

ABSTRACT

LAYFIELD, JOHNATHON BLAKE. Characterization of Hybrid Strains of *Saccharomyces pastorianus* for Desiccation Tolerance and Fermentation Performance. (Under the direction of John D. Sheppard.)

Dry yeast can be utilized in both ale and lager beer production to provide an inexpensive source of large amounts of viable cells for fermentation. This study examines the desiccation tolerance of different strains of *S. pastorianus* and the subsequent fermentation performance in comparison to *S. cerevisiae*. The use of active dry brewer's yeast (ADY), *Saccharomyces cerevisiae*, as a starter for the production of ales has been gaining popularity within the brewing industry, spurring manufactures to also produce active dry lager yeast (ADLY), *S. pastorianus*. The drying process is known to have a greater negative effect on the cell viability and physiology of ADLY than that of ADY, possibly due to the fastidious growth, low production temperature and poor thermotolerance of *S. pastorianus*. This may result in lower cell viability and concentration of ADLY starter cultures, which could lead to stuck or slow fermentations. *S. pastorianus* is a hybrid organism resulting from a cross between *S. cerevisiae* and *S. bayanus*. It has been proposed that it can be categorized into two distinct groups: Group 1 (*S. pastorianus*- Saaz type) has lost a significant amount of the genomic content contained within *S. cerevisiae* and is therefore closer to *S. bayanus*; while Group 2 (*S. pastorianus*- Frohberg type) has retained almost all of the genomic content of *S. cerevisiae*. To investigate whether these two groups differ in their tolerance to desiccation, both groups of *S. pastorianus* were spray dried at 140°C and rehydrated in phosphate buffer at 25°C for 30 minutes. The viability of the rehydrated cultures was determined using microscopic and viable cell counts. The fermentation performance of the cultures was tested by inoculating equal counts of viable rehydrated cells into brewer's wort and monitoring changes in cell count, carbohydrate and alcohol concentration until completion. The findings suggest that the *S. pastorianus*- Frohberg type is less tolerant to desiccation than either *S. cerevisiae* or *S. pastorianus*- Saaz type. The Frohberg type shows evidence of membrane damage which could delay the onset of fermentation. Utilization of the correct strain of ADLY could reduce the possibility of contamination or extended lag phases leading to stuck fermentations.

Key Words: *Saccharomyces pastorianus*, *Saccharomyces cerevisiae*, Active dry yeast, Dehydration, Beer fermentation, Saaz, Frohberg, Cellular Injury

Characterization of Hybrid Strains of *Saccharomyces pastorianus* for Desiccation Tolerance and Fermentation Performance

by
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A thesis submitted to the Graduate Faculty of
North Carolina State University
in partial fulfillment of the
requirements for the Degree of
Master of Science

Food, Bioprocessing & Nutrition Sciences

Raleigh, N.C.

2009

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ACKNOWLEDGEMENTS

I would like to thank Dr. Sheppard for the opportunity to perform research under his tutelage. I would also like to thank the Dr. Phister, Dr. Klaenhammer, Dr. Jinjun Gong, Mara Massel, Lucas Vann, Melissa Ivey, Michael Leonardelli and Dr. Klaenhammer's lab group for all their encouragement, help and support throughout the research process. I would also like to thank my family and friends for all their love and support over the years. Lastly, if beer is proof that God loves us and wants us to be happy, then I must thank God for the knowledge to produce beer.

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LITERATURE REVIEW

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Introduction to the Brewing Process

The word beer stems from the Latin infinitive '*bibere*' meaning "to drink." The old High German word for beer was '*beor*' or '*bior*' corresponding to an old English word '*beer*'. The production of beer is both an art and science that is thought to have been established around 6000 B.C. in ancient Babylonia. Brewing spread from the Middle East into Western Europe and then to the British Isles. Early breweries were often in monasteries and brewing itself was considered an art, carried out by skilled craftsmen. European monasteries used practical knowledge and brewing experience to develop a tradition of expertise and innovation. The first use of hops and bottom fermenting yeast are linked to these early monk brewmasters. Over time the beer manufacturing grew from small, craft-oriented production into a large brewing industry. The traditional brewing process utilizes 4 main ingredients: water, malted barley, hops, and yeast. Although the basic ingredient list is fairly small, each ingredient plays a crucial role in the production and final quality of the beer (McCabe, 1999; Hutkins, 2006).

Water, malted barley, hops and yeast are the four main ingredients used in the manufacture of beer and these ingredients are utilized in four distinct stages to produce (Figure 1.1). Malting is the first stage in which barley is converted to malted barley or "malt". The second stage is "mashing" where a highly nutritive growth medium is formed by substrate extractions and enzymatic reactions. The third stage is fermentation wherein the sugars in the nutritive medium, "wort," are fermented into beer. The last stage, post-fermentation, is an often overlooked but extremely important step in which the beer is clarified, filtered, carbonated, and packaged for consumption (Hutkins, 2006). Using a process similar to Figure 1.1, many different types of beer are produced in countries around the world (Table 1.1).

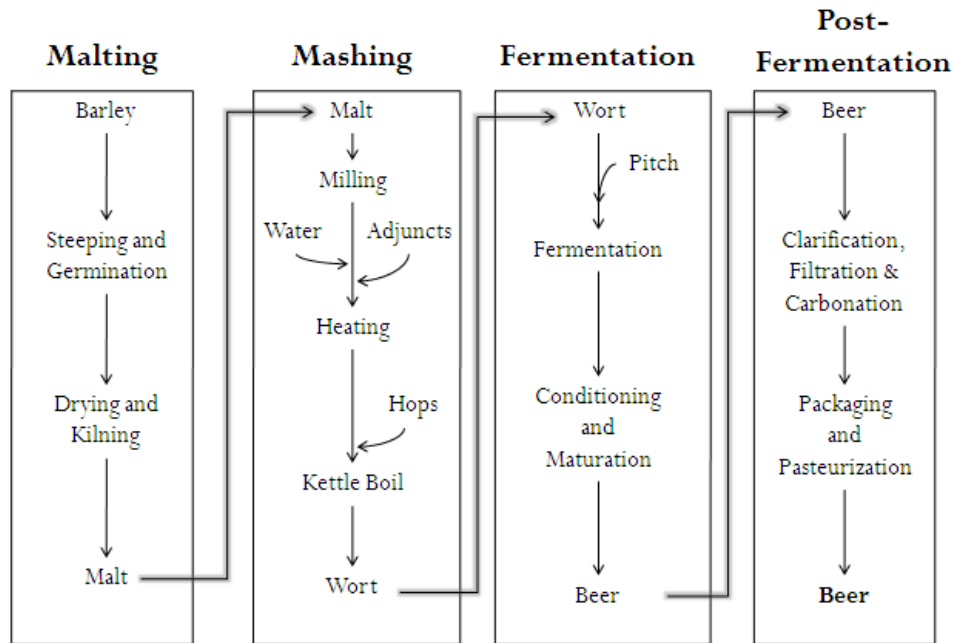


Figure 1.1. Manufacturing of beer (Adapted from Hutkins, 2006)

