Then, diploid generations are sporulated and spores are segregated. Finally, the haploid cells are regenerated and germinated to screen for the desired phenotypes. Selected hybrid strains can then be used for starting strains for the next round of mating.

Hybrid strains obtained through this method were reported to be stable haploids and amenable to further improvement by mutagenesis and mating (Harner, Wen et al. 2015). However, mating-based genome shuffling is limited to species with well-known and easily manipulated sexual cycles (Biot-Pelletier and Martin 2014). Well-optimized mating protocol is crucial for efficient utilization of this method to develop enhanced strains as the success of this method relies on mating frequency of parental strains (Harner, Bajwa et al. 2015). Most studies which applied this method developed intraspecific hybrids, *S. cerevisiae* being the most frequently used yeast strain. Recent reports based on mating-based genome shuffling have investigated improved ethanol tolerance in pretreated lignocellulosic biomass (Table 2.3).

Acid and heat tolerance of a xylose-fermenting *S. cerevisiae* was recently developed for use in simultaneous saccharification and co-fermentation of lignocellulosic biomass (Inokuma, Iwamoto et al. 2017). Mutants of acid-tolerant *S. cerevisiae* Sun049T-Z and thermotolerant *S. cerevisiae* Sun224T-K were shuffled using spore mating and screened for improved tolerance to acetic acid and formic acid at 38 °C. Before genome shuffling, drug resistance markers were integrated into parental strain DNA. This enables the elimination of unmated cells by selecting for double resistance (Zheng, Wu et al. 2011). One of the hybrids obtained exhibited enhanced xylose consumption and ethanol production rates compared to both parents. Also, DNA microanalysis showed 13 genes in the hybrid strain were upregulated more than in both parents under co-stress conditions. It should be noted that genetic stability of the hybrid was not investigated and only one round of genome shuffling was conducted.

**Table 2.3.** Recent studies on recursive mating-based genome shuffling

Parental Strain(s)	Phenotype of Interest	Source of Diversity	No. of GS rounds	Industry	Reference
<i>S. cerevisiae</i> Sun049T-Z <i>S. cerevisiae</i> Sun224T-K	Acid and heat tolerance	UV mutagenesis	1	Biofuel	Inokuma, Iwamoto et al. (2017)
Industrial <i>S. cerevisiae</i> strains	Ethanol tolerance, ethanol productivity	Whole genome transfer	3	Biofuel	Snoek, Picca Nicolino et al. (2015)
<i>P. tannophilus</i> NRRL Y-2460	Inhibitor tolerance	UV mutagenesis	3	Biofuel	Harner, Wen et al. (2015)
<i>S. cerevisiae</i> ZTW1	Ethanol tolerance	MBC treatment	3	Biofuel	Zheng, Chen et al. (2014)
Recombinant <i>S. cerevisiae</i> (Ethanol Red)	Inhibitor tolerance	EMS mutagenesis	1	Biofuel	Demeke, Dietz et al. (2013)
<i>P. stipitis</i> NRRL Y-7124	Inhibitor tolerance	UV mutagenesis	4	Biofuel	Bajwa, Pinel et al. (2010)

In another study, three rounds of genome shuffling was applied to various industrial *S. cerevisiae* strains using random mating (Snoek, Picca Nicolino et al. 2015). For random mating, they first created and pooled eight parental strains to allow them to mate randomly. After one round of genome shuffling, ethanol tolerance and ethanol productivity of the hybrid strains did not continue to increase, which they suggested this may be due to the decrease in probability of inheriting beneficial alleles with each round (Snoek, Picca Nicolino et al. 2015). In contrast, *S. cerevisiae* ZWT1 was genome-shuffled for enhanced ethanol tolerance under very high gravity conditions and, increased ethanol tolerance and ethanol productivity at each round of mating for three rounds (Zheng, Chen et al. 2014).

On the other hand, more rounds of genome shuffling may be needed for hybridization of different genera. In one study an improved strain of *Pachysolen tannophilus* NRRL Y-2460 was developed for better inhibitor tolerance for fermenting hardwood spent sulfide liquor (HW SSL) (Harner, Bajwa et al. 2015). After three rounds of mating, one of the hybrid strains produced 37% more ethanol than the parental strain. *P. tannophilus* NRRL Y-2460 was reported to have had low mating frequency, which limited the number of starting strains for next rounds of mating, therefore genetic diversity was limited (Harner, Bajwa et al. 2015). In another study, inhibitor tolerance of *P. stipitis* in HW SSL showed progressive improvement up to four rounds of mating (Bajwa, Pinel et al. 2010).

Mating-based genome shuffling can also be combined with evolutionary engineering. One round of genome shuffling followed by multiple rounds of directed evolution was applied to recombinant an industrial strain, Ethanol Red *S. cerevisiae*, to obtain a hybrid strain with increased tolerance to inhibitor-rich lignocellulose hydrolysates for effective xylose utilization. (Demeke, Dietz et al. 2013). The best performing hybrid had 32% higher ethanol yield compared to xylose-fermenting Ethanol Red strain, due to improved xylose consumption under inhibitor stress. The hybrid maintained its xylose fermenting ability for 50 generations.

### 2.2.3 Electroporation-based Genome Shuffling

Electroporation-based genome shuffling is the most recent development in this field of strain improvement. This method is less laborious and time-consuming than recursive protoplast fusion- and mating-based genome shuffling (Zhang and Geng 2012). However, this method requires expensive lab equipment for electroporation.

In this method, genomic DNA of one parental strain is extracted and transferred into the host strain using electroporation. Then, the recombinants are screened for desired phenotype and are used as the host strains for the next round of electroporation. Genomic DNA extraction randomly creates small fragments of the DNA. Transferring these small fragments through electroporation improves gene transfer and recombination efficiency compared to protoplast fusion, however there is no control over the size of the DNA fragments and separation locations of the DNA fragments (Zhang and Geng 2012). Unlike protoplast fusion, the cell wall is not removed but made permeable for DNA to pass through so this method does not require cell fusion and regeneration of cell walls. To our knowledge, only three studies with this method applied to yeast have been published to date and mainly focused on improvement of ethanol productivity and xylose utilization for bioethanol production from lignocellulosic feedstock (Zhang and Geng 2012, Ren, Wang et al. 2016, Jutakanoke, Tolieng et al. 2017). The genetic diversity of the recombinant strains obtained in these studies was limited to the genetic information of the initial strains, since genetic diversity was not increased through mutagenesis.

Electroporation-based genome shuffling was first applied to a wild-type *S. cerevisiae* ATCC 24860 to develop a xylose-fermenting recombinant strain for efficient bioethanol production from lignocellulosic biomass (Zhang and Geng 2012). Genomic DNA of *P. stipitis* CBS 6054, was transferred into *S. cerevisiae* ATCC 24860. The recombinant strains were selected on agar plates containing xylose and ethanol for improved xylose utilization and ethanol tolerance. After two rounds of electroporation, the best performing recombinant strain had 38% increased xylose consumption and 49% increased ethanol productivity, compared to *P. stipitis* CBS 6054. RAPD analysis showed that genetic information of the recombinant was closer to that of *P. stipitis* CBS 6054. Moreover, RAPD

analysis of the recombinant strain at different times over the course of a year was unchanged and had similar profiles, which confirmed the genetic stability of the recombinant strain.

A thermo-tolerant, xylose-fermenting recombinant strain was developed for simultaneous fermentation of glucose and xylose from pretreated corn stover (Ren, Wang et al. 2016). The recombinant strain was obtained by transferring genomic DNA of xylose-fermenting yeast *C. intermedia* strain 23 into thermo-tolerant yeast *S. cerevisiae* and screening for growth on xylose plates at 37 °C. One of the recombinants obtained had 28% higher xylose consumption rate than that of *C. intermedia* strain 23. RAPD analysis confirmed the recombinant nature of the strain. However, only one round of electroporation was performed, thus, it is not known whether more rounds further would have improved xylose consumption.

In another study, three rounds of electroporation was performed for improved ethanol productivity from xylose (Jutakanoke, Tolieng et al. 2017). The aim of this study was to develop a recombinant strain for simultaneous fermentation of xylose and glucose from pretreated sugarcane leaves. Genomic DNA of *P. stipitis* JCM 10742 was introduced into a cellobiose-fermenting yeast, *K. marxianus* G2-16-1. After screening for improved ethanol tolerance, one of the recombinants obtained had ethanol yield from xylose 10% higher than that of *K. marxianus* G2-16-1. Glucose and xylose fermentation capacity were stable for up to 12 generations. It was reported that two more rounds of electroporation after the first round did not further improve ethanol productivity.

## Chapter 3

### 3 Comparison of Xylose-Fermenting Yeasts

#### 3.1 Introduction

Natural xylose-fermenting yeasts have received increasing attention due to their ability to convert mixed carbohydrates in lignocellulosic biomass to ethanol. Lignocellulosic hydrolysates mainly contain a mixture of disaccharide (cellobiose), hexose (glucose) and pentose (xylose) carbohydrates. Xylose-fermenting yeasts can ferment lignocellulosic hydrolysates completely in which xylose is the second most abundant carbohydrate. Co-utilization of cellobiose, glucose and xylose can improve ethanol productivity. Brewing yeast is the workhorse of the beer industry but it is not capable of metabolizing xylose. Therefore, a yeast with efficient cellobiose, glucose and xylose co-utilization ability is needed for complete brewing of hemp extract.

In this chapter, two xylose-fermenting yeast, *Pichia stipitis* DSM3651 and *S. passalidarum* MYA4345 were evaluated. Firstly, the growth of xylose-fermenting yeasts, then, the xylose-fermenting yeasts were evaluated based on their capacity at different temperatures and aeration levels to ferment a mixture of cellobiose, glucose and xylose in which xylose was the most abundant carbohydrate as well as in synthetic hemp extract which contained mostly glucose.

*Pichia stipitis* DSM3651, is a haploid xylose-fermenting yeast that was isolated from larvae of wood-inhabiting insects (Toivola, Yarrow et al. 1984). *P. stipitis* is able to utilize most of the carbohydrates in lignocellulosic biomass such as glucose, mannose, galactose, cellobiose and oligomers of xylan and mannan (Agbogbo and Coward-Kelly 2008). It is one of the most studied xylose-fermenting yeast for ethanol production from lignocellulose. *Spathaspora passalidarum* MYA4345 was isolated from wood-boring beetles (Nguyen, Suh et al. 2006). It has the ability to simultaneously utilize cellobiose, glucose, xylose under aerobic conditions (Long, Su et al. 2012). It has been long known that glucose represses activity of enzymes for xylose consumption and competes with

xylose for transport through membrane proteins. On the other hand, co-utilization of cellobiose and xylose is easier as cellobiose does not compete with xylose (Ha, Galazka et al. 2011).

Beer is fermented in oxygen-limited environment and usually at low temperatures. Brewing temperature depends on the type of beer that is desired to be produced. Ale beer requires a temperature between 12-25 °C, while lower temperatures (4-15 °C) are necessary for lager beer (Willaert 2006). Although wort is initially aerated during yeast pitching to promote cell growth, fermentation is conducted under anaerobic conditions. Temperature and aeration are the factors which mainly determine the fermentation performance. Therefore, it is necessary to investigate xylose-fermenting yeast strains in brewing conditions.

Ethanol accumulation during fermentation can have inhibitory effects on yeast cells. High ethanol concentration decreases cell viability and vitality and, limits carbohydrate uptake, ethanol productivity and yield (Stewart 2017). Xylose-fermenting yeasts tend to have preferential uptake of glucose over xylose during lignocellulosic ethanol production. Xylose utilization needs to continue when ethanol concentration is high. Therefore, studying the ethanol tolerance of xylose-fermenting yeasts can help understand their potential usage in brewing of lignocellulosic biomass.

## 3.2 Materials and Methods

### 3.2.1 Strains and Media

Yeast strains used in this study were listed in Table 3.1. Pure cultures of each strains were stored in 20% glycerol at -80 °C. *S. pastorianus* WLP830 was used as the control strain.

For growth assessment, ethanol tolerance and shake flask fermentation experiments, seed cultures were propagated in YM media (yeast extract, 3 g/L; malt extract, 3 g/L; glucose, 10; g/L peptone, 5 g/L) in 125-mL flasks at 20 °C and 130 rpm by inoculating from cultures stored at -80 °C. The media used for flask fermentation and ethanol tolerance experiments

contained yeast nitrogen base (YNB) without amino acids (ammonium sulfate), 13.4 g/L; cellobiose, 5 g/L; glucose, 5 g/L; xylose, 20 g/L, unless stated otherwise. Varying glucose, xylose and YNB concentrations were used for growth assessment experiments. Media containing YNB were sterilized using 0.2 um filtration and, flasks were autoclaved at 121 °C for 15 min.

**Table 3.1.** Strains used in this study.

Organism	Strain	Type	Source
<i>Spathaspora passalidarum</i>	MYA4345	Xylose-fermenting yeast	Cedarlane
<i>Pichia stipitis</i>	DSM3651	Xylose-fermenting yeast	DSMZ
<i>Saccharomyces pastorianus</i>	WLP830	Lager yeast	Toronto Brewing

For synthetic hemp extract fermentation, the medium contained cellobiose, 2.2 g/L; glucose, 66 g/L; xylose, 11 g/L; casamino acids, 5 g/L; yeast extract, 1 g/L; peptone, 1 g/L and supplemented with vitamins, minerals and salts. Vitamin and mineral solutions were prepared based on the essential requirements for yeast, and listed in Appendix A (Table A.1, Table A.2, 3) The medium was prepared by combining two separate solutions due different sterilization requirement of the compounds in synthetic hemp extract. The solution containing carbohydrates, casamino acids and the vitamin and mineral solutions were sterilized using 0.2 um filtration. The solution containing yeast extract, peptone and salts were autoclaved at 121 °C for 20 min.

### 3.2.2 Growth Assessment

Seed cultures were grown overnight in YM media at 20 °C, then centrifuged at 3500 rpm and 4 °C for 15 min. Inoculum was made by pouring off the supernatant and the cell pellets were washed once with 10 mL sterile water and resuspended in sterile water with the same volume as the media removed. Initial OD<sub>600</sub> was approximately 0.4. Growth on glucose-only (20 g/L), xylose-only (20g/L) were assessed in a 96-well plate at 25 °C. All media contained 13.4 g/L of YNB without amino acids (containing ammonium sulfate). Each



column ( $n = 8$  wells) of the 96-well plate was used to evaluate one variable. Level of oxygen was altered for each plate, by changing the length of mixing and covering on the 96-well plate. Each well contained 300  $\mu\text{L}$  of growth media and 20  $\mu\text{L}$  of inoculum. Tecan M200 PRO plate reader was used to measure the optical density at 600 nm. Measurements were taken every 30 minutes over 24 hours. Data was recorded to a spreadsheet by i-control™ Microplate Reader Software from Tecan. Specific growth rates ( $\text{h}^{-1}$ ) were calculated using R package, named Growthcurver (Sprouffske K.), by fitting the data to a logistic equation shown in Equation 3.1 where  $K$  is the carrying capacity,  $N_t$  the initial population size,  $r$  is the intrinsic growth rate ( $\text{h}^{-1}$ ) and  $t$  is time (h).

$$N_t = \frac{K}{1 + \left(\frac{K - N_0}{N_0}\right) e^{-rt}}$$

**Equation 3.1.** Logistic Equation for cell growth

### 3.2.3 Flask Fermentations

Effect of temperature at 17, 21 and 25 °C in aerobic and anaerobic conditions were assessed. *P. stipitis* and *S. passalidarum* were evaluated in YNB-CGX (cellobiose, glucose, xylose) medium. Control experiments were conducted in YNB-G using *S. pastorianus*. All experiments were conducted in triplicate 125-mL flasks with a working fermentation volume of 50 mL. Flasks were autoclaved at 121 °C for 15 min.