Table 1.1. Classical beer types brewed around the world (Adapted from McCabe, 1999)

TYPE	CHARACTER	ORIGIN	ALCOHOL (%W/V)	FLAVOR FEATURES
---Bottom Fermented---				
Bock	Lager	Bavaria, US, Canada	6	Full bodied
Doppe Bock	Lager/Ale	Bavaria, US, Canada	7 to 13	Full bodied, Estery
Dortmunder	Lager	Dortmund	5+	Light Hops, Dry, Estery
Light Beers	Lager	US	4 to 5	Light Hops, Light Body
Munchner	Lager/Ale	Munich	4 to 4.5	Malty, Dry, Mod Bitter
Pilsner	Lager	Pilsen	4.5 to 5	Full bodied, Hoppy
Vienna (marzen)	Lager	Vienna	5.5	Full bodied, Hoppy
---Top Fermented---				
Ales	Ale	UK, US, Canada, Australia	2.5 to 5	Hoppy, Estery, Bitter
Alt	Ale	Dusseldorf	4	Estery, Bitter
Barley Wine	Ale/Wine	United Kingdom	8 to 10	Rich, Full, Estery
Kolsh	Ale	Cologne	4.4	Light, Estery, Hoppy
Porter	Stout	London, US, Canada	5 to 5.7	Very malty, Rich
Provisie	Ale	Belgium	6	Sweet, Ale-like
Saisons	Ale	Belgium, France	5	Light, Hoppy, Estery
Stout (Bitter)	Stout	Ireland	4 to 7	Dry, bitter
Stout (Mackeson)	Stout	United Kingdom	3.7 to 4	Sweet, Mild, Lactic Sour
Strong/Old Ale	Ale	United Kingdom	6 to 8.4	Estery, Heavy, Hoppy
Trappiste	Ale	Belgium-Dutch abbeys	6 to 8	Full bodied, Estery
---Wheat-Malt Beers---				
Gueuze-Lambic	Acidic Ale	Brussels	5+	Acidic, Estery
Hoegaards wit	Ale	East of Brussels	5	Full bodied, Bitter
Berliner Weissen	Lager	Berlin	2.5-3	Lightly flavored, Mild
S. Ger Weizenbier	Lager/Ale	Bavaria	5 to 6	Full bodied, Low hops

Water and Barley Malt

The quality of water used in a brewery is of prime importance, due to the fact that water comprises about 90-96% of beer. Water quality is based on mineral content, pH, and iconicity. Typically brewing water should have a medium hardness, containing approximately 100ppm of calcium and magnesium salts and around 50 ppm or less of bicarbonate/carbonate. Insoluble calcium phosphate complexes are formed upon heating of wort, which results in a decrease of the mash pH. This decrease in mash pH increases the activity of enzymes that are active at low pH and may also increase the thermal stability of α -amylase. Calcium availability also improves yeast growth and flocculation. Magnesium is required in water to support the activity and stability of yeast produced enzymes. A high concentration of bicarbonate will increase the pH of the mash or wort which can decrease amylase and protease stability. Sulfates may also be found in brewing water at low concentrations, as they are required for the biosynthesis of sulfur-containing amino acids, which are essential to yeasts. Traditionally brewing water differs markedly from site to site with the prime comparison being between the high calcium sulfate water from Burton-on-Trent for bitter pale ales, high bicarbonate water from Dublin for sweeter dark lagers and stouts, and soft low-alkaline water from Pilsen for pale lagers with delicate hop flavors (McCabe, 1999; Hutkins, 2006).

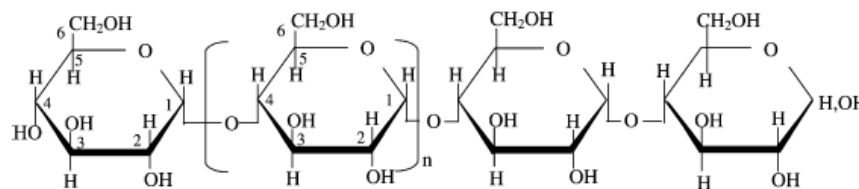
Malted barley, or malt, is the principle ingredient other than water used in the production of beer and is a member of the Graminia (grass family) of plants. Cereal grains, usually barely, are converted into malt through a series of biochemical events that are enzymatic in nature. Barley is typically found in two forms, six-row or two-row. Six-row malted barley (*Hordeum vulgare*) contains less starch, more protein, and a higher level of starch degrading enzymes than two-row barley (*Hordeum distichon*). The barley, which is essentially a seed, is germinated so that it synthesizes a plethora of enzymes that it would need were it to grow into a plant. The germination is arrested once the root system begins to develop. The barely is then slowly dried at a series of different temperatures to stabilize and preserve enzyme activity. The dried grains are then heated for further drying to reduce water activity levels in a process called kilning. Kilning is the step where non-enzymatic browning and associated heat-generated flavor reactions occur, which is readily apparent in finished beers such as stouts. The now malted barley is combined with water during the mash step of brewing. The purpose of the mash is to transform non-fermentable starch into various sugars that the yeast can ferment. The product of the mash is wort, a nutrient rich

medium of fermentable sugars, non-fermentable sugars, proteins, free amino acids and minerals (McCabe, 1999; Hutkins, 2006).