Seed cultures were grown in 20 mL YM media overnight at 20 °C and 130 rpm in 125-ml flask. Then, the seed cultures were transferred to sterile 500-ml flasks and the volumes were scaled up to 200 ml by adding fresh YM media. The temperature was increased to 25 °C and shaking to 145 rpm. When the  $\text{OD}_{600}$  reached between 1.0-1.2, the seed cultures were transferred into 50-mL centrifuge tubes, one per fermentation flask (approximately 25 mL per flask). This resulted in an initial cell concentration represented by an  $\text{OD}_{600}$  of 0.5 in the fermentation flasks. The 25 ml of cells were pelleted by centrifugation at 3500 rpm and 4 °C for 15 minutes and washed once with 25 ml sterile water to remove YM media. The supernatant was discarded. The cell pellets were resuspended in 50 ml total

volume of fermentation medium. Aerobic flasks were covered with tin foil for passive gas transfer from the environment. Airlocks and stoppers were placed on anaerobic flasks. Airlocks were filled with water to maintain the anaerobic environment. The total fermentation duration was 120 hours. Samples were taken every 24 hours to determine carbohydrate, ethanol, by-product and biomass concentrations.

For fermentation in synthetic hemp extract, seed cultures were grown in 10 mL YM media overnight at 20 °C and 130 rpm in 125-ml flask. Then, the seed cultures were scaled up to a final volume of 100 mL in sterile 500-ml flasks. Seed cultures were grown to OD<sub>600</sub> 1.2 - 1.4. Then, 50 mL of seed cultures per fermentation flask was pelleted at 4 °C and 3500 rpm for 15 min and the supernatant was removed. The cell pellets were washed once with sterile water and resuspended with the fermentation medium. The working fermentation volume was 100 mL. The experiment was conducted in duplicate at 23 °C with shaking at 145 rpm in aerobic and anaerobic conditions. Samples were taken every 24 hours to determine carbohydrate, ethanol, by-product and biomass concentrations. *S. pastorianus* was used as the control strain.

Ethanol productivities were calculated at the time of maximum ethanol concentration. Consumption rates were calculated using linear regression between time points of maximum consumption. For calculation of biomass concentrations, calibration curves of OD<sub>600</sub> versus dry cell weight were used (Figure A.1).

### 3.2.4 Analytical methods

A Waters HPLC system was used with an isocratic pump (1515) and degasser, autosampler and refractive index detector (RID). The column was an Agilent HiPlex H Column and guard. The operating parameters were: flow rate 0.6 ml/min; mobile phase 5 mM H<sub>2</sub>SO<sub>4</sub>; a column temperature of 65°C and a RID temperature of 50 °C. Cellobiose, glucose, xylose, galactose, arabinose, xylitol, lactic acid, glycerol, acetic acid, levulinic acid and ethanol concentrations were measured. Control standards used to prepare calibration curves were run often as unknown samples to validate the method. Biomass was monitored

spectrophotometrically by measuring OD<sub>600</sub>. Samples were diluted so that the OD<sub>600</sub> values were in the range of the calibration curves and then multiplied by dilution ratios.

### 3.2.5 Ethanol Tolerance

Seed cultures were grown overnight in YM at 20 °C, then centrifuged at 3500 rpm and 4 °C for 15 min. Cell pellets were washed once with 10 mL sterile water and resuspended in sterile water with the same volume of media removed. The growth medium was YNB-CGX which was modified with 100% ethanol to obtain concentrations from 0 to 194 g/L (0-25% v/v) ethanol. The wells of the 96-well plate were filled with 240 µL of growth medium containing varying amounts of ethanol and 20 µL inoculum. Target initial OD<sub>600</sub> was approximately 0.5. Plates were covered with a gas impermeable transparent film and 3 holes per well were punched with a sterile needle to have an aerobic environment. Growth was monitored using TECAN M200 plate reader with continuous shaking at 25 °C. Measurements were taken every 30 minutes over 24 hours. Each column of the 96-well plate was used to assess one variable (n=8). Control wells had YNB-CGX without ethanol and were inoculated with the strains evaluated for this study.

The area under the OD<sub>600</sub> versus time curve of the control was compared to the areas of the tests. The relative amount of growth to the control was defined as the fractional area ( $f_a$ ) which was the ratio of the test area to the control area (Equation 3.2).

$$f_a = \frac{area_{test}}{area_{control}}$$

#### **Equation 3.2.** Fractional Area

The plots were analyzed using a modified Gompertz function of decay (Equation 3.3), where A is the lower asymptote of , B is a slope parameter, C is the distance between the upper and lower asymptote, and M is the log<sub>10</sub> ethanol concentration of the inflexion point. A, C, B and M parameters were obtained by nonlinear least squares regression. Then, non-inhibitory (NIC) (Equation 3.4) and minimum inhibitory (MIC) (Equation 3.5)

concentration parameters were calculated. Parameter estimation and NIC and MIC calculations were done using MATLAB.

$$f_a = A + C e^{-e^{B(x-M)}}$$

**Equation 3.3.** Modified Gompertz Function of Decay

$$NIC = 10^{\left(M + \frac{1}{B}\right)}$$

**Equation 3.4.** Non-inhibitory Concentration

$$MIC = 10^{\left(M - \frac{1.718}{B}\right)}$$

**Equation 3.5.** Minimum Inhibitory Concentration

### 3.3 Results and Discussion

#### 3.3.1 Growth Assessment

The growth of two xylose-fermenting yeast strains on glucose and xylose separately were assessed on a 96-well plate. The level of oxygen was altered using a lid versus a film with holes. One plate for each aeration level was prepared for assessment. The number of holes on the transparent film and the shaking duration determined the level of oxygen transfer to the growth medium. Each level was categorized as anaerobic, microaerobic and aerobic, based on the level of oxygen transfer and the shaking duration. The anaerobic environment was ensured by placing a lid on the plate and shaking for 2 min before measurement. A transparent film was applied to the microaerobic and aerobic plates. One hole was punched on the microaerobic plate and was shaken for 2 min before measurement while the aerobic plate had three holes and was shaken continuously.

Logistic equation (Equation 3.1) was used to calculate the growth rates. Logistic equation is commonly used in ecology and evolution for growth assessment (Rockwood 2015). An R package, named Growthcurver, was used to fit the growth data to Equation 3.1, using a non-linear least-squares Levenberg-Marquardt method (Sprouffske and Wagner 2016). The growth rates were reported in Table 3.2.

**Table 3.2.** Specific growth rates ( $\text{h}^{-1}$ ) on 20 g/L glucose and 20 g/L xylose with three different aeration levels at 25 °C. Each value shows the mean values of eight wells. Deviation from the mean was less than 4% in all samples.

<b>Strain</b>	<b>Medium</b>	<b>Medium</b>
<b>Aeration</b>	<b>Specific Growth Rate (<math>\text{h}^{-1}</math>)</b>	<b>Specific Growth Rate (<math>\text{h}^{-1}</math>)</b>
<b><i>P. stipitis</i> DSMZ3651</b>	<b>20 g/L Glucose</b>	<b>20 g/L Xylose</b>
Anaerobic	0.17	0.13
Microaerobic	0.20	0.15
Aerobic	0.29	0.26
<b><i>S. passalidarum</i> MYA4345</b>	<b>20 g/L Glucose</b>	<b>20 g/L Xylose</b>
Anaerobic	0.23	0.17
Microaerobic	0.25	0.19
Aerobic	0.28	0.22

**Anaerobic:** Lid with shaking for 2 min before measurement; **Microaerobic:** Gas impermeable film with 1 hole and shaking for 2 min before measurement; **Aerobic:** Gas impermeable film with 3 holes and shaking continuously

For both *P. stipitis* and *S. passalidarum*, the growth on glucose and xylose were improved with a more aerobic environment. Overall, *S. passalidarum* had higher growth rates in glucose than *P. stipitis*. Growth on xylose was slower than the growth on glucose for both strains in all aeration levels. Similar results on the growth of xylose-fermenting yeasts on xylose were previously reported by Hou (2012), as well. In xylose-only media, the growth rate of *P. stipitis* in the most aerobic condition ( $0.26 \text{ h}^{-1}$ ) was 2-fold faster than the growth rate ( $0.13 \text{ h}^{-1}$ ) in the anaerobic condition and was also faster than the growth rate of *S.*

*passalidarum* ( $0.22 \text{ h}^{-1}$ ) in the most aerobic condition. However, *S. passalidarum* had a higher growth rate ( $0.17 \text{ h}^{-1}$ ) in the anaerobic environment than *P. stipitis* ( $0.13 \text{ h}^{-1}$ ). This was likely due to NADH-dependent XR that *S. passalidarum* uses in oxygen-limited conditions, which enables cofactor regeneration without using oxygen for proton transport. Since *P. stipitis* only has the NADPH-preferred enzyme, oxygen limitation affects the growth of *P. stipitis* on xylose more than that of *S. passalidarum*.

### 3.3.2 Effect of aeration and temperature on *P. stipitis* and *S. passalidarum*

Shake flask fermentation experiments were conducted to analyze the impact of aeration and temperature on *P. stipitis* and *S. passalidarum* when xylose is the most abundant carbohydrate in the mixed carbohydrate medium, YNB-CGX. Fermentation performance of *P. stipitis* and *S. passalidarum* were assessed at three temperature levels (17, 21 and 25 °C) in aerobic and anaerobic conditions. Cellobiose, glucose and xylose consumption in both conditions were shown in Figure 3.1.

Cellobiose consumption of *P. stipitis* started after glucose was completely consumed in 24 h in all aeration and temperature conditions (Figure 3.1). The cellobiose and xylose was coutilized after glucose was depleted. As the temperature decreased from 25 to 17 °C, cellobiose consumption was reduced (Table 3.3). Cellobiose consumption rates of *P. stipitis* in aerobic environment were faster than the rates in the anaerobic environment (Table 3.4). At 25 °C, cellobiose consumption in the aerobic fermentation was consumed at a rate 3-fold faster than the rate in the anaerobic fermentation. *P. stipitis* utilized cellobiose 100% in the aerobic fermentation at 25 °C whereas 82.4 and 62.1% of cellobiose were utilized in the aerobic fermentation experiments at 21 and 17°C, respectively.

In the anaerobic fermentation experiments, the cellobiose consumption of *P. stipitis* at 25 °C was faster, while *P. stipitis* consumed cellobiose at the same rate at 21 °C and 17 °C (Table 3.4). *P. stipitis* was not able to use cellobiose completely in any of the anaerobic experiments in 120 h. Cellobiose utilization in the anaerobic experiments at 25, 21 and 17 °C remained at 47.3, 25.0 and 25.9%, respectively.

Cellobiose consumption of *S. passalidarum* is shown in Figure 3.1. Overall, *S. passalidarum* was faster at cellobiose utilization than *P. stipitis*. At 25 °C, *S. passalidarum* utilized cellobiose simultaneously with glucose and xylose. However, at 21 °C and 17 °C, cellobiose utilization in aerobic fermentation did not start until after glucose was consumed. *S. passalidarum* was able to consume cellobiose completely in the aerobic condition at 25 and 21 °C in 24 and 48 h, respectively. Cellobiose consumption rates of *S. passalidarum* decreased as the temperature decreased but were faster than the rates of *P. stipitis* at 25 and 21 °C (Table 3.3). *S. passalidarum* and *P. stipitis* consumed cellobiose in the aerobic fermentation at 17 °C at the same rate. Cellobiose utilization in the aerobic fermentation at 17 °C remained at 36.6% at the end of 120 hours because cellobiose uptake stopped after 72 hours.

In the anaerobic fermentations, *S. passalidarum* consumed cellobiose completely at all temperature levels. Similar to the cellobiose consumption in the aerobic fermentations, *S. passalidarum* utilized cellobiose simultaneously with glucose and xylose at 25 °C and after glucose depletion at 21 and 17 °C. Increasing temperature also improved cellobiose consumption rates (Table 3.4). However, cellobiose consumption rates of *S. passalidarum* in anaerobic fermentation were slower than the rates in aerobic fermentation, except in the fermentation at 21 °C. Aeration at 21 °C did not affect the cellobiose utilization of *S. passalidarum*. Cellobiose was consumed at the same rate ( $0.097\pm 0.001$  g/L/h) in both aerobic and anaerobic fermentation at 21°C.

*P. stipitis* consumed xylose at a slow rate initially. The consumption rates increased after glucose was consumed completely in 24 hours. In both aerobic and anaerobic fermentation at 17 °C, *P. stipitis* started consuming xylose after glucose was depleted. Xylose consumption rates of *P. stipitis* in aerobic fermentation were  $0.19\pm 0.01$ ,  $0.186\pm 0.02$  and  $0.0219\pm 0.03$  g/L/h, for 17, 21 and 25 °C, respectively. An increase in temperature improved the xylose consumption rates (Table 3.3). In the aerobic fermentation at 25 °C, *P. stipitis* consumed xylose completely whereas at 21 °C and 17 °C, 95.4 and 82.0 % of xylose was utilized by *P. stipitis*, respectively.

**Table 3.3.** Aerobic fermentation of mixed carbohydrates by *P. stipitis* and *S. passalidarum* at 17, 21 and 25 °C. Each value shows mean±std. dev values of triplicate flasks.

Strain	17 °C	21 °C	25 °C
<b><i>P. stipitis</i> DSMZ3651</b>			
Max Ethanol Concentration (g/L)	1.1±0.3	3.9±0.2	4.8±1.0
Max Ethanol Yield (g/g)	0.05±0.01	0.14±0.01	0.17±0.03
Ethanol Productivity <sup>a</sup> (g/L/h)	0.013±0.001	0.038±0.003	0.06±0.02
Xylose Consumption Rate <sup>b</sup> (g/L/h)	0.19±0.01	0.186±0.002	0.219±0.02
Cellobiose Consumption Rate <sup>b</sup> (g/L/h)	0.021±0.002	0.0326±0.0002	0.047±0.003
Biomass <sup>c</sup> (g/L)	9.2±0.1	5.0±0.1	6.0±0.4
<b><i>S. passalidarum</i> MYA4345</b>			
Max Ethanol Concentration (g/L)	1.5±0.1	8.0±0.2	9.5±0.3
Max Ethanol Yield (g/g)	0.10±0.01	0.27±0.01	0.35±0.02
Ethanol Productivity <sup>a</sup> (g/L/h)	0.0209±0.0002	0.167±0.005	0.40±0.01
Xylose Consumption Rate <sup>b</sup> (g/L/h)	0.11±0.02	0.420±0.002	0.72±0.01
Cellobiose Consumption Rate <sup>b</sup> (g/L/h)	0.029±0.003	0.097±0.001	0.197±0.002
Biomass <sup>c</sup> (g/L)	4.4±0.5	12.2±1.2	13.1±0.3

<sup>a</sup> Ethanol productivities were calculated at the time of maximum ethanol concentration.

<sup>b</sup> Consumption rates were calculated as linear regression between time points of maximum consumption.

<sup>c</sup> For biomass concentrations, samples were diluted to keep the OD<sub>600</sub> measurements in the range of the calibration curves and then multiplied by the dilution ratio.

Xylose consumption rates of *P. stipitis* in the anaerobic fermentation (Table 3.4) at 25 °C, 21 °C and 17 °C were 0.13±0.01, 0.14±0.01, and 0.105±0.001 g/L/h, respectively, and slower than the rates in the aerobic fermentation. Similar to the xylose utilization in the aerobic fermentation, xylose uptake in the anaerobic fermentation was also slow initially due to glucose presence. After glucose was consumed in 24 h, xylose was consumed at faster rates. However, due to slower consumption rates compared to the rates in the aerobic fermentation, the percent xylose utilization at 120 h for 25 °C, 21 °C and 17 °C remained at 73.8, 70.1 and 55.1%, respectively.