The carbohydrate fraction of malt is mostly starch. This starch is primarily composed of amylose and amylopectin, which represent approximately 58% of the dry weight (Table 1.2). Malted barley would be considered a ‘normal’ starch with approximately 25% of its starch comprised of α -1, 4 linked amylose (Figure 1.2), and about 75% being α -1, 4 and α -1, 6 linked amylopectin (Figure 1.3). The main starch-degrading enzymes found in malted barley are α -amylase and β -amylase. α -amylase (1, 4- α -D-glucan glucanohydrolase) catalyses the hydrolysis of internal (endoenzyme) α -glucosidic linkages in starch and other related oligo- and polysaccharides (Kadziola et al. 1994). The primary products formed by α -amylase are short chain α -1, 4 dextrans and branched, short chain α -1, 4 and α -1, 6 limit dextrans. β -amylase (1, 4- α -glucan maltohydrolase) catalyzes the liberation of β -maltose from the non-reducing ends (exoenzyme) of 1, 4- α -glucans (Eglinton et al. 1998). The primary products of β -amylase are maltose and small chain dextrans (Hutkins, 2006; Tester, 2004).

Table 1.2. Approximate composition of malt (Adapted from Hutkins, 2006)

Component	% dry weight
Starch	58
Sucrose	4
Reducing Sugars	4
Hemicellulose	6
Cellulose	5
Lipid	2
Protein	12
Amino acids/peptides	1
Minerals	2
Other	6



Amylose: α -(1 \rightarrow 4)-glucan; average n = ca. 1000. The linear molecule may carry a few occasional moderately long chains linked α -(1 \rightarrow 6).

Figure 1.2. Amylose: The linear α -1, 4 linked polymer consisting of glucose (Tester et al. 2004)

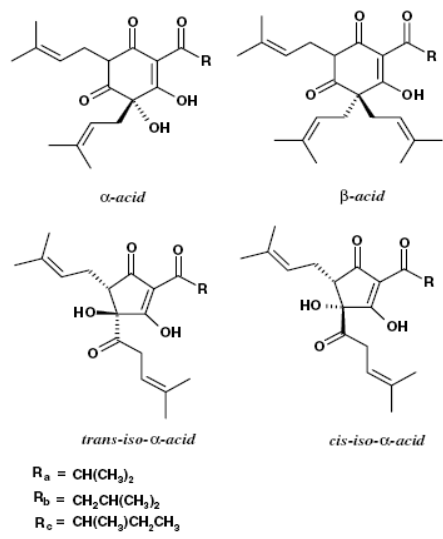


Figure 1.4. The most important hop bittering acids in beer (Vanderhaegen et al. 2006)

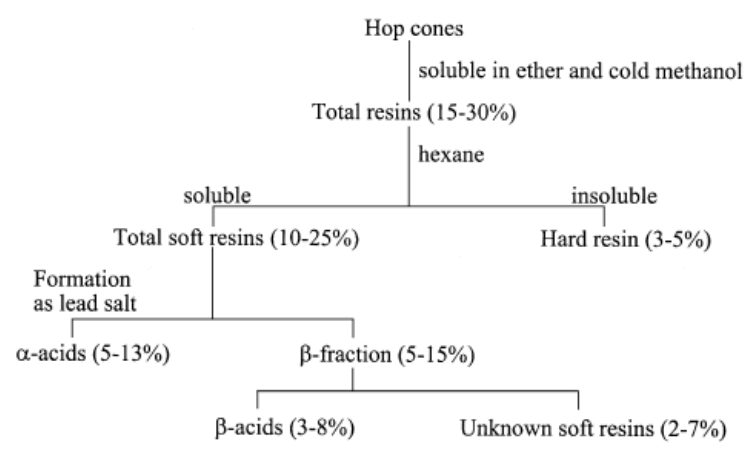


Figure 1.5. Fractionation of Hop Resins (Sakamoto et al. 2003)

Hop iso- α -acids are also one of the most important anti-microbial factors found in beer. Hop β -acids have a higher overall antibacterial activity than iso- α -acids but they are poorly soluble in wort and beer and thus are present in low amounts in beer. Beer has long been known as a safe beverage that has a high level of microbial stability. The reason that beer is an unfavorable medium for many microorganisms is due to the presence of ethanol (0.5-10% w/w), hop bitter compounds (~17-55 ppm of iso- α -acids), high carbon dioxide content (~0.5% w/w), low pH (3.8-4.7), very low oxygen content (<0.1 ppm) and only trace amounts of nutritive substances such as

glucose, maltose and maltotriose. Both Gram positive and negative bacteria are contaminants in beer (Table 1.3). Hop acids, act like a weak acid preservatives in that they are inhibitory due to the ability to decrease intracellular pH and to disrupt proton gradients (Sakamoto et al. 2003).

Table 1.3. Beer spoilage bacteria (Sakamoto et al. 2003)

Beer spoilage bacteria		
	Rod-shaped	Cocci
Gram-positive bacteria	<i>Lactobacillus</i> spp.	<i>Pediococcus</i> spp.
	<i>Lb. brevis</i>	<i>P. damnosus</i>
	<i>Lb. brevisimilis</i>	<i>P. dextrinicus</i>
	<i>Lb. buchneri</i>	<i>P. inopinatus</i>
	<i>Lb. casei</i>	
	<i>Lb. coryneformis</i>	<i>Micrococcus</i> sp.
	<i>Lb. curvatus</i>	<i>M. kristinae</i>
	<i>Lb. lindneri</i>	
	<i>Lb. malefermentans</i>	
	<i>Lb. parabuchneri</i>	
Gram-negative bacteria	<i>Pectinatus</i> spp.	<i>Megasphaera</i> sp.
	<i>P. cerevisiiphilus</i>	<i>M. cerevisiae</i>
	<i>P. frisingensis</i>	
	<i>P. sp. DSM20764</i>	
	<i>Selenomonas</i> sp.	<i>Zymomonas</i> sp.
	<i>S. lacticifex</i>	<i>Z. mobilis</i>
	<i>Zymophilus</i> sp.	
	<i>Z. raffinosisivorans</i>	

There are two genera of yeast that are commonly used in the brewing industry, each responsible for one of the two major categories of beer: ale and lager. Brewing yeasts are drastically different from the yeast strains found in a typical laboratory (Table 1.4). These two types differ physiologically, biochemically, and genetically, and many of the differences play a significant role in the yeasts ability to conduct the beer fermentation (Table 1.5). Ale is made using the top fermenting yeast, *Saccharomyces cerevisiae*, while lager beer is produced by bottom-fermenting yeast, *Saccharomyces pastorianus* (also known as *S. uvarum* or *S. carlsbergensis*). Top or bottom fermentation does not mean that the yeast, in wort, is confined to a specific area of the fermentor. Rather, upon growth, top-fermenting yeasts tend to form low density clumps or flocculations that trap CO₂ and rise to the surface. Lager yeast, in contrast, flocculates and settles to the bottom of the fermentor. Another major difference between ale and lager yeasts is the temperature of fermentation. Ale yeasts typically ferment from 18°C to 27°C, whereas lager yeasts ferment around or below 15°C. For this reason ales are historically more prominent in warmer

regions such as Great Britain, and lager yeast are more common in colder regions such as Germany (Hutkins, 2006).

Table 1.4. Differences between lab and brewing strains of *Saccharomyces* (Adapted from Hutkins, 2006)

Lab Strains	Brewing Strains
Haploid and diploid	Polyploid and aneuploid
Sporulating	Sporulate poorly
Spores viable	Spores mostly non-viable
Able to mate (a and α mating types)	Mating Rare

Table 1.5. Differences between ale and lager yeasts (Adapted from Hutkins, 2006)

Ale (<i>Saccharomyces cerevisiae</i>)	Lager (<i>Saccharomyces pastorianus</i>)
Flocculated yeast rises to the top	Flocculated yeast settles to the bottom
Optimum growth temperature > 30°C	Optimum growth temperature < 30°C
Minimum growth temperature = 15°C	Minimum growth temperature = 7°C
Maximum growth temperature = 40°C	Maximum growth temperature = 34°C
Cannot metabolize melibiose	Able to metabolize melibiose
Slow assimilation of maltotriose	Efficient assimilation of maltotriose
Sporulating (at low frequency)	Non-sporulating

To produce an ale type beer, the fermentation utilizes *S. cerevisiae* at temperatures ranging from 20°C to 25°C, followed by a short period of aging, or none at all. The production of a lager type beer uses *S. pastorianus*/*S. carlsbergensis*/*S. uvarum* at temperatures from 8°C to 15°C, followed by a long low-temperature period of maturation (this type of aging is called “lagering”). There is much diversity in beer despite the use of only two major (ale or lager) categories. Both ales and lagers have colors ranging from pale to black, alcohol contents ranging from one to ten percent by volume, and both can be lightly or strongly flavored. Ale brewing strains constitute a broad spectrum of *Saccharomyces* strains, some of which are similar to laboratory strains. Lager yeast strains are more conserved and are likely to be closely related to the first lager strain, *S. carlsbergensis* CBS 1513 (IFO 11023), which was discovered in 1883 by Carlsberg laboratory’s Emil Chr. Hansen (Kodama et al. 2005). Yeasts are a rich source of vitamins of the vitamin B-complex, especially thiamine (vitamin B₁), riboflavin (vitamin B₂), and nicotinic acid (niacin).

Yeasts also contain ergosterol or pro-vitamin D which is converted to commercial vitamin D by exposure to ultra-violet light (Clerck, 1957).

Starter Cultures

Traditional or natural fermentation methods are initiated by endogenous flora or by re-pitching, to yield products that have unique or singular quality attributes. Many European wines are made via natural fermentations, relying on the naturally occurring yeast present on the grape surface and winery equipment. This concept is fine on a small scale basis, given the inherent flexibility in terms of quality and time. In contrast, large scale fermentations of food and beverages demands consistent production quality and predictable production schedules, as well as stringent quality control to ensure food safety. Table 1.6 describes the inherent differences between traditional and modern fermentations of food and beverages (Hutkins, 2006).

Table 1.6. Fermented foods industry: past and present (Adapted from Hutkins, 2006)

Traditional	Modern
Small scale (craft industry)	Large scale (in factories)
Non-sterile medium	Pasteurized or heat-treated medium
Septic	Aseptic
Open	Contained
Manual	Automated
Insensitive to time	Time-sensitive
Significant exposure to contaminants	Minimal exposure to contaminants
Varying quality	Consistent quality
Safety a minor concern	Safety a major concern

Starter cultures consist of microorganisms that are inoculated directly into food materials to overwhelm the existing flora and bring about desired changes in the finished product. Changes may include novel functionality, enhanced preservation, reduced food safety risks, improved nutritional or health value, enhanced sensory qualities, and increased economic value. Although some fermented foods and beverages can be made without a starter culture, as mentioned above, the addition of high concentrations of microorganisms ensures that products are manufactured on a timely and repeatable schedule, with consistent and predictable product qualities. For large scale fermentations, such as cheese fermentations, there is no easy way to produce the amount of culture

necessary without the use of a concentrated starter culture. For all practical purposes, starter cultures are considered an essential component of nearly all commercially-produced fermented foods and beverages (Hutkins, 2006).

Yeast starter cultures are utilized primarily by bread manufactures but also by the wine and brewing industries. Yeast starters can come in a variety of forms from moist yeast cakes, used solely by the baking industry, to active dry yeast (ADY) packages sold at retail to home brewers and winemakers. Brewing starter cultures are selected based on a variety of desirable properties: rapid fermentation, production of desired properties, preservation tolerance and stability, flocculation, lack of off-flavors, proper attenuation, growth at a wide temperature range, and tolerance to osmotic, temperature, and handling stresses. If a brewery does not use a starter culture it is likely that it maintains and propagates its own proprietary yeast culture (Hutkins, 2006). The largest supplier of active dry yeast, Lallemand, supplies specific ale and lager yeast strains to hundreds of breweries in the United States, Europe, Australia, and Asia. Lallemand also supplies ADY tailored to the wine and distilling industry. ADY from Lallemand have 5×10^9 living cells per gram with less than 1 living cell of wild yeast or bacteria per 10^6 yeast cells (Lallemand, 2009). Quain and Boulton (2006) report active dry yeast to deliver a viability of $80 \pm 7\%$ for ale yeast and $64 \pm 7\%$ for lager yeast. The lower viabilities of ADY and active dry lager yeast (ADLY), compared to traditional propagation methods (re-pitching), deliver acceptable fermentations and products but the lower cell viability must be compensated for with the use of additional cells. Additional cell numbers can be achieved by pitching yeast at a higher cell density than usual. Cyr et al, (2007) tested two commercially produced active dry lager strains and assessed total viable cell count and viability with noteworthy results. The two strains (A & B) tested were significantly different in viability and viable cell concentration per gram (Table 1.7). Dry yeast fermentation performance also appeared to be significantly affected compared with freshly prepared inoculums of yeast of the same strain and identical pitching rate, based on viable cell count. The difference was at least 26 hours in the time required to reach the desired final gravity (Figure 1.6) (Cyr et al. 2007).