The xylose consumption rates of *S. passalidarum* in the aerobic fermentation improved as the temperature increased. In the aerobic fermentation at 25 °C and 21 °C, *S. passalidarum* consumed xylose within 48 h whereas at 17 °C xylose consumption started after glucose depleted and stopped after 72 h. The xylose consumption rate of *S. passalidarum* in the aerobic fermentation at 17 °C ( $0.11\pm 0.01$  g/L/h) was also very slow compared to the rate at 25 °C ( $0.72\pm 0.01$  g/L/h) and 21 °C ( $0.420\pm 0.002$  g/L/h) (Table 3.3). *S. passalidarum* was able to utilize xylose completely at 25 °C and 21 °C while only 42.9% of xylose was utilized at 17 °C in 120 hours.

In the anaerobic fermentation, the xylose consumption rates of *S. passalidarum* were slower than the rates in the aerobic fermentation (Table 3.4). A temperature increase also improved the consumption rates. Unlike xylose utilization in the aerobic fermentation at 17 °C, xylose uptake did not stop, and xylose was consumed at a rate of  $0.131\pm 0.003$  g/L/h, which was the rate before xylose uptake stopped in the aerobic fermentation. In the anaerobic fermentation at 25 °C and 21 °C, *S. passalidarum* depleted xylose in 72 and 96 h, respectively, whereas, at 17 °C, 78.6% of xylose was utilized by *S. passalidarum* in 120 h. Xylose consumption rates at 25 °C and 21 °C were  $0.28\pm 0.01$  and  $0.201\pm 0.004$ , respectively.

Both strains produced more xylitol during anaerobic fermentation than during aerobic fermentation (Figure 3.1). In the aerobic fermentation, *P. stipitis* produced xylitol only at 25 °C with a maximum xylitol concentration of  $0.03\pm 0.02$  g/L. Anaerobic xylitol production was higher than the aerobic xylitol production. *P. stipitis* did not consume the xylitol produced during anaerobic fermentation. Maximum xylitol concentration in the anaerobic fermentation was obtained at 21 °C ( $0.13\pm 0.01$  g/L), followed by at 25 °C ( $0.12\pm 0.02$  g/L) and 17 °C ( $0.07\pm 0.02$  g/L). *S. passalidarum* produced xylitol in all experiments. However, *S. passalidarum* consumed xylitol after xylose depletion in all fermentation experiments, except in the aerobic fermentation at 17 °C. The maximum xylitol production by *S. passalidarum* occurred in the anaerobic fermentation at 25 °C ( $0.71\pm 0.08$  g/L), followed by the anaerobic fermentations at 21 °C ( $0.50\pm 0.02$  g/L) and 17 °C ( $0.45\pm 0.04$  g/L).

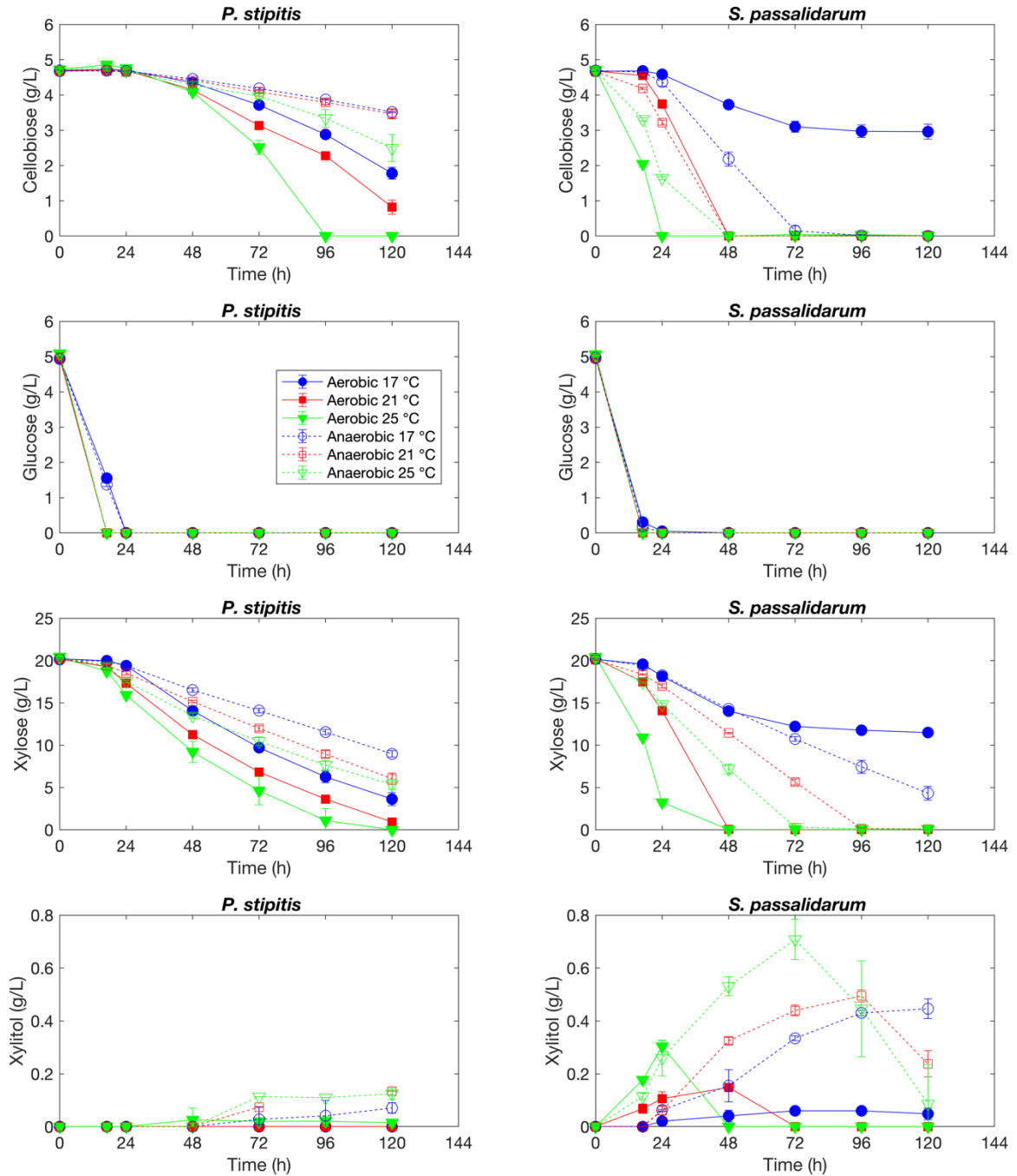
**Table 3.4.** Anaerobic fermentation of mixed carbohydrates by *P. stipitis* and *S. passalidarum* at 17, 21 and 25 °C. Each value shows mean±std. dev values of triplicate flasks.

Strain	17 °C	21 °C	25 °C
<b><i>P. stipitis</i> DSMZ3651</b>			
Max Ethanol Concentration (g/L)	4.3±0.3	7.7±0.3	8.9±0.2
Max Ethanol Yield (g/g)	0.25±0.02	0.38±0.02	0.40±0.01
Ethanol Productivity <sup>a</sup> (g/L/h)	0.039±0.004	0.062±0.002	0.074±0.002
Xylose Consumption Rate <sup>b</sup> (g/L/h)	0.105±0.002	0.14±0.01	0.13±0.01
Cellobiose Consumption Rate <sup>b</sup> (g/L/h)	0.0103±0.0002	0.012±0.0001	0.023±0.004
Biomass <sup>c</sup> (g/L)	2.8±0.7	2.4±0.3	1.8±0.3
<b><i>S. passalidarum</i> MYA4345</b>			
Max Ethanol Concentration (g/L)	10.7±0.5	12.5±0.5	13.7±0.5
Max Ethanol Yield (g/g)	0.42±0.01	0.42±0.02	0.46±0.01
Ethanol Productivity <sup>a</sup> (g/L/h)	0.102±0.002	0.143±0.002	0.185±0.003
Xylose Consumption Rate <sup>b</sup> (g/L/h)	0.131±0.003	0.201±0.004	0.28±0.01
Cellobiose Consumption Rate <sup>b</sup> (g/L/h)	0.082±0.002	0.097±0.001 <sup>a</sup>	0.098±0.001
Biomass <sup>c</sup> (g/L)	2.6±0.1	4.3±0.2	3.7±0.1

<sup>a</sup> Ethanol productivities were calculated at the time of maximum ethanol concentration.

<sup>b</sup> Consumption rates were calculated as linear regression between time points of maximum consumption.

<sup>c</sup> For biomass concentrations, samples were diluted to keep the OD<sub>600</sub> measurements in the range of the calibration curves and then multiplied by the dilution ratio.



**Figure 3.1.** Carbohydrate consumption in mixed of *P. stipitis* and *S. passalidarum* in mixed carbohydrate medium under aerobic (solid lines) and anaerobic conditions (dashed lines) at 17 (blue), 21 (red) and 25 (green) °C.

The ethanol yields and volumetric ethanol productivities of *S. stipitis* in the aerobic fermentations (Table 3.3) were lower than in the anaerobic fermentations (Table 3.4). An increase in temperature improved maximum ethanol yields and ethanol productivities in both aerobic and anaerobic fermentation. The ethanol productivity of *P. stipitis* in the aerobic fermentation at 17 °C was  $0.013\pm 0.002$  g/L/h and ethanol yield was  $0.05\pm 0.01$  g/g. When the temperature increased to 21 °C, the ethanol productivity and ethanol yield improved by approximately 3-fold. At 25 °C, the productivity and ethanol yield were  $0.06\pm 0.01$  g/L/h and  $0.17\pm 0.02$  g/g, respectively. The maximum ethanol concentration ( $4.8\pm 1.0$  g/L) was obtained at 25 °C in 96 hours (Figure 3.2). However, after 96 hours, the ethanol was consumed by *S. stipitis*.

In anaerobic fermentation, *P. stipitis* produced ethanol at faster rates and obtained higher concentrations than in aerobic fermentation. As the temperature increased from 17 °C to 25 °C, the ethanol yield improved from  $0.25\pm 0.02$  to  $0.40\pm 0.01$  g/g and the ethanol productivity increased from  $0.039\pm 0.003$  to  $0.074\pm 0.002$  g/L/h.

For *S. passalidarum*, ethanol productivities at all temperatures under aerobic conditions were initially faster than the ethanol productivities of *P. stipitis* (Table 3.3). However, at 25 and 21 °C, *S. passalidarum* respired ethanol after the carbohydrates were depleted (Figure 3.1, Figure 3.2). The maximum ethanol concentrations at 25 °C ( $9.5\pm 0.2$  g/L) and 21 °C ( $8.0\pm 0.3$  g/L) were obtained in 24 and 48 hours, respectively, whereas the maximum ethanol concentration at 17 °C was lower ( $1.5\pm 0.1$  g/L) because ethanol production at 17 °C stopped after 48 hours due to the discontinuation of carbohydrate utilization.

*S. passalidarum* had higher fermentation performance in anaerobic conditions than *P. stipitis*. In anaerobic conditions, an increase in temperature from 17 °C to 25 °C improved ethanol productivities from  $0.102\pm 0.004$  to  $0.185\pm 0.003$  g/L/h (Table 3.4) while ethanol yields slightly increased from  $0.42\pm 0.01$  to  $0.46\pm 0.01$  g/g. Unlike in the aerobic fermentation, carbohydrate uptake did not stop at 17 °C. Ethanol respiration also did not occur in the anaerobic fermentations at any temperature levels (Figure 3.1).

Ethanol respiration during aerobic fermentation increased the cell growth of both strains after carbohydrates were consumed (Figure 3.2). For *P. stipitis*, carbohydrate conversion

at 17 °C led to higher biomass production than at 25 °C and 21 °C under aerobic conditions. *P. stipitis* at 25 °C and 21 °C grew at a similar pace and slower than at 17 °C. Therefore, the final biomass concentration at 17 °C ( $9.2\pm 0.1$  g/L) was higher than the final biomass concentrations at 21 °C ( $5.0\pm 0.1$  g/L) and 25 °C ( $6.0\pm 0.4$  g/L).

The anaerobic condition resulted in lower biomass production for *S. stipitis* compared to the aerobic condition. Final biomass concentrations at 25 °C, 21 °C and 17 °C were  $1.8\pm 0.3$  g/L,  $2.4\pm 0.3$  g/L and  $2.8\pm 0.7$  g/L, respectively. Similar to the aerobic fermentation at 17 °C, carbohydrates in the medium were mostly converted into biomass instead of ethanol during the anaerobic fermentation at 17 °C.

For *S. passalidarum*, the biomass concentration in the aerobic fermentation at 17 °C was  $4.4\pm 0.5$  g/L whereas the biomass concentrations at 21 °C and 25 °C were higher,  $12.2\pm 1.2$  g/L and  $13.1\pm 0.3$  g/L, respectively. Since carbohydrate uptake stopped after 48 hours during the aerobic fermentation at 17 °C, biomass concentration did not increase as much as at 21 °C and 25 °C. Final biomass concentration in the anaerobic fermentations at 17, 21 and 25 °C were  $2.6\pm 0.2$ ,  $4.3\pm 0.2$ ,  $3.7\pm 0.1$  g/L, respectively and were lower than in the aerobic fermentation. Most of the carbohydrates converted into ethanol in anaerobic conditions due to lack of oxygen in the medium which led to lower biomass concentrations and higher ethanol yields.

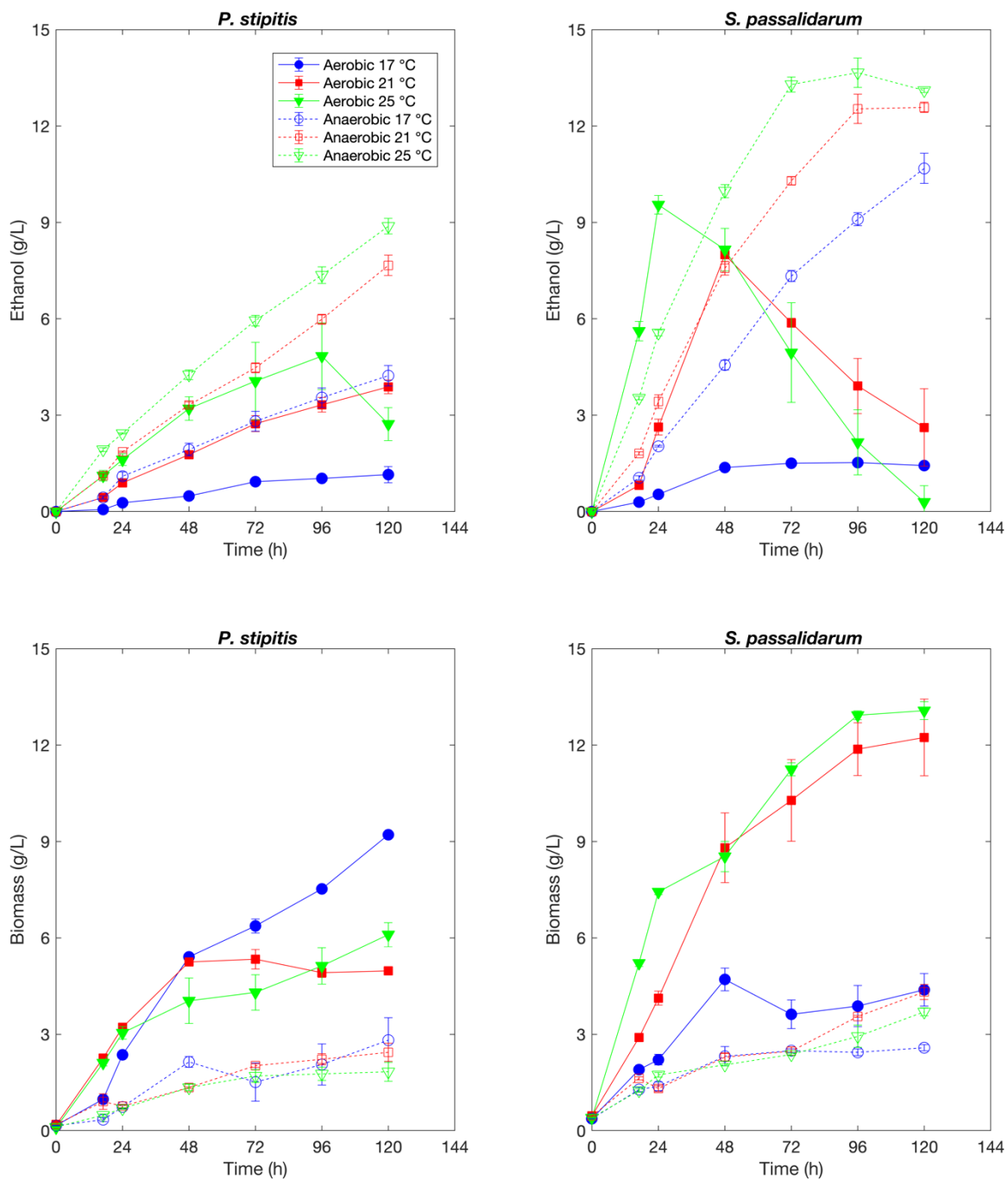
In both aeration conditions, *S. passalidarum* was able to simultaneously utilize cellobiose and xylose when the xylose was the most abundant carbohydrate in the medium. *P. stipitis* did also utilize cellobiose and xylose simultaneously but at slower rates. Simultaneous consumption of carbohydrate sources could reduce the cost of hemp brewing process as it would decrease the time and energy requirement for fermentation.