Table 1.7. Active Dry Lager Yeast Product Analysis (Cyr et al. 2007)

Strain	Viable Cell Concentration (g ⁻¹)	Viability (%)
A	$5.00 \pm 0.36 \times 10^9$	25.2 ± 2.0
B	$1.40 \pm 0.03 \times 10^{10}$	71.1 ± 2.0

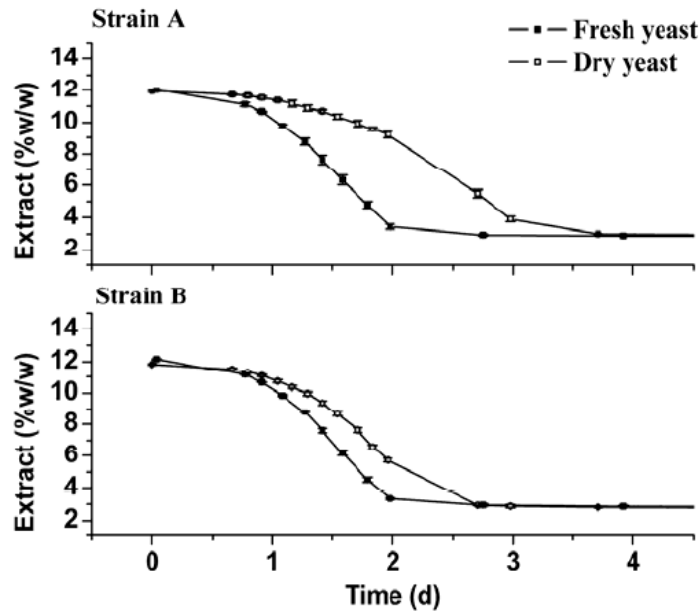


Figure 1.6. Extract consumption by fresh and dry yeast strains A and B (Cyr et al. 2007)

Using starter cultures not only ensures a consistent and predictable fermentation, it also addresses a more fundamental problem, namely how to produce enough cells to accommodate the inoculation demands of large-scale fermentations. For most fermented foods the first requirement of a starter culture is that it initiates the fermentation promptly and rapidly. While a short lag phase may be acceptable or even expected, a long lag phase is generally a sign that the culture has suffered a loss of cell viability. Thus for a starter culture to function effectively, it must contain a large number of viable microorganisms. For brewing, as the volume of wort increases, either larger starter culture volumes or greater starter culture cell concentrations are required. Future starter cultures for brewing can be genetically modified such that they produce beers with specific compositional characteristics (low carbohydrate, low ethanol), that have specific performance characteristics (attenuation, flocculating properties), or that simply produce better tasting beer (no diacetyl or hydrogen sulfite) (Hutkins, 2006). Brewing strains could also be modified in order to

better survive drying to provide superior viability in starter cultures. The drawbacks to the use of yeast with genetic improvements are the adverse public opinions of using genetically modified organisms (GMOs) in foods and regulatory restrictions on their use.

***Saccharomyces* Brewing Yeast**

Yeasts are unicellular, eukaryotic organisms that are typically spherical, or oval in shape, and 5-10 μm in diameter (Figure 1.7). The cell wall of yeast is composed of hemicelluloses, yeast gum, chitin, and 'fungal cellulose' which contains nitrogen in the molecule. The cell wall constitutes 19-20% of the fresh weight of yeast and is only permeable to low molecular weight substances and impermeable to complex proteins (Clerck, 1957). The cytoplasmic membrane (or plasma membrane) is a thin structure, about 8nm thick. This vital structure is a barrier separating the inside of the cell from its environment. If the membrane is broken, the integrity of the cell is destroyed, the internal contents leak into the environment, and the cell will die. The cytoplasmic membrane is also a highly selective barrier, enabling a cell to concentrate specific metabolites and excrete waste materials. The cytoplasmic membrane is essentially a phospholipid bilayer when fatty acids point inward toward each other in a hydrophobic environment, and the hydrophilic portions remain exposed to the aqueous external environment (Figure 1.8) (Madigan et al. 2003). The protoplast generally contains the nucleus, a large vacuole, and other inclusions. The moisture content of pressed yeast is approximately 75%. The dry matter mainly consists of glycogen, protein, yeast gum, lipids and mineral salts. Glycogen ($\text{C}_6\text{H}_{10}\text{O}_5$)_n, is the reserve carbohydrate of yeast. Under starvation conditions the yeast consumes glycogen, and this amount ranges from 0-30%. The protein content found in yeast is typically 45% but can be as high as 70%. The protein content has an inverse correlation with glycogen, when glycogen decreases, the protein content increases as a percent of total cell mass. Yeast contain both thermo-stable and thermo-labile proteins. Yeast typically contain 2-5% of lipids, under heavy aeration yeast lipid content can be as high as 10-20%. Yeast lipids contain phospholipids and sterols, such as ergosterol. Yeasts usually contain around 8% ash, primarily composed of phosphate (Clerck, 1957).

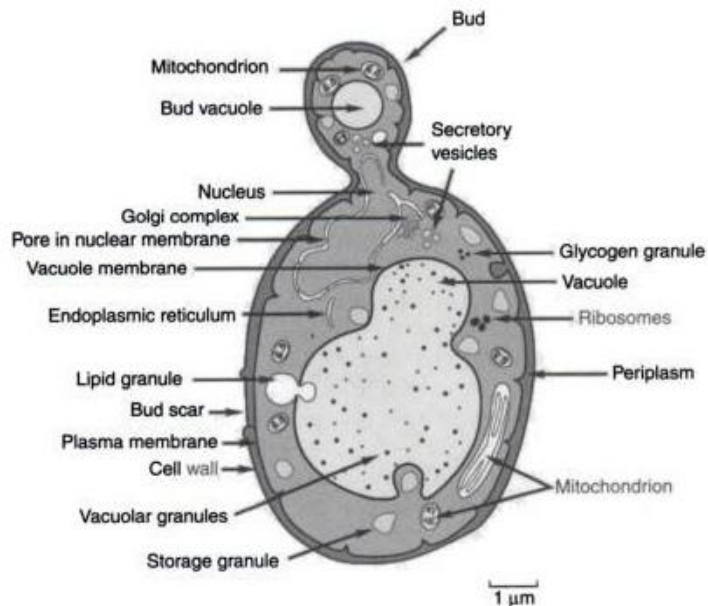


Figure 1.7. Main features of a typical yeast cell (Priest et al. 2006)