*S. passalidarum* produced ethanol very fast in aerobic conditions, however, *S. passalidarum* also quickly consumed ethanol almost completely in aerobic conditions. On the other hand, aerobic conditions improved xylose utilization. Ethanol respiration is not a desired property in brewing as the main product in beer is consumed by yeast. In addition, oxidative products formed during aerobic fermentation can cause off-flavor formation because oxygen presence and high temperature improve cell growth which results in an

increase in the utilization of nitrogenous compounds, but this leads to the formation of compounds that gives off-flavor (Russell 2009). Therefore, aerobic fermentation is not preferred for brewing.

*S. passalidarum* had a higher fermentation performance under anaerobic conditions, which are essential in brewing. Xylose consumption in an anaerobic environment has been a challenge in lignocellulosic ethanol fermentation due to different cofactor requirements of XR and XDH in xylose conversion, which leads to by-product accumulation. XR depends on NADPH or NADH to convert xylose to xylitol while XDH depends on NAD<sup>+</sup> to convert xylitol to xylulose. XR of *P. stipitis* has a higher affinity to NADPH than NADP, which causes a cofactor imbalance and impede xylose conversion (Agbogbo and Coward-Kelly 2008). *S. passalidarum*, on the other hand, expresses NADH-preferred XR in anaerobic conditions (Hou 2012), thus, NADH can be regenerated without requiring oxygen as the electron acceptor and cofactor imbalance does not occur.

Discontinuation of carbohydrate, utilization may be due to the oxidative stress response of *S. passalidarum* at low temperature. In aerobic conditions, yeast produce reactive oxygen species (ROS), such as hydrogen peroxide, hydroxyl radical and superoxide anion, which can damage proteins, lipids, carbohydrates and DNA (Herrero, Ros et al. 2008). Increased intracellular ROS levels likely inactivated enzymes and transport proteins of *S. passalidarum* which stopped carbohydrate uptake and therefore fermentation. Under aerobic conditions, *S. passalidarum* expresses NADPH-dependent XR which likely has poor activity at low temperatures. *P. stipitis*, on the other hand, continued fermentation which indicated that oxidative stress response may be strain specific among xylose-fermenting yeast.



**Figure 3.2.** Ethanol production and cell growth of *P. stipitis* and *S. passalidarum* in mixed carbohydrate medium under aerobic (solid lines) and anaerobic (dashed lines) conditions at 17 (blue), 21 (green) at 25 (red) °C.

### 3.3.3 Fermentation in synthetic hemp extract

The purpose of this experiment was to evaluate the fermentation performance of *P. stipitis* and *S. passalidarum* in synthetic hemp extract. *S. pastorianus* was used in control flasks. The experiment was designed to target the carbohydrate profile of the hemp extract produced by Province Brands of Canada. A synthetic media was prepared using this carbohydrate profile and casamino acids were added as the nitrogen source along with vitamins, minerals and essential salts. Vitamin and mineral solutions were prepared based on the essential requirements for yeast. Fermentation in synthetic hemp extract was performed in aerobic and anaerobic conditions at 23 °C, which was the temperature that Province Brands of Canada was testing for Ale brewing during this research work.

#### **Aerobic Fermentation**

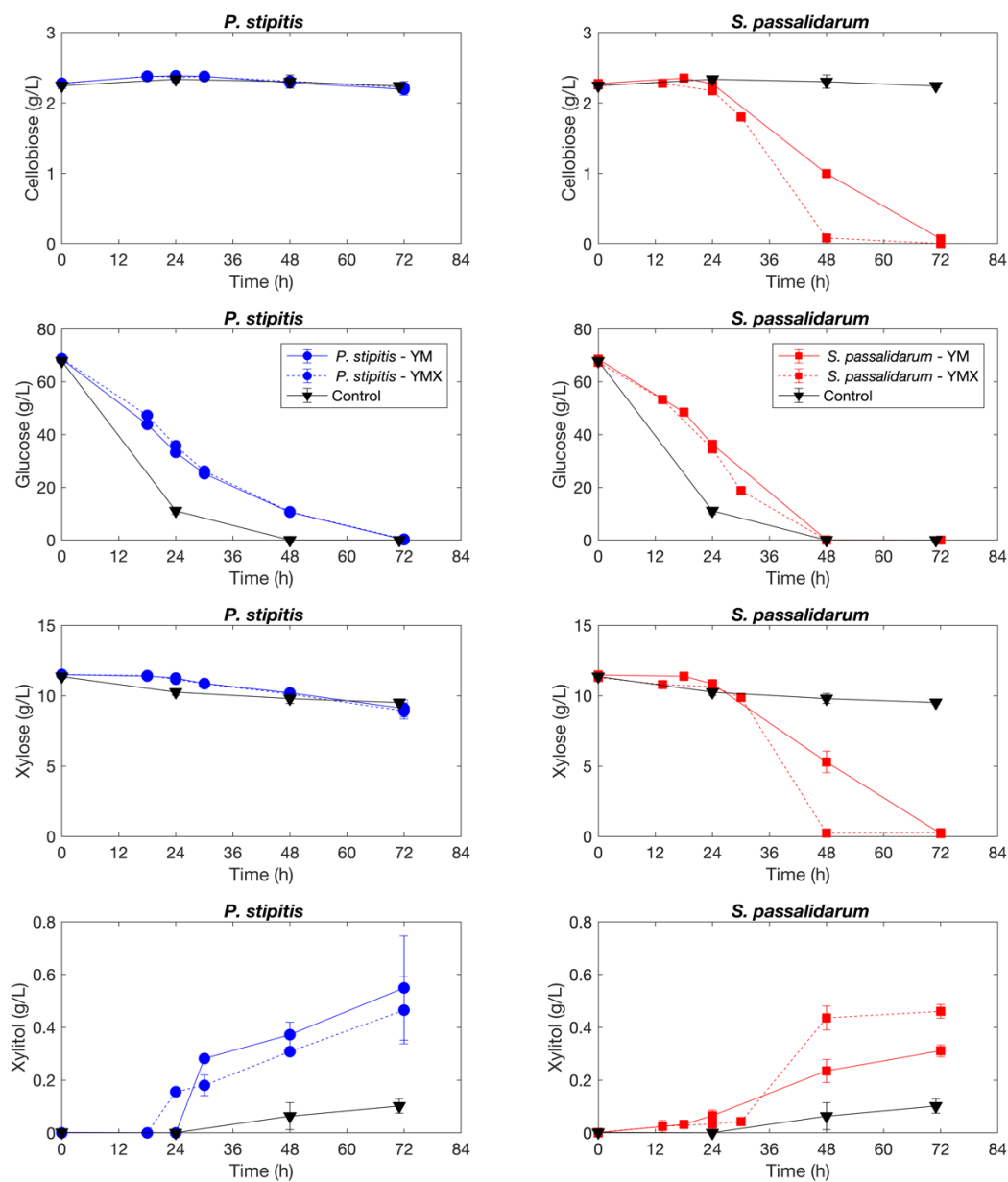
Carbohydrate consumption of *P. stipitis* and *S. passalidarum* were shown in (Figure 3.3). Under aerobic conditions, *S. passalidarum* consumed glucose completely in 48 hours while *P. stipitis* consumed it in 72 hours. Cellobiose consumption of *S. passalidarum* was delayed initially. *S. passalidarum* started consuming cellobiose after 18 hours at a rate of  $0.053 \pm 0.003$  g/L/h and depleted cellobiose in 72 hours whereas *P. stipitis* did not consume cellobiose.

Glucose repressed xylose uptake of both strains. Both strains utilize glucose at rates faster than xylose consumption rates (Table 3.5). Xylose consumption was very slow initially. The rates increased after 24 hours. For *S. passalidarum*, xylose consumption rate increased by approximately 9-fold from  $0.026 \pm 0.002$  to  $0.23 \pm 0.03$  g/L/h. However, xylose consumption of *P. stipitis* was delayed for 24 hours. *S. passalidarum* consumed xylose completely in 48 hours whereas *P. stipitis* was very slow at using xylose and most of the xylose remained in the fermentation medium at the end of the experiment. Glucose presence repressed xylose utilization of *P. stipitis* more than that of *S. passalidarum*.

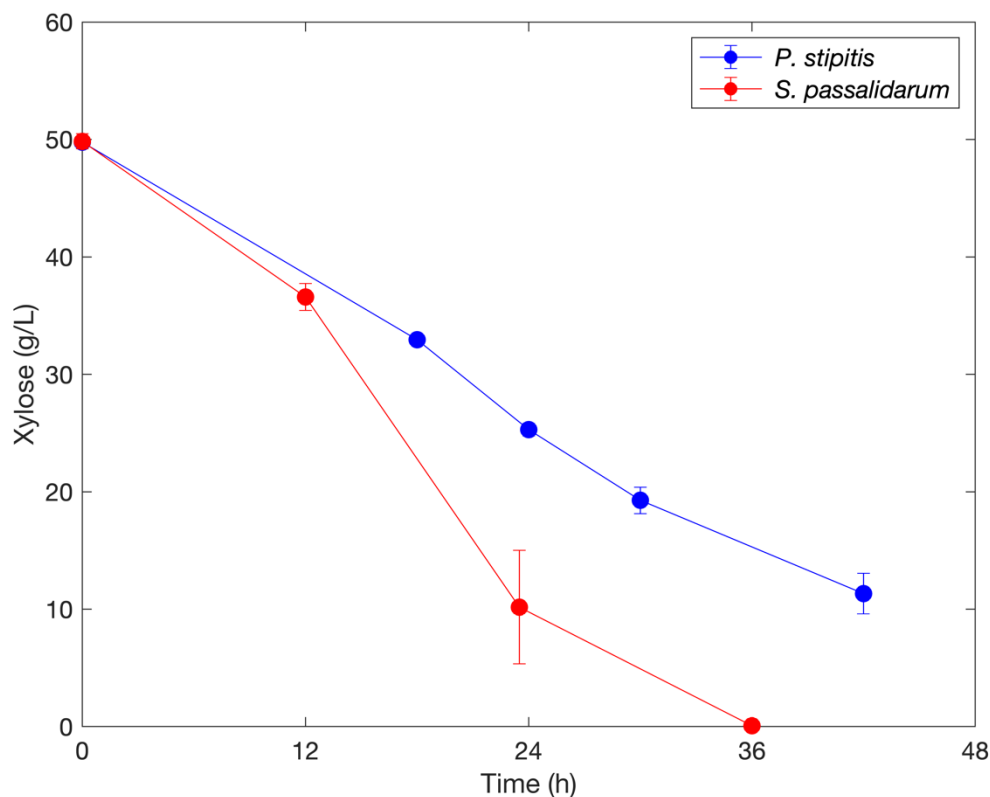
Another experiment was conducted with a higher xylose concentration (50 g/L) and without glucose. Xylose was completely fermented in 36 – 42 hours by both xylose-fermenting yeast (Figure 3.4). Xylose consumption rates in high-xylose medium were



0.92±0.04 and 1.38±0.02 g/L/h for *P. stipitis* and *S. passalidarum*, respectively, which were as fast as the glucose consumption rates in the aerobic fermentation of synthetic hemp extract (Table 3.5).



**Figure 3.3.** Carbohydrate consumption and xylitol production of glucose-grown (YM-G) and xylose-grown (YM-X) xylose-fermenting yeast (*P. stipitis* (blue), *S. passalidarum* (red)) in synthetic hemp extract under aerobic conditions at 23 °C. Solid lines show the seed cultures propagated in YM-G. Dashed lines show the seed cultures propagated in YM-X.



**Figure 3.4.** Xylose consumption of *P. stipitis* (blue) and *S. passalidarum* (red) in high xylose medium (50 g/L) under aerobic conditions at 23 °C.

This experiment showed that xylose utilization was very fast when there was no glucose present. Based on these results, it was hypothesized that gene expression for xylose fermentation is affected by the environmental conditions. In both experiments, xylose-fermenting yeast were initially propagated in glucose media. The genes responsible for producing the enzymes required for xylose metabolism may be regulated by the presence or absence of xylose and influenced by environmental and growth conditions. To test this hypothesis, *P. stipitis* and *S. passalidarum* were propagated in YM media with xylose, instead of glucose. The fermentation flasks contained synthetic hemp extract.

Xylose-grown *P. stipitis* did not consume cellobiose, however, xylose-grown *S. passalidarum* consumed cellobiose completely in 48 hours. Similar to the previous experiment, cellobiose consumption was repressed for 24 hours, then, it was consumed by

xylose-grown *S. passalidarum* at a faster rate (0.087 g/L/h) than the cellobiose consumption rate of glucose-grown *S. passalidarum*.

Glucose consumption rates of xylose-grown *P. stipitis* and *S. passalidarum* were 0.95 and 1.43 g/L/h, respectively. When both strains were propagated in glucose medium, the glucose consumption rates were 0.95 and 1.40 g/L/h for *P. stipitis* and *S. passalidarum* respectively. Propagation on xylose did not affect the glucose utilization of either strain.