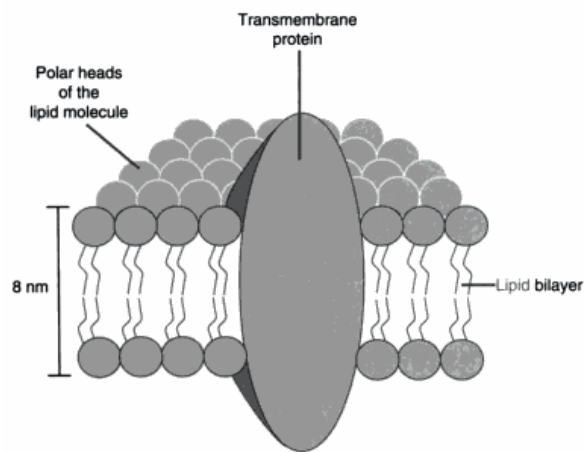


Figure 1.8. Yeast cytoplasmic (plasma) membrane (Priest et al. 2006)

The fundamental physiological characteristic of beer-brewing yeast is their ability to degrade carbohydrates, usually six-carbon molecules (C_6) and some disaccharides, such as glucose and maltose, into two-carbon (C_2) components, like ethanol, without completely oxidizing them to CO_2 , even in the presence of oxygen. Yeasts which produce or accumulate ethanol in the presence of oxygen are called Crabtree-positive yeast. The Crabtree effect is an alcoholic fermentation

where the degradation of hexose sugars, in the presence of oxygen, is due to insufficient capacity, saturation, or repression of the respiratory metabolism, leading to pyruvate overflow. Thus during ethanol production, the energy for growth is provided by the glycolysis and fermentation pathways rather than by the respiration pathways (Figure 1.9). For *S. cerevisiae* the Crabtree effect relies, largely, on a glucose-repression circuit, which in the presence of glucose or other C₆ carbohydrates, represses respiration (Piskur et al. 2006). In normal aerobic respiration a gram of hexose yields 674 kilo-calories of energy, whereas anaerobic fermentation only gives 66 kilo-calories, of which a large part is lost as heat. The amount of energy available to yeast in fermentation is therefore small, and growth and development are greater in the presence of oxygen (Clerck, 1957).

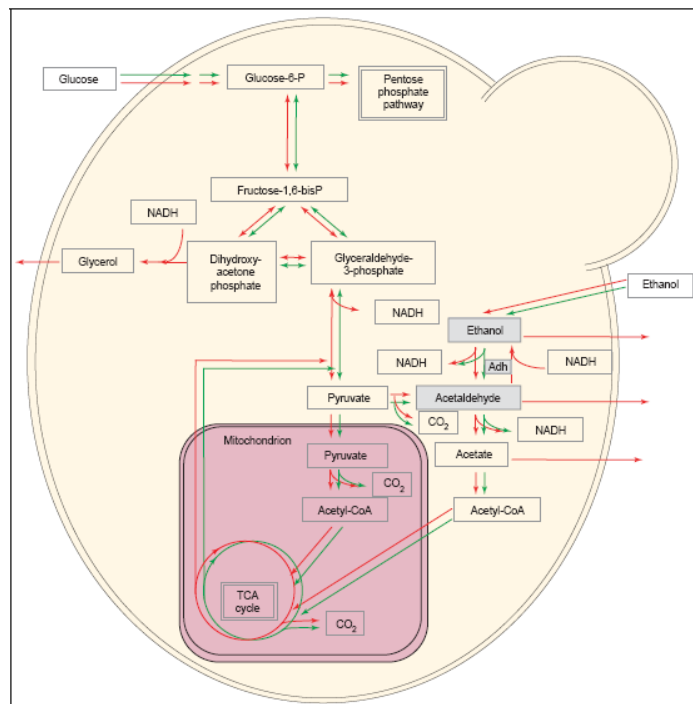


Figure 1.9. A scheme of the pathways involved in glucose and ethanol assimilation under aerobic conditions to distinguish the differences between Crabtree-positive yeast, *S. cerevisiae* (Red arrows), and Crabtree-negative yeast, *Kluyveromyces lactis* (Green arrows). Alcohol dehydrogenase (Adh) catalyzes the conversion between acetaldehyde and ethanol and is marked in gray (Piskur et al. 2006)

During fermentation, yeast recycles NADH in the acetaldehyde-to-ethanol conversion, and will convert accumulated ethanol back to acetaldehyde if oxygen becomes available (Figure 1.9).

Thus yeast can utilize ethanol, a product, as a substrate in the presence of oxygen. Alcohol dehydrogenase (Adh) catalyzes the acetaldehyde-to-ethanol reaction in both directions (acetaldehyde-to-ethanol or ethanol-to-acetaldehyde), at different catalytic efficiencies. Adh activity is encoded by two genes, *ADH1* which is expressed constitutively, and *ADH2* which is expressed only when internal sugar concentrations drop. The Adh genes and the ability to ferment glucose and accumulate ethanol even in the presence of oxygen, give *Saccharomyces* yeast a distinct advantage over other microbial competitors in the natural environment. The “make-accumulate-consume” strategy of ethanol production kills *Saccharomyces* competitors by producing ethanol, which is toxic to most other microorganisms, and then the generated ethanol can be consumed as an energy source (Piskur et al. 2006).

Brewing Yeast Nutrients

Although yeast can assimilate ethanol, the main nutrient classes impacting brewing yeast performance are carbohydrates and nitrogenous compounds. Brewing strains of *Saccharomyces* can utilize various carbohydrates (glucose, sucrose, fructose, maltose, galactose, raffinose and maltotriose), with a major distinguishing characteristic of lager yeast being the ability to ferment melibiose. The uptake of carbohydrates follows the simplest route in that monosaccharides (glucose and fructose) are utilized first, followed in order of complexity by disaccharides (maltose) and trisaccharides (maltotriose), excluding larger dextrans (DP) that are DP4 and above (Lodolo et al. 2008). Glucose chains longer than three are commonly given the annotation “DP” and then listing the number of glucose molecules on the chain (ex. DP4= a dextrin with 4 glucose units).