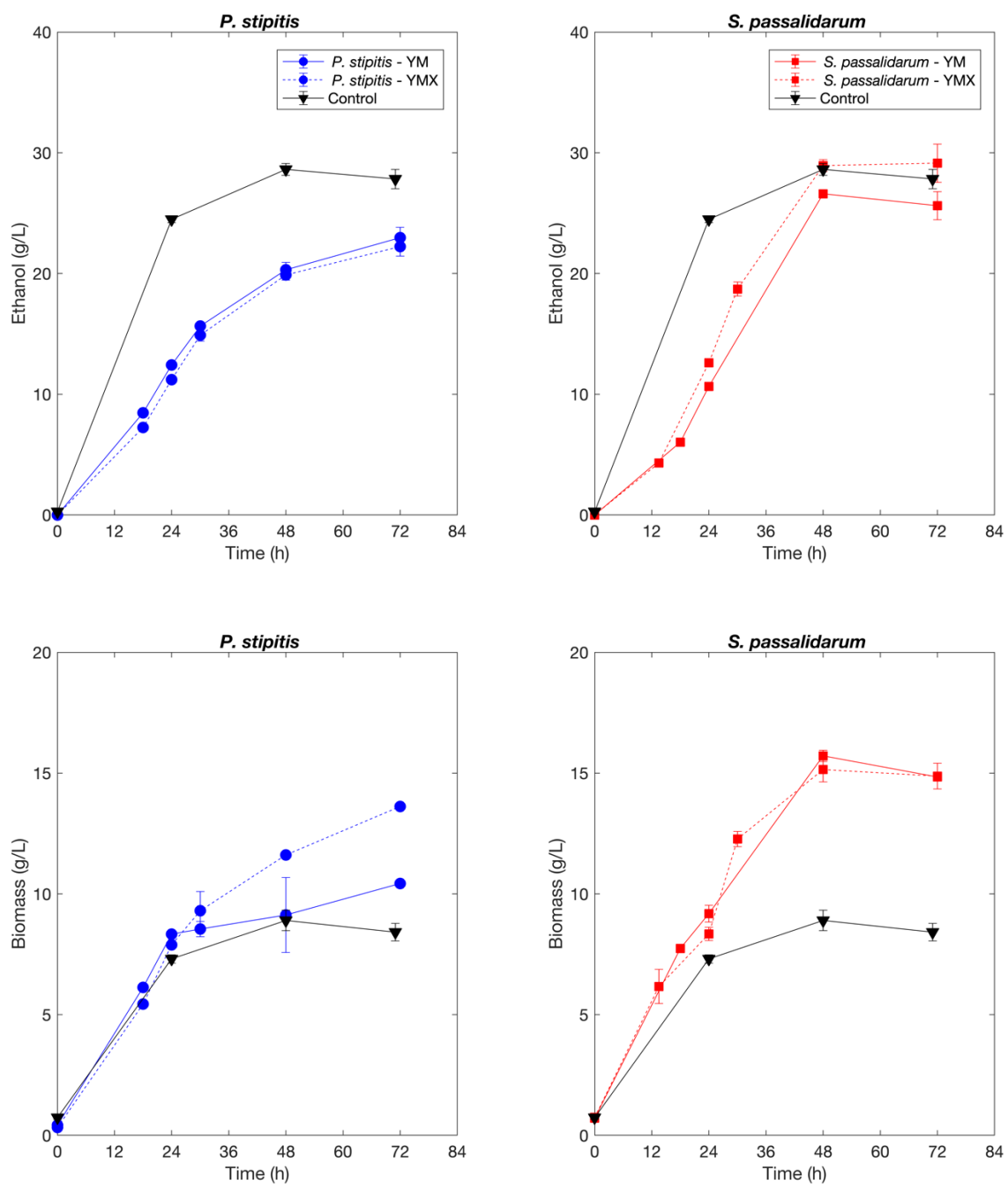
Xylose-grown *S. passalidarum* had improved xylose utilization capacity. Similar to the previous experiment in synthetic hemp extract, initial xylose consumption was slow. After 18 hours, xylose consumption rate increased by 16-fold, from 0.027 to 0.43 g/L/h. When compared to glucose-grown *S. passalidarum*, xylose utilization improved by approximately 2-fold. Xylitol production of xylose-grown *S. passalidarum* was also slightly higher than that of glucose-grown *S. passalidarum*. Growing cells in xylose initially increased xylose utilization rate of *S. passalidarum*, however, did not overcome the slow xylose uptake at the beginning of fermentation. On the other hand, xylose utilization of *P. stipitis* did not improve when it was propagated in xylose medium (Figure 3.3).

Xylose is transferred into cells by low-affinity facilitated diffusion or high-affinity active transport (Hahn-Hägerdal, Karhumaa et al. 2007). Facilitated diffusion occurs through membrane proteins which require a carbohydrate gradient and active transport occurs by high-affinity xylose transporters on the cell membrane. When the glucose is present and at high concentrations glucose enters to cells via the facilitated diffusion which blocks the uptake of xylose. Glucose has also been known to compete against xylose for the high-affinity transporters when these carbohydrates are present together. In addition, intracellular glucose inhibits the induction of XR and XDH enzymes (Hou 2012). Therefore, high glucose concentration repressed xylose transport and enzyme activity regardless of initial propagation of *P. stipitis* on xylose.

Oxygen transfer to the medium may also have been insufficient for *P. stipitis* to utilize xylose. As the fermentation progresses, the cell population becomes larger which increases the competition for oxygen. Slininger, Branstrator et al. (1990) previously reported that

limited oxygen availability per cell makes cultures essentially anaerobic. In our experiment, the aerobic environment was maintained passively by covering the top of the flasks with aluminum foil, which may impacted the oxygen transfer to the medium. Thus, oxygen availability per cell may have become very limited as the cell growth increased. As mentioned in Section 3.3.2, xylose utilization of *P. stipitis* is more oxygen-dependent than that of *S. passalidarum* due to different co-factor dependencies of XR that *P. stipitis* have. Therefore, in addition to the cofactor imbalance and glucose repression of xylose uptake and enzyme activity, limited availability of dissolved oxygen in the fermentation medium may have further repressed xylose utilization ability of *P. stipitis*.

In terms of ethanol productivity, since using xylose as the sole carbon in seed culture medium did not affect the carbohydrate consumption rates of *P. stipitis*, productivity and maximum ethanol yield of *P. stipitis* did not improve. Ethanol productivity remained at 0.31 g/L/h while maximum ethanol yield was 0.32 g EtOH/g total carbohydrate. *P. stipitis* produced approximately 23 g/L ethanol in both experiments. For *S. passalidarum*, however, productivity slightly improved from 0.55 g/L/h to 0.60 g/L/h and was higher than the productivity of *P. stipitis* in both experiments. Maximum ethanol yield of xylose-grown *S. passalidarum* was 0.37 g EtOH/g total carbohydrate. Xylose-grown *S. passalidarum* produced slightly more ethanol (approx. 30 g/L) than glucose-grown *S. passalidarum* (approx. 27 g/L) (Figure 3.5).



**Figure 3.5.** Ethanol production and cell growth of glucose-grown (YM-G) and xylose-grown (YM-X) xylose-fermenting yeast (*P. stipitis* (blue), *S. passalidarum* (red), *S. pastorianus* (control)(black)) in synthetic hemp extract under aerobic conditions at 23 °C. Solid lines show the seed cultures propagated in YM-G. Dashed lines show the seed cultures propagated in YM-X.

**Table 3.5.** Fermentation performance of *P. stipitis* and *S. passalidarum* in synthetic hemp extract under aerobic and anaerobic conditions at 23 °C. Results show the mean of duplicate flasks. Deviation from the mean was less than 5% for all samples.

Seed Culture	Strain	Aeration	Time Interval (h)	Cellobiose (g/L/h)	Glucose (g/L/h)	Xylose (g/L/h)	Ethanol Productivity (g/L/h)	Max Ethanol Yield (g/g tot carb.)	Final Biomass Concentration (g/L)
YM-G	<i>P. Stipitis</i>	Aerobic	0-24	0	0.95	0.010	0.31	0.32	10.4
			24-48	0		0.044			
	<i>S. passalidarum</i>	Aerobic	0-24	0	1.43	0.026	0.55	0.35	
			24-48	0.053		0.23			
YM-X	<i>P. Stipitis</i>	Aerobic	0-24	0	0.95	0.014	0.31	0.31	13.6
			24-48	0		0.044			
	<i>S. passalidarum</i>	Aerobic	0-24	0	1.40	0.027	0.60	0.37	
			24-48	0.087		0.43			
YM-X	<i>P. Stipitis</i>	Anaerobic	0-213	0	0.26	0.008	0.11	0.42	3.2
YM-X	<i>S. passalidarum</i>	Anaerobic	0-162	0 <sup>a</sup>	0.48	0.017	0.17	0.43	8.0
			162-213	0.016 <sup>b</sup>		0.17			

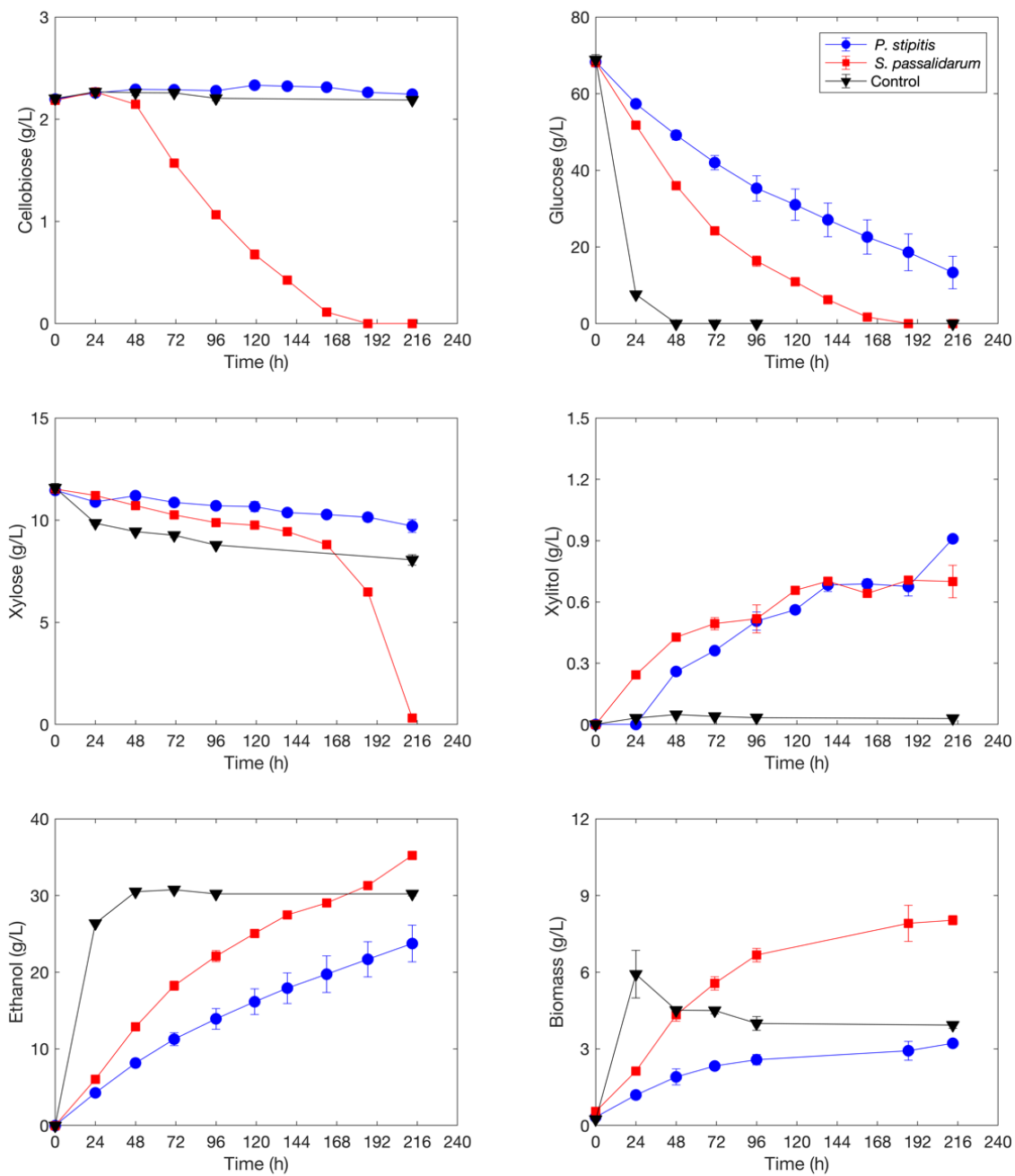
Consumption rates and ethanol productivities were calculated using the slope of the straight line between the time points in the indicated time interval and the concentration values that at those time points. Ethanol yields were calculated at the end of the fermentations. <sup>a</sup>Time Interval 0-48 h, <sup>b</sup>Time Interval 48-186.5 h.

### **Anaerobic Fermentation**

The same experiment using hemp extract (YM-X grown cells) was performed under anaerobic conditions at 23 °C, as well. Airlocks and stoppers were placed on the flasks to maintain the anaerobic environment. Both strains were initially cultured in xylose medium.

*P. stipitis*, consumed glucose at a slower rate (0.26 g/L/h) than *S. passalidarum* but did not consume cellobiose and xylose consumption was very slow (0.008 g/L/h) (Figure 3.6). Cellobiose consumption of *S. passalidarum* under anaerobic conditions was delayed for 48 hours, then, *S. passalidarum* consumed cellobiose at a rate of 0.016 g/L/h, approximately 5-fold slower than the rate under aerobic conditions. Unlike the simultaneous consumption of cellobiose and xylose under anaerobic conditions when the xylose was the most abundant carbohydrate in the medium (Section 3.3.2), *S. passalidarum* consumed glucose first and then switched to xylose, when glucose was the most abundant carbohydrate. Glucose was consumed at rate of 0.48 g/L/h while xylose consumption was very slow (0.017 g/L/h) for 162 hours. Once the glucose was depleted within 162 hours, xylose was rapidly utilized, at a rate (0.17 g/L/h) 10-fold higher and was consumed completely. Xylitol production was very low for both strains. At the end of the fermentation, *S. passalidarum* had slightly higher final xylitol concentration (0.91 g/L) than *P. stipitis* (0.7 g/L) (Figure 3.6).

Hou (2012) also previously demonstrated the sequential uptake of glucose and xylose by *S. passalidarum* under anaerobic conditions. The reason that the very slow fermentation compared to the aerobic fermentation was due to carbohydrate uptake. Under anaerobic conditions, the only way for carbohydrate uptake to occur is through low-affinity facilitated diffusion as active transport deplete ATP (Hou 2012). Since the glucose gradient was very high relative to xylose, glucose was transferred first via facilitated diffusion. Once the glucose was consumed completely, xylose was rapidly utilized as the enzymes have been already expressed. Thus, *S. passalidarum* was able to consume all carbohydrates in the synthetic hemp extract within 213 hours, therefore, had higher productivity, maximum ethanol yield and final biomass concentration than *P. stipitis*.