The monosaccharides are transported across the cellular membrane by common membrane transporters in coupled facilitated diffusion and active transport process. Of the four putative identified for glucose uptake, two glucose uptake systems are recognized as the most important: low affinity (constitutively expressed) and a high affinity transporter, which is repressed in the presence of high glucose concentrations (Bisson et al. 1993). Catabolite repression (the repression of the high affinity transporter) is a trait only found in fermentative yeast strains. The disaccharide maltose, which accounts for 50-55% of the total carbohydrates found in wort, has an uptake mechanism that involves two systems (Stewart, 2006). The first is an energy-dependent maltose permease (ATP → ADP) which transports the maltose intact across the cellular membrane where the second system, a maltase (α -glucosidase), hydrolyses maltose internally to yield two glucose

units. The trisaccharide maltotriose, which accounts for 10-14% of the total carbohydrate in wort, has an independent energy-dependent permease for intact transport, but shares the α -glucosidase to hydrolyze maltotriose into three units of glucose. Three maltose utilization genes (*MAL* genes) are involved in the operation of the high-affinity maltose transporter (Lodolo et al. 2008)

Another critical nutrient for yeast is nitrogen, which originates from the malt used in wort production. The main sources of nitrogen in wort are amino acids, ammonium ion and some di- and tripeptides. In most wort the free amino nitrogen (FAN) is utilized by yeast for protein formation (structural and enzymatic), required for yeast growth and other functions such as osmoregulation. The level and composition of FAN in wort can have a big impact on higher alcohol, ester, vicinal diketone (VDK), and H₂S formation due to the role of amino acid metabolism in the formation of these flavor compounds (Lodolo et al. 2008). Two VDKs, diacetyl (2,3-butanedione) and 2,3-pentanedione, are fermentation byproducts that both possess very low flavor threshold, 0.1 and 0.9 mg L⁻¹, respectively, and impart a buttery flavor. VDKs are undesirable in most beers, especially lagers which are supposed to have a cleaner organoleptic profile, and are caused by the biosynthesis of valine, leucine, and isoleucine (Figure 1.10) (Cyr et al. 2007).

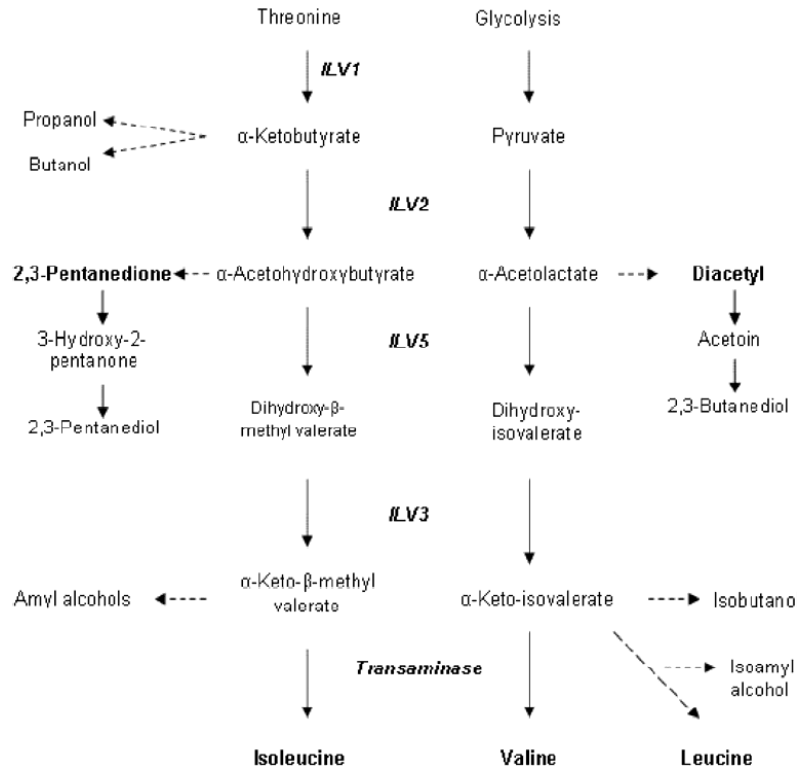


Figure 1.10. Pathway for isoleucine, valine, and leucine biosynthesis (Cyr et al. 2007)

Amino acids are utilized by yeast in a sequence that appears to be independent of fermentation conditions (Pierce, 1987). The group A amino acids are utilized first (arginine, asparagines, aspartate, glutamate, glutamine, lysine, serine, and threonine), followed by group B amino acids (histidine, isoleucine, methionine and valine. Group C amino acids (alanine, glycine, phenylalanine, tyrosine, tryptophan and ammonia) are only absorbed after the complete depletion of the group A amino acids. Group D amino acids (proline) are very poorly utilized because proline has no free amino group and thus cannot be transaminated (Lodolo et al. 2008; Perpete et al. 2005).

In addition to major carbohydrate and nitrogen requirements, minerals (trace elements) are also necessary for adequate fermentation performance. Calcium availability is important in the multifunction roles as a second messenger in the modulation of growth and metabolic responses of yeast cells to external stimuli. Calcium is also required for yeast flocculation, which is commonly used to differentiate between ale and lager yeast, and will be covered in detail later. Trace amounts of copper and iron are also necessary for yeast growth as they act as cofactors in several enzymes

involved in the respiratory chain. Iron also serves as the building block for heme formation and is involved in cellular redox homeostasis, and oxidative stress resistance. Magnesium, the most abundant intracellular divalent cation, acts primarily as an enzyme cofactor. Zinc is essential for the structure and function of over 300 enzymes, including alcohol dehydrogenase (Lodolo et al. 2008).

Dissolved oxygen (DO) is necessary in the beginning of a yeast fermentation to achieve a balanced fermentation that results in the desired end product(s). Oxygen has many roles in yeast physiology and various genes are expressed based on the oxygen in the environment to regulate cellular metabolism (Figure 1.11) (Lodolo et al. 2008). The primary role of oxygen in the initial stages of fermentation is the generation of sufficient amounts of biomass to efficiently carry out the fermentation. Oxygen is also essential for the biosynthesis of ergosterol and unsaturated fatty acids (UFA). Ergosterol and UFAs are integrated into the membrane bilayer structure of growing cells. Yeast cell membranes are very important in that the uptake of nutrients occurs across the membrane barrier. Yeast withheld from an adequate oxygen supply will have inferior membranes with a reduced transport capability and a reduced ability to withstand osmotic stresses and high exterior ethanol levels. The most common way to provide oxygen for fermentation is to aerate the wort or yeast slurry before pitching. This provides oxygen to the yeast to jump start the fermentation and avoids the negative metabolic processes, from too much oxygen, that can lead to reactive oxygen species, such as H_2O_2 , and hydroxyl radicals (OH^-) (Lodolo et al. 2008).