**Figure 3.6.** Anaerobic fermentation of synthetic hemp extract at 23 °C by *P. stipitis* (blue), *S. passalidarum* (red), and *S. pastorianus* (black)

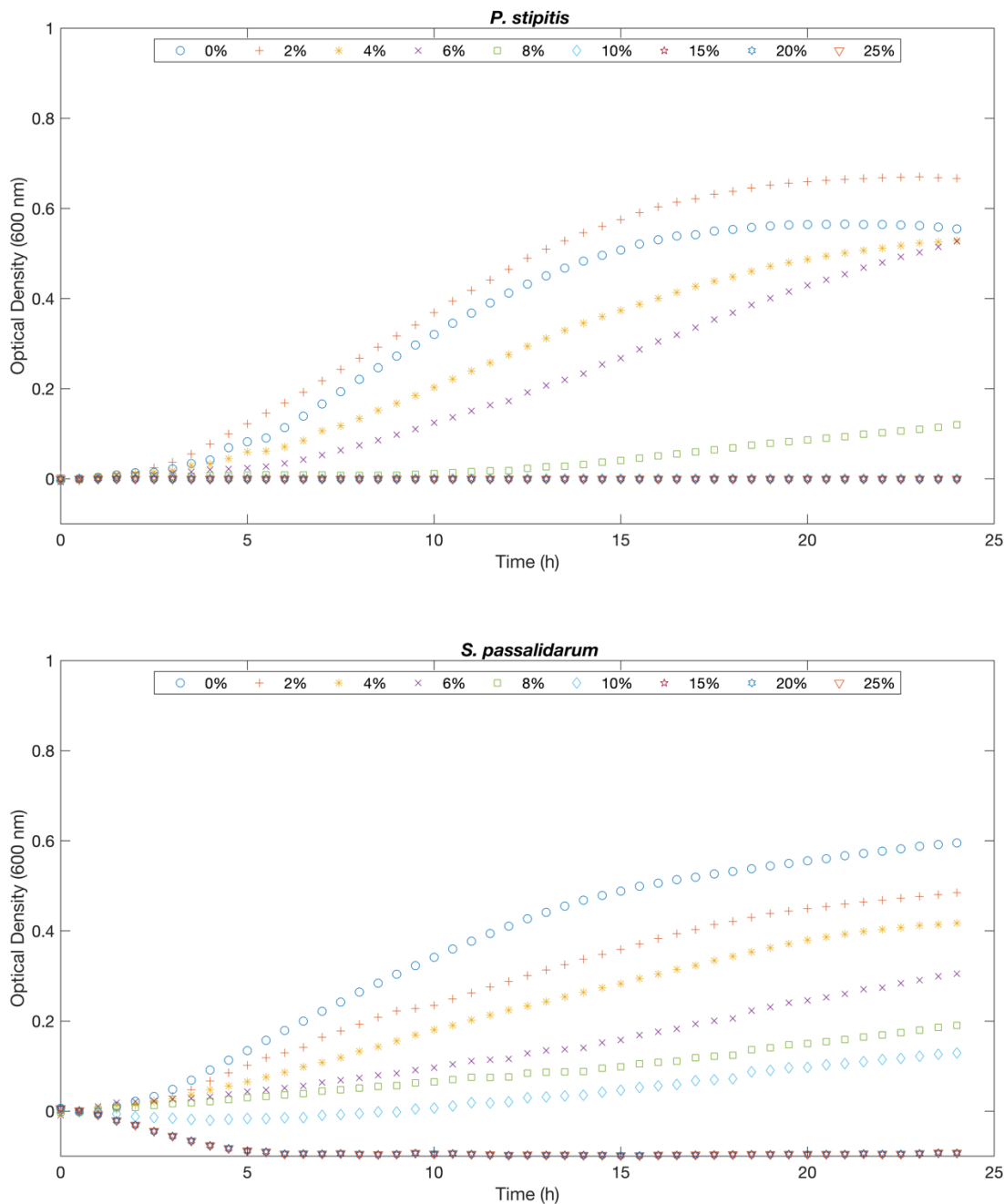


### 3.3.4 Ethanol Tolerance

Ethanol concentration was varied between 0 to 25% v/v in order to evaluate the effect of ethanol on the growth of xylose-fermenting yeast. Each column of the 96-well plate was used to assess one level of ethanol concentration. Growth curves of *P. stipitis* and *S. passalidarum* were analyzed and the effect of ethanol was quantified (Figure 3.7).

The control had no ethanol in the medium and the tests had increasing concentrations of ethanol. The area under the OD<sub>600</sub> versus time curve of the control was compared to the areas of the tests. As the ethanol concentration in the medium increased, growth was inhibited, thus, the area under the OD<sub>600</sub> versus time curve relative to the control decreased. The areas were calculated using numerical integration and curve fitting was performed via MATLAB. Initial OD<sub>600</sub> values were used as the baseline and areas under the baselines were subtracted from the areas under each curve. The relative amount of growth to the control was defined as the fractional area ( $f_a$ ) which was the ratio of the test area to the control area (Equation 3.2). Then, non-inhibitory (NIC) and minimum inhibitory concentrations (MIC) were estimated based on the technique developed by Lambert and Pearson (2000). NIC is the concentration above which the ethanol starts to inhibit growth and MIC is the concentration above which no growth is observed.

Parameter estimations are listed in Table 3.6. The plots of  $f_a$  versus  $\log_{10}$  of ethanol concentration for both strains (Figure 3.8, Figure 3.9) showed a sigmoidal function, similar to the results reported in literature ((Arroyo-López, Salvadó et al. 2010).  $R^2$  values for curve fittings were 0.974 and 0.985 for *P. stipitis* and *S. passalidarum*, respectively. Coefficient A was found to be statistically insignificant, therefore, was not used in curve fitting (Table 3.6).



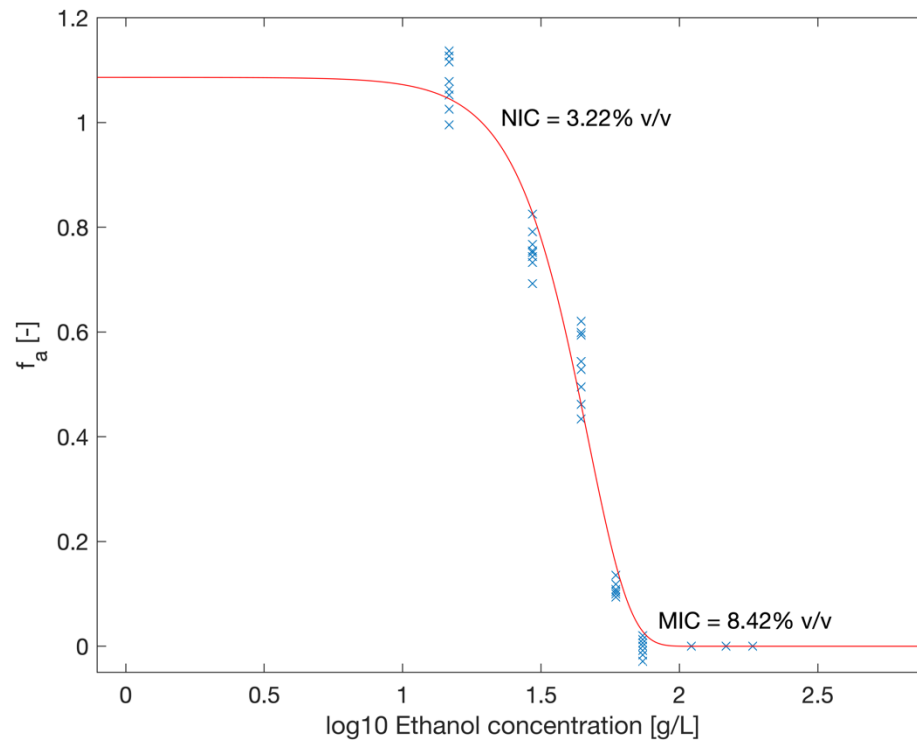
**Figure 3.7.** Effect of ethanol on the growth of *P. stipitis* and *S. passalidarum* in mixed carbohydrate medium (cellobiose, 5 g/L; glucose 5 g/L; xylose, 20 g/L, YNB w/o amino acids, 13.4 g/L) at 25 °C. Level of ethanol increased from 0 to 25% v/v. Curves show the average of eight wells. Initial OD<sub>600</sub> measurements were subtracted to normalize the growth curves.

**Table 3.6.** Parameter estimation of Gompertz function for *P. stipitis* and *S. passalidarum*

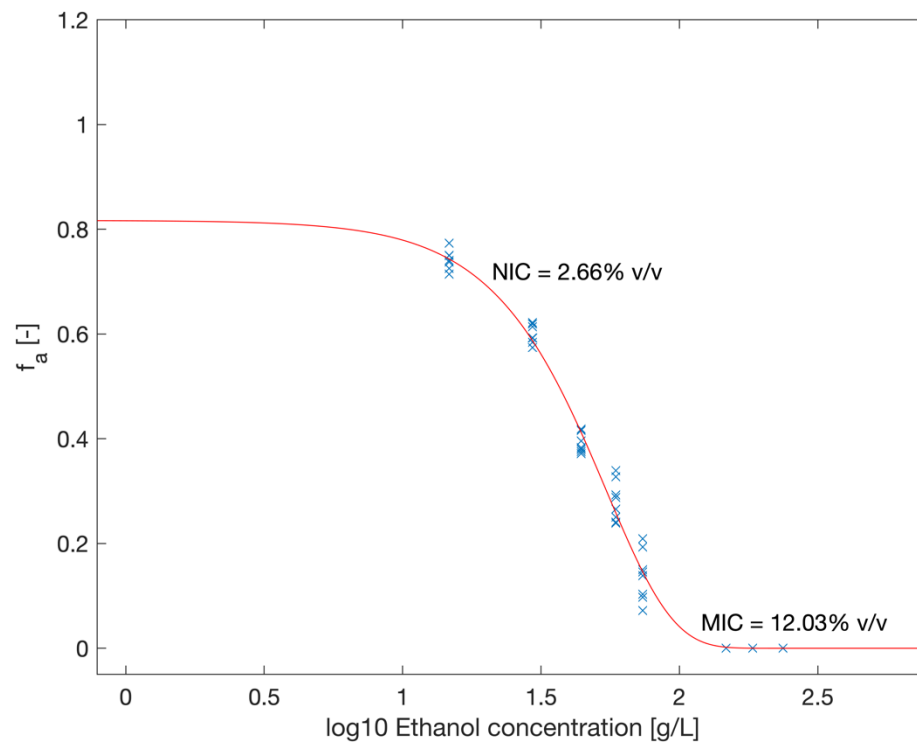
Strain	Coeff.	Lo. Conf (Limit)	Up. Conf (Limit)
<i>P. stipitis</i>			
A	-0.0363	-0.088	0.01541
C	1.0862	1.019	1.153
B	6.5157	4.935	7.374
M	1.6688	1.65	1.687
SSE	0.1814		
R-squared	0.9755		
Deg. of Freedom	40		
Adj-squared	0.9742		
RMSE	0.0673		
<i>S. passalidarum</i>			
A	-0.047	-0.03595	0.02655
C	0.8169	0.7689	0.8649
B	4.1496	3.567	4.732
M	1.7365	1.717	1.756
SSE	0.0422		
R-squared	0.9845		
Deg. of Freedom	42		
Adj-squared	0.9838		
RMSE	0.0317		

95% confidence level

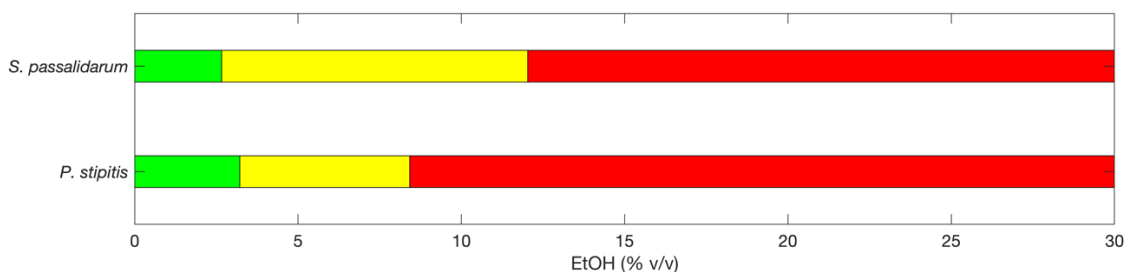
Arroyo-López, Salvadó et al. (2010) described the sigmoid curves by dividing them into three sections which were indicated as non-inhibitory region (NIR), progressive inhibitory region (PIR) and no growth region (NGR) (Figure 3.8). NIR corresponds to the ethanol concentrations from zero to NIC, where  $f_a$  is approximately 1. PIR is the region between NIC and MIC where the growth is inhibited progressively. NGR is the region above MIC where the yeast cannot grow, thus,  $f_a$  is approximately 0. These regions for both strains were illustrated in Figure 3.10.



**Figure 3.8.** Curve fitting of modified Gompertz function for *P. stipitis*.



**Figure 3.9.** Curve fitting of modified Gompertz function for *S. passalidarum*.



**Figure 3.10.** Non-inhibitory region (NIR), progressive inhibitory region (PIR) and no-growth region (NGR) of xylose-fermenting yeast. Green: NIR, Yellow: PIR, Red: NGR

*P. stipitis* (3.22% v/v) had a slightly higher NIC than *S. passalidarum* (2.66% v/v). In terms of MIC, *S. passalidarum* (12.03% v/v) exhibited higher tolerance to ethanol than *P. stipitis* (8.42% v/v). It was previously reported that brewing yeast strains can tolerate ethanol concentrations up to approximately 8% v/v without any inhibition on their growth (Arroyo-López, Salvadó et al. 2010). This indicated that xylose-fermenting yeasts are more sensitive to ethanol than brewing yeast.

### 3.4 Conclusions

*P. stipitis* and *S. passalidarum* were evaluated based on their fermentation performance in mixed carbohydrates of cellobiose, glucose and xylose at temperatures between 17 and 25 °C. Overall, *S. passalidarum* had higher fermentation ability in mixed carbohydrates under anaerobic conditions and was more tolerant to ethanol than *P. stipitis*. However, it should be noted that, *S. passalidarum* respired ethanol under aerobic conditions. Low temperature and oxygen-limitation decreased xylose utilization rates of both strains. Propagation in xylose-containing seed cultures improved gene expression for xylose metabolism which improved xylose utilization rate of *S. passalidarum*. Under anaerobic conditions, *S. passalidarum* sequentially consumed glucose and xylose, while glucose presence at a high concentration decreased xylose uptake of *P. stipitis*. Therefore, *S. passalidarum* was chosen as the DNA donor strain due to superior carbohydrate utilization ability in mixed carbohydrate medium under anaerobic conditions.

## Chapter 4

### 4 Electroporation-based Transformation

#### 4.1 Introduction

In 2019, the legalization of edible cannabis-derived products occurred which expanded the rapidly-growing cannabis market in Canada. In the beverage industry, there are companies working on cannabis-infused beer. However, they first brew beer from barley and then infuse the psychoactive compounds from the cannabis plant so they are not “brewing cannabis”. Alternatively, cannabis plant material, containing cellulose and hemicellulose can be pretreated and hydrolyzed to release the lignocellulosic carbohydrates for brewing.

Hemp is a type of cannabis plant that contains low amount of psychoactive compounds (THC content < 0.3% by dry weight). Hemp, contains polymers of glucose and significant amount of xylose, a pentose carbohydrate. The knowledge from the biofuel industry can be transferred, however, brewing process needs to be redesigned for brewing as it is a food-grade product. Stalk, stem, and roots of the cannabis plant is processed very differently, requires chopping and cutting, washing, cooking and, hydrolysis with much more complex cellulose and hemicellulose enzyme complexes. Brewing yeast cannot utilize pentose carbohydrates as it cannot produce necessary enzymes to metabolize them. Using a brewing yeast will leave the fermentation incomplete.

Recombinant brewing yeast strains can be developed for efficient fermentation of hemp carbohydrates while producing flavor-active compounds. Classical strain improvement methods such as mutagenesis, laboratory evolution and cross-mating are used to develop improved microorganisms but they are time-consuming and labor intensive. Genome shuffling offers a rapid production of improved microorganisms through recursive genomic recombination with higher gene recombination efficiency, however the changes are random and the results subject to chance (Zhang, Perry et al. 2002). Electroporation-based genome shuffling is the latest method in the field of strain improvement, which is a fast

and easy method compared to protoplast fusion and mating-based genome shuffling (Zhang and Geng 2012).

## 4.2 Materials and Methods

### 4.2.1 Strains and Media

*Spathaspora passalidarum*, MYA4345, an insect associated yeast, was obtained from Cedarlane. *S. passalidarum* was streak plated on YM agar containing (g/L): yeast extract, 3; malt extract, 3; glucose, 10; peptone, 5, agar 10, at pH 6.8. *S. passalidarum* was subcultured from the ampoule received. Subcultures were stored in YM media containing 20% (v/v) glycerol at -80 °C in sterile 2-ml cryogenic vials. *Saccharomyces cerevisiae* WLP001 and *Saccharomyces pastorianus* WLP830 were obtained from Toronto Brewing as a liquid culture. A volume of 1-ml liquid culture was inoculated in YM media, incubated overnight at 25 °C and 150 rpm. *S. cerevisiae* and *S. pastorianus* was streak plated on YM agar. Isolated colonies were used to prepare subcultures, for purity and to eliminate any contamination, and stored in YM media containing 20% (v/v) glycerol at -80 °C. For genomic DNA extraction, *S. passalidarum* was cultured in YPX media containing (g/L): yeast extract, 10; peptone 20; xylose 20 at pH 5.5. The host strains *S. cerevisiae* and *S. pastorianus* were cultured in YPD media containing (g/L): yeast extract, 10; peptone 20; glucose 20 at pH 5.5.

### 4.2.2 Genomic DNA extraction

For genomic DNA extraction of *S. passalidarum*, a protocol from Yeast Protocols book was adapted (Biss, Hanna et al. 2014). *S. passalidarum* was cultured in 10 mL YPX at 30 °C and 130 rpm overnight. The yeast cells were harvested by centrifugation at 3500 rpm and 4 °C for 10 minutes then were washed with sterile water two times. After the last centrifugation, the supernatant was removed and the cells were resuspended in 1 mL sterile

water. The cell suspension was vortexed and transferred to 2-ml microcentrifuge tube. The cell suspension was centrifuged for 5 minutes at 13000 rpm and room temperature, then, the supernatant was removed. The cells were resuspended in 230  $\mu$ L lysis buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 0.1 M NaCl, 1% (v/v) SDS, 2% (v/v) Triton X-100). Acid-washed 0.5-mm glass beads (0.4 g) and 500  $\mu$ L of phenol:chloroform:isoamyl (25:24:1 (v/v/v)) alcohol were added. The mixture was vortexed at maximum speed for 3 minutes. The mixture was, then, centrifuged for 5 min at 5000 rpm and room temperature. The aqueous layer (upper layer) was carefully withdrawn and transferred to a 1.5-ml microcentrifuge tube. Six hundred  $\mu$ L ice-cold 95% (v/v) ethanol was added to the aqueous layer and mixed by inversion. Then, the mixture was stored at -20  $^{\circ}$ C for 30 minutes to precipitate the genomic DNA. After that, the genomic DNA was pelleted for 15 min at 13000 rpm and room temperature and the supernatant was discarded carefully. The DNA pellet was air-dried upside down at room temperature for 30 minutes. The DNA pellet was resuspended in 200  $\mu$ L of TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA, pH 8.0). Five  $\mu$ L of RNase A was added to the suspension and incubated at 37  $^{\circ}$ C for 10 min. Then, 8  $\mu$ L of 5 M NaCl and two times the total volume of 95% ice-cold ethanol was added. The suspension was stored in -20  $^{\circ}$ C for 30 minutes. After that, the genomic DNA was pelleted at 13000 rpm and room temperature for 15 minutes and the supernatant was discarded. The genomic DNA pellet was air-dried upside down for 30 minutes then resuspended in 200  $\mu$ L sterile water. The genomic DNA was stored at -20  $^{\circ}$ C until use.

### 4.2.3 Transformation

The host strains, *S. cerevisiae* and *S. pastorianus*, were propagated in 9.5 ml YPD media by inoculating 500  $\mu$ L from cryogenic storage cultures. The seed culture was incubated overnight at 30  $^{\circ}$ C with shaking at 130 rpm. Fifty mL of pre-warmed YPD media and  $2.5 \times 10^8$  cells from the seed culture were transferred to a 500-ml flask to give  $5 \times 10^6$  cells/ml ( $OD_{600} \sim 0.5$ ) and further cultured. When the cell concentration was between  $1.2 - 1.4 \times 10^7$  cells/ml ( $OD_{600} \sim 1.2 - 1.4$ ), after approximately 3 hours of incubation, the cultures were transferred to a 50-ml centrifuge tube. The cells were harvested by centrifugation at 3500 rpm and 4  $^{\circ}$ C for 15 minutes. The cells were washed once with ice-cold sterile water and



resuspended in 25 mL pre-treatment solution (0.1 M lithium acetate, 10 mM dithiothertiol, 10mM TE (Tris HCL:EDTA = 10:1 (v/v))). The cell suspension was incubated at room temperature for 1 hour. Then, the cells were pelleted and washed twice with 20 mL of ice-cold sterile water and once with 20 mL of ice-cold 1 M sorbitol. Finally, the cells were resuspended in 100  $\mu$ L ice-cold sorbitol and kept on ice along with a 0.2-cm electroporation cuvette. Approximately 4  $\mu$ g genomic DNA in 5  $\mu$ L was added into a 1.5-ml microcentrifuge tube. Then, 40  $\mu$ L of the cell suspension was transferred into the microcentrifuge tube and incubated on ice for 5 minutes. After incubation, the cell suspension was transferred into an ice-cold 0.2-cm electroporation cuvette. The cuvette was electroporated at 1.5 kV for 5 ms. After electroporation, 1 ml of 1 M sorbitol was immediately added into the cuvette. The cuvettes were incubated at 30 °C for 2 hours. Then, the cell suspension was transferred into a 50-mL centrifuge tube containing 5 mL of YPD. The centrifuge tube was incubated at 30 °C with shaking at 150 rpm for 3 hours. The cells were pelleted at 3500 rpm for 30 seconds and the supernatant was discarded. The cell pellet was washed once with sterile water and resuspended in 200  $\mu$ L of sterile water. The cell suspension was serially diluted and 100  $\mu$ L was spread on YNB-X selective plates, containing YNB without amino acids (ammonium sulfate), 6.7 g/L and xylose, 20 g/L. The host strain was also electroporated without genomic DNA and spread on YNB-X plates as the negative control. The plates were incubated at 30 °C for 7-10 days. Transformation efficiency was calculated using (Equation 4.6). Putative recombinants were evaluated in flasks containing 50 ml YNB-X under aerobic conditions and at 25 °C for 5 days. The same protocol was applied to the other host strain, *S. cerevisiae*, as well.

$$\text{Trans. Eff.} = \frac{\text{cfu} \times \text{Resuspension Volume } (\mu\text{L})}{\text{Volume plated } (\mu\text{L}) \times \text{Dilution Factor} \times \text{DNA Amount} (\mu\text{g}) \times 10^8 \text{ cells}}$$

**Equation 4.6.** Transformation Efficiency

### 4.3 Results and Discussion

A transformation method that was previously developed (Zhang and Geng 2012) was used to recombine the genomes of *S. pastorianus* and *S. cerevisiae* with the extracted genomic DNA of *S. passalidarum*. The whole genomic DNA of *S. passalidarum* was transferred into the host strains using an electric pulse. Approximately  $10^8$  cells in 45  $\mu$ L were electroporated at 1.5 kV. For *S. pastorianus*, 1.8 kV was used, as *S. pastorianus* was previously reported to have low transformation efficiency (Bernardi, Kayacan et al. 2019). Electroporated cells were spread on selective plates containing xylose as the sole carbon source and wrapped with parafilm. These selective plates were incubated at 30 °C for 7-10 days in a separate incubator from other plates to prevent cross-feed of ethanol vapours.

**Table 4.1.** Transformation efficiency of electroporation with *S. pastorianus* and *S. cerevisiae*

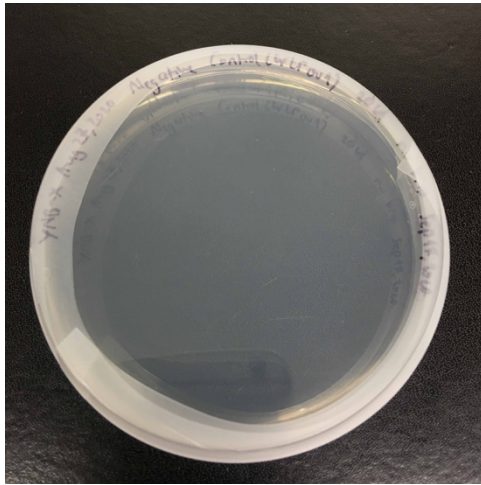
Host Strain	Donor Strain	Voltage (kV)	LiAc (M)	Transformation Efficiency (cfu/ $\mu$ g DNA/ $10^8$ cells)
<i>S. pastorianus</i> WLP830	<i>S. passalidarum</i> MYA4345	1.8	0.1	$5.19 \times 10^3$
<i>S. pastorianus</i> WLP830	<i>S. passalidarum</i> MYA4345	1.8	0.3	$1.41 \times 10^4$
<i>S. cerevisiae</i> WLP001	<i>S. passalidarum</i> MYA4345	1.5	0.1	$3.76 \times 10^4$

Approximately 4  $\mu$ g of *S. passalidarum* DNA was used in each experiment.

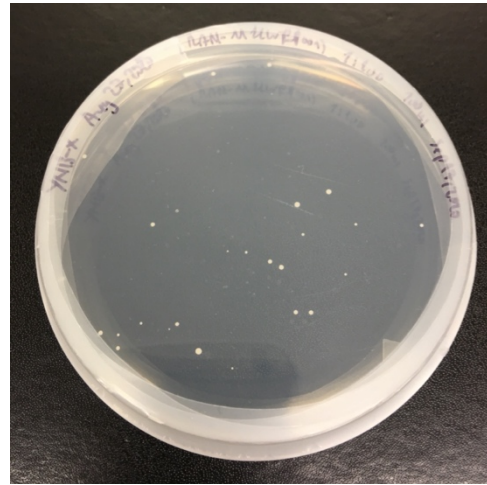
Transformation efficiency for *S. pastorianus* was  $5.19 \times 10^3$  cfu/ $\mu$ g DNA/ $10^8$  cells. Increasing LiAc concentration in the pretreatment solution to 0.3 M and electroporating the cells with 1.8 kV improved the transformation efficiency by 1 order of magnitude to  $1.41 \times 10^4$  cfu/ $\mu$ g DNA/ $10^8$  cells. For *S. cerevisiae* WLP001, 2.5-fold higher transformation efficiency was obtained at lower electroporation voltage (1.5 kV) and lower LiAc concentration (0.1 M) in the pretreatment solution.

A total of 21 *S. pastorianus* recombinant colonies of various sizes grew on the selective plates, which were round and white (Figure 4.1). The largest five *S. pastorianus* recombinant colonies were further evaluated for their xylose utilization ability. It was assumed that these colonies consumed xylose faster, therefore, grew faster and were larger in size. The colonies were inoculated in 5 ml YPX 1-2 days and mixed with glycerol for cryopreservation and grew to an OD<sub>600</sub> of approximately 2. These cultures were also used as seed cultures for the evaluation of recombinants.

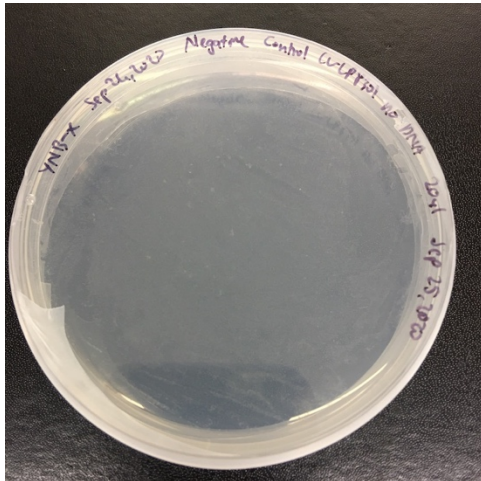
A 96-well plate, containing xylose or glucose, was prepared to evaluate the growth of the largest 5 putative recombinants. All 5 putative recombinants grew well in glucose, as fast as the host strain. However, no growth was observed in any of the wells that contained xylose as sole carbohydrate (Figure 4.2). The experiment was repeated in 125-ml flasks containing 50 ml of YNB-X with tin foil on top to provide better aeration to the medium. However, the recombinants did not grow in flasks, either (Figure 4.3).



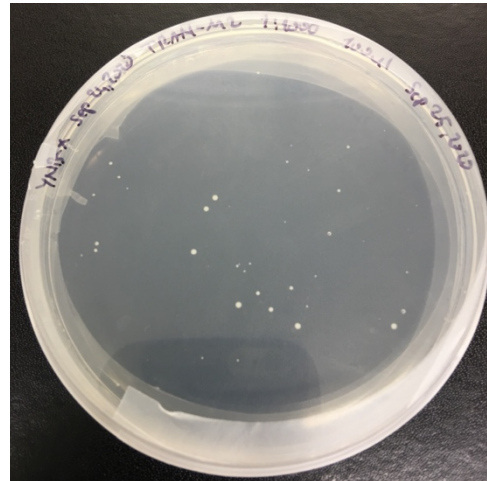
Negative Control  
(*S. cerevisiae* WLP001)



*S. cerevisiae* WLP001  
Putative Recombinants

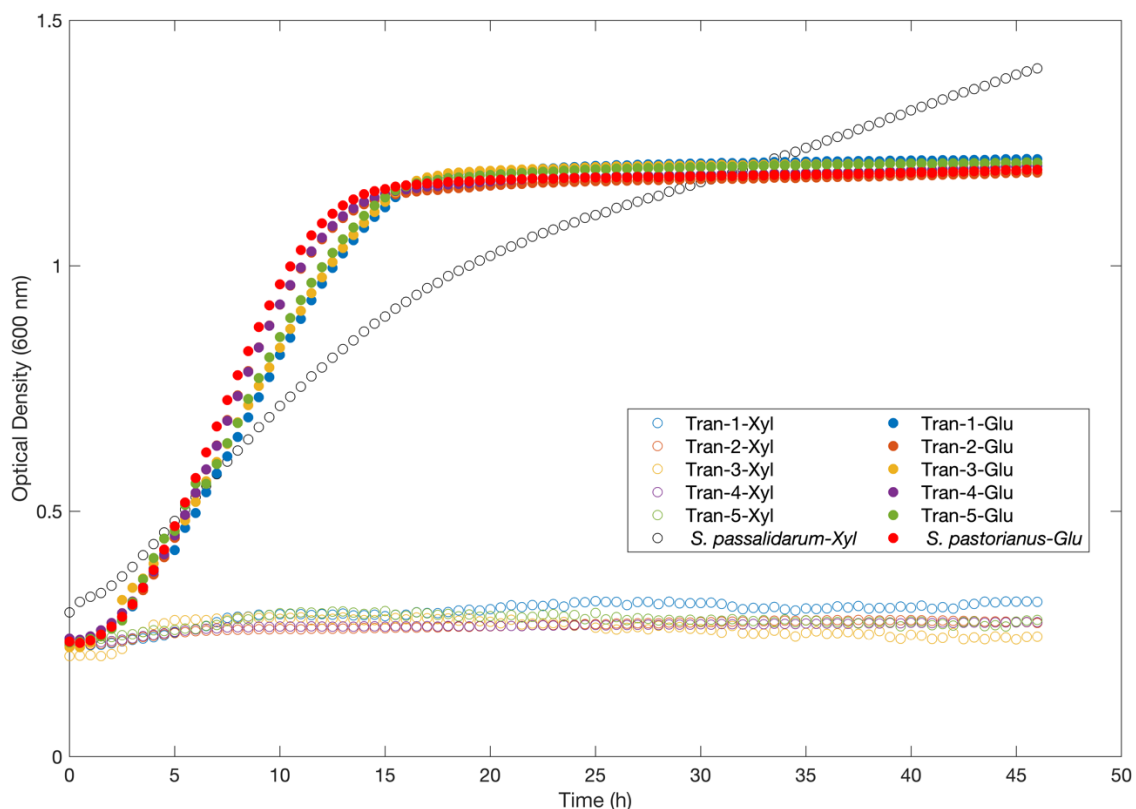


Negative Control  
(*S. pastorianus* WLP830)



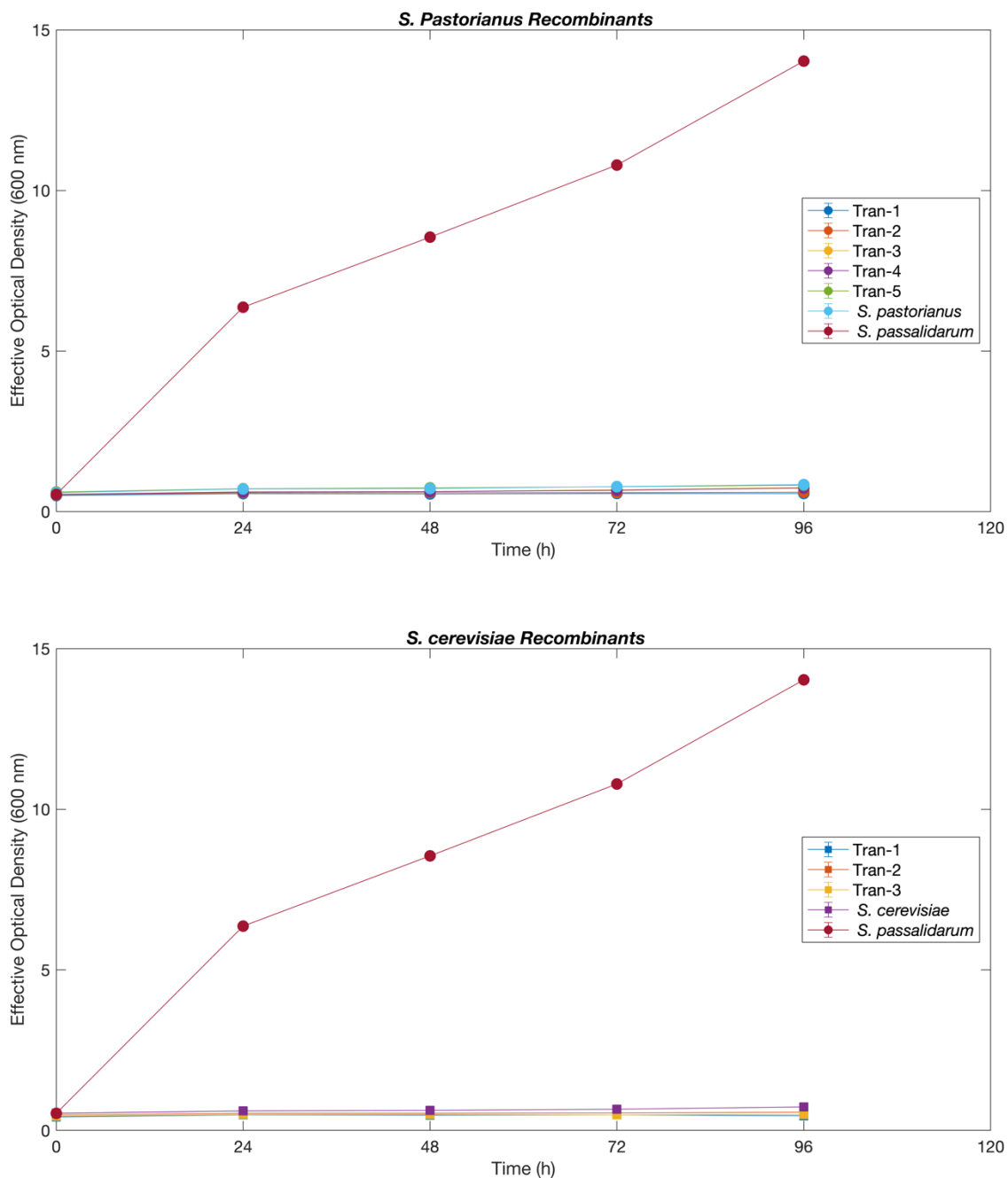
*S. pastorianus* WLP830  
Putative Recombinants

**Figure 4.1.** *S. pastorianus* WLP830 and *S. cerevisiae* WLP001 putative recombinants obtained on YNB-X selective plates containing, YNB, 6.7 g/L; xylose, 20 g/L; agar 15 g/L. For negative control, cells were electroporated without genomic DNA and then spread on the selective plates.



**Figure 4.2.** Growth assessment of recombinants in 96-well plate. Growth medium contained 13.4 g/L YNB and 20 g/L xylose or glucose. Each curve shows the average of 4 wells. *S. pastorianus* WLP830 and *S. passalidarum* MYA4345 were used as control strains.

The putative recombinant strains were able to grow on selective plates, containing xylose as the sole carbon source, but not in liquid medium in 96-well plates or flask cultures. It was hypothesized that the putative recombinant strains did not express the genes obtained from *S. passalidarum*. To allow electroporated cells to repair and integrate DNA to their genome, a recovery period was added to the transformation protocol. Electroporated cells were incubated in YPD medium at 30 °C for 3 hours prior to spreading on selective plates and washed once with sterile water. The recombinants were then evaluated in 125-ml flasks containing liquid selective medium. However, the putative recombinants obtained using this revised protocol did not grow in liquid YNB-X and no further experiments were performed with these recombinants. Transformation was also performed using *S. cerevisiae* WLP001 as the host strain. However, when the putative recombinants, after culturing and stored, were sub-cultured and inoculated in selective medium containing xylose (either 96-well plates or flask fermentations), they did not grow (Figure 4.3).



**Figure 4.3.** Growth of putative recombinants in YNB-X medium containing, YNB, 13.4 g/L; xylose, 20 g/L. Curves show the average growth in duplicate flasks.

It was suggested that the extracted genomic DNA did not integrate with the genomic DNA of the brewing host strains, therefore, they lost the xylose utilization ability transferred from the xylose-fermenting yeast strains after the selection plates. Several studies also

reported genetic instability after hybridization for artificial hybrids of industrial brewing yeast strains ((Pérez-Través, Lopes et al. 2012, Selmecki, Maruvka et al. 2015, Peris, Moriarty et al. 2017). However, this was a different method than the transformation method used in this chapter. Another suggestion is that the strains (xylose-fermenting and host brewing yeast) selected for this transformation procedure, may not be suitable for this method. The researchers who developed this method used a diploid *S. cerevisiae* as the host strain (Zhang and Geng 2012) and obtained a stable recombinant strain. While Jutakanoke, Tolieng et al. (2017) used non-*saccharomyces* strain, *K. marxianus* G12-16-1, and Ren, Wang et al. (2016) used a thermotolerant mutant of *S. cerevisiae* strain as the host strains. The ploidy of these two strains were not reported. The strains used in these three studies of electroporation-based transformation were industrial strains and obtained recombinants were genetically stable. However, *S. pastorianus* WLP830 and *S. cerevisiae* WLP001 used in this study were aneuploid industrial strains (Vaughan Martini and Kurtzman 1985, Large, Hanson et al. 2020). Brewing strains have very complex genomes and tend to lose chromosomes, due to their aneuploid nature, making them stable for the brewing industry (Kumaran, Yang et al. 2013). Also chromosomal instability increases as the genome size increase due to hybridization (Peris, Alexander et al. 2020). Sheltzer, Blank et al. (2011) reported that aneuploidy in yeast decreases homologous recombination efficiency and most strains had increased chromosome loss and defective DNA damage repair. Therefore, it is suggested that stable recombinants could not be developed using industrial brewing strains *S. pastorianus* and *S. cerevisiae*.

#### 4.4 Conclusions

Transformations of brewing strains of *S. cerevisiae* and *S. pastorianus* with *S. passalidarum* were performed via Li-Ac/electroporation to obtain xylose-fermenting brewing yeast strain. The putative recombinants did not utilize xylose in liquid medium after they were isolated, cultured and stored from the selective plates. The genetic instability of the putative recombinants was likely due to aneuploid nature of the brewing yeasts strains.

## Chapter 5

### 5 Conclusions and Recommendations

This chapter summarizes the major findings from this thesis and recommendations for future work.

#### 5.1 Conclusions

##### **Anaerobic Fermentation Performance**

*S. passalidarum* had higher fermentation performance of mixed carbohydrates of cellobiose, glucose and xylose under anaerobic conditions between temperatures 17 and 25 °C, compared to *P. stipitis*. Cellobiose and xylose consumption rates of *S. passalidarum* was higher than that of *P. stipitis*. In anaerobic fermentation of synthetic hemp extract, *S. passalidarum* consumed cellobiose and glucose simultaneously while xylose utilization was slow until glucose was depleted. *S. passalidarum* consumed xylose quickly after glucose depletion. High glucose concentration in synthetic hemp extract decreased xylose utilization rates of *P. stipitis* under both aerobic and anaerobic conditions.

##### **Aerobic fermentation performance**

*S. passalidarum* simultaneously consumed cellobiose, glucose and xylose under aerobic conditions. Propagation on xylose improved the xylose utilization rate of *S. passalidarum* under aerobic conditions. Aerobic conditions improve xylose uptake. Under aerobic conditions, carbohydrate consumption rates of both strains were faster than the rates under anaerobic conditions. However, aerobic fermentation is not preferred in brewing due to off-flavor and poor shelf life of oxidative products.

##### **Ethanol Respiration**

*S. passalidarum* and *P. stipitis* respired ethanol under aerobic conditions as soon as the carbohydrates were depleted but not under anaerobic conditions. If ethanol is the product



of interest, then the industrial fermentation needs to be monitored and stopped as soon as the xylose is exhausted.

### **Simultaneous carbohydrate utilization**

Simultaneous utilization of carbohydrates can decrease energy requirements and duration of brewing as the ethanol can be produced in shorter time, which will help develop a more economic brewing process.

### **Ethanol Tolerance**

*S. passalidarum* was more tolerant to ethanol than *P. stipitis*. Using an ethanol-tolerant yeast in brewing will allow producing a low-calorie beer with high ethanol content as these strains will be able to utilize carbohydrates in high ethanol concentrations.

### **Transformation**

Based on the fermentation performance of *S. passalidarum* under anaerobic conditions, transformation of *S. cerevisiae* and *S. pastorianus* were performed by transferring the extracted genomic DNA of *S. Passalidarum* using electroporation. Transformation of *S. cerevisiae* had higher transformation efficiency than that of *S. pastorianus*. Recombinants of *S. cerevisiae* and *S. pastorianus* were deemed to be unstable and lost the ability of xylose utilization due to aneuploid nature of these brewing yeast strains.

## 5.2 Recommendations

Based on the findings of this work, future research should address the following areas:

- Discontinuation of xylose utilization by *S. passalidarum* under aerobic conditions and at low temperature requires more investigation.
- Hemp extract should be analysed for inhibitory components.
- Fermentation ability of *S. passalidarum* and *P. stipitis* should be evaluated in an actual hemp extract in order to evaluate the effects of any inhibitory compounds, such as acetic acid, on carbohydrate utilization.

- Transformation should be performed using a haploid or diploid brewing yeast as the host strain.
- Genome shuffling should be performed once transformation is successful.
- After obtaining a stable recombinant, its fermentation ability should be evaluated in synthetic hemp extract under beer brewing conditions.
- Ethanol tolerance and utilization of carbohydrates other than cellobiose, xylose and glucose should be analysed. This will show that the recombinant also gained other traits from both host and DNA donor strain.

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## Appendices

### Appendix A: Supplementary Information for Chapter 3

#### Vitamins, Trace Elements and Salts in Synthetic Hemp Extract

**Table A.1.** Vitamin Concentration Delivered per Litre

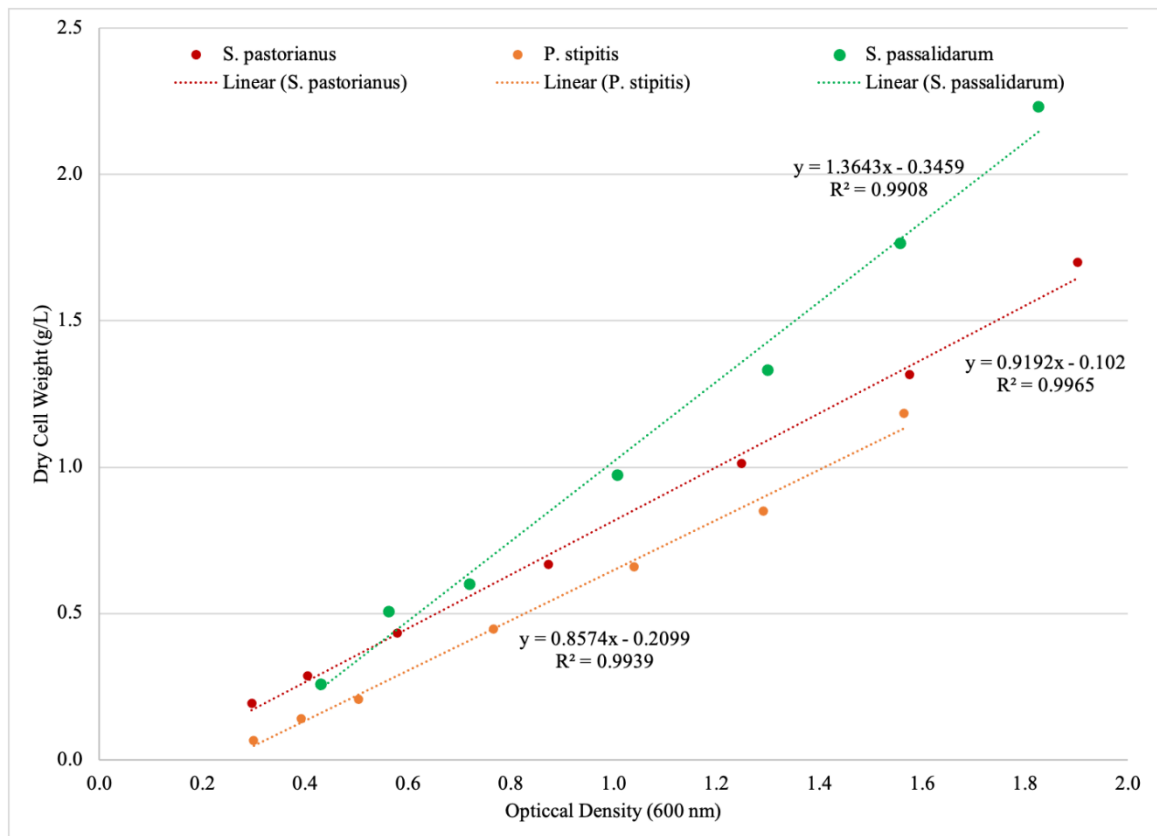
<b>Vitamins on a per litre basis</b>	<b>2x YNB</b>
Biotin ( $\mu\text{g}$ )	4
Calcium pantothenate ( $\mu\text{g}$ )	800
Folic acid ( $\mu\text{g}$ )	4
Inositol ( $\mu\text{g}$ )	4,000.00
Nicotinic acid ( $\mu\text{g}$ )	800
p-Aminobenzoic acid	400
Pyridoxine HCl ( $\mu\text{g}$ )	800
Roboflavin ( $\mu\text{g}$ )	400
Thiamine ( $\mu\text{g}$ )	800

**Table A.2.** Trace Element Concentration Delivered per Litre

<b>Separate Trace Elements</b>	<b>2x YNB</b>
Boric acid ( $\mu\text{g}$ )	1000
Copper Sulfate ( $\mu\text{g}$ )	80
Potassium Iodide ( $\mu\text{g}$ )	200
Ferric Chloride ( $\mu\text{g}$ )	400
Manganese Sulfate ( $\mu\text{g}$ )	800
Sodium Molybdate ( $\mu\text{g}$ )	400
Zinc Sulfate ( $\mu\text{g}$ )	800

**Table A.3.** Essential Salts Concentration Delivered per Litre

Separate Salts	1x YNB
Potassium phosphate monobasic (g)	1
Magnesium sulfate (g)	0.5
Sodium Chloride (g)	0.1
Calcium Chloride (g)	0.1

**Figure A.1.** Calibration Curves for *P. stipitis*, *S. passalidarum* and *S. pastorianus*

## Curriculum Vitae

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