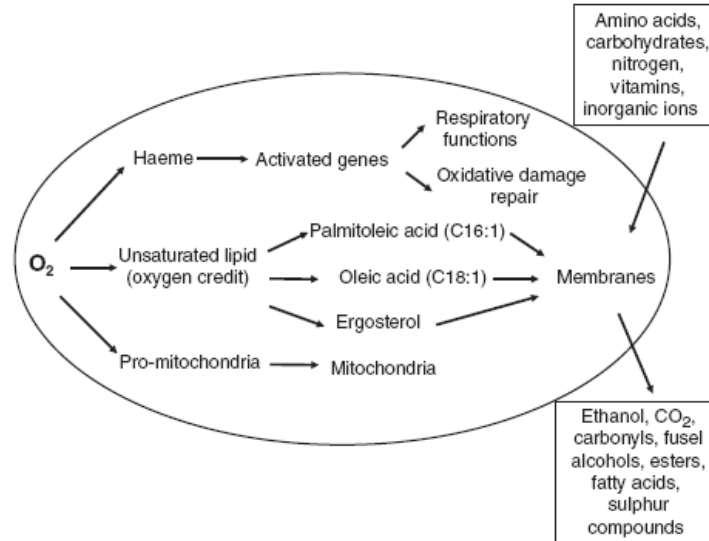


Figure 1.11. Summary of the multifaceted roles of oxygen in yeast metabolism (Lodolo et al. 2008)

Brewing Yeast Flocculation and Flavor Production

Yeast flocculation, which is commonly used to differentiate between ale and lager yeast is a reversible, asexual and calcium dependent process in which cells adhere together to form flocs consisting of thousands of cells. Upon formation, flocs rapidly separate from the bulk medium by sedimentation (lager yeast), or by rising to the surface (ale yeast). Flocculation is a complex process in which lectin-like proteins (flocculins), are activated by calcium in the medium, stick out of the cell wall of flocculent cells and bind to mannose residues present in the cell walls of adjacent cells (Figure 1.12).

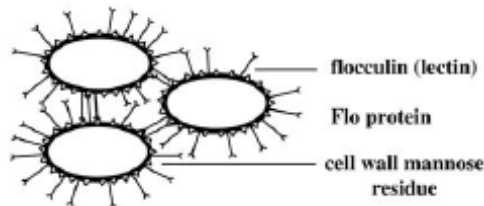


Figure 1.12. The lectin model for flocculation (Verstrepen et al. 2003)

The flocculins are encoded by specific genes, *FLO* genes, which form flocculins when activated and allow flocculation to take place. Lager yeasts contain an *Lg-FLO1* gene, which is not found in ale yeast, which is believed to encode a flocculin that can bind to both mannose and glucose. Flocculation is not a simple biochemical process, it also needs physical interaction: cells need to collide in order to bind with each other. Factors that increase cell to cell interaction (agitation, increase in cell surface hydrophobicity or cell-surface charge) could play just as important a role in flocculation as the expression of the *FLO* genes and activation of the Flo proteins. Factors affecting flocculation can be divided into three groups: the genetic background of the strain, environmental factors that influence *FLO* gene expression and activation, and factors that act upon the physical interactions between yeast cells (Figure 1.13) (Verstrepen et al. 2003).

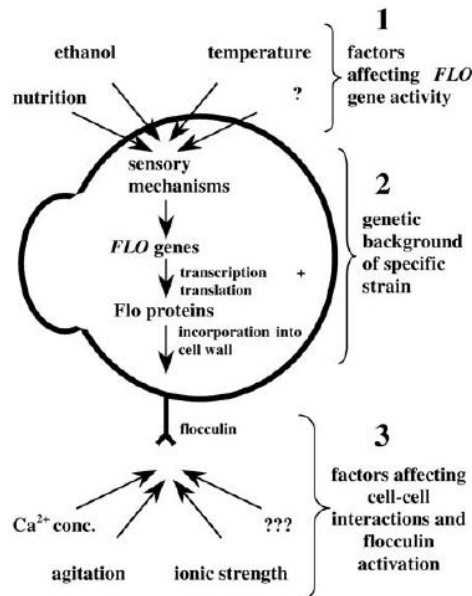


Figure 1.13. Factors affecting flocculation. Three categories of factors, some may act through more than one mechanism (Verstrepen et al. 2003)

The biochemical pathways that are active during fermentation contribute significantly to the unique flavors associated with beer. Flavor compounds are intermediates in pathways leading from the catabolism of wort components (sugars, nitrogenous compounds and sulphur compounds), to the synthesis of components necessary for yeast growth (amino acids, proteins, nucleic acids, lipids, ect.) (Figure 1.14). Yeast derived flavor-active compounds can be generally listed as ethanol, CO₂, carbonyls (aldehydes/ketones), higher/fusel alcohols, esters, VDKs, fatty and

organic acids, and sulphur compounds, with the primary by-products being ethanol and CO₂. Aldehydes as part of anabolic or catabolic pathways are part of higher alcohol formation during fermentation (Lodolo et al. 2008). Acetaldehyde is a major aldehyde, as an intermediate in the formation of ethanol and acetate, which can cause a “grassy” off-flavor at a threshold of 10-20 mg L⁻¹ (Meilgaard, 1975). Higher alcohols contribute to the overall beer flavor and can be synthesized by two routes either anabolic or catabolic. The first anabolic pathway is the synthesis of pyruvate from the wort sugars. The second catabolic process is by the Ehrlich pathway, which is the conversion of branched amino acids to higher alcohols by transamination, decarboxylation, and reduction. Esters are responsible for many of the beer flavors such as fruity (ethyl acetate), banana (isoamyl acetate), and apple/aniseed (ethyl caproate). Ester formation is linked to lipid metabolism, alcohol acetyl transferases (*ATF* genes), and cellular growth, it is also a fermentation product. Organic acids (pyruvate, citrate, malate, acetate, etc.) impart sour flavors and contribute to pH reduction during fermentation. In particular α-acetolactate and α-acetohydroxy acids are interesting due to their roles as precursors of diacetyl and 2,3-pentanedione. The primary sulphur components impacting beer flavor are sulphur dioxide (SO₂), hydrogen sulphide (H₂S), dimethyl sulphide (DMS) and mercaptans. Reduced sulphur is a critical part of proteins (sulphur-containing amino acids, like cysteine and methionine), coenzymes (CoA, biotin, thiamine and pyrophosphate) and other cellular metabolites (glutathione, sulphides, and thiols) (Lodolo et al. 2008).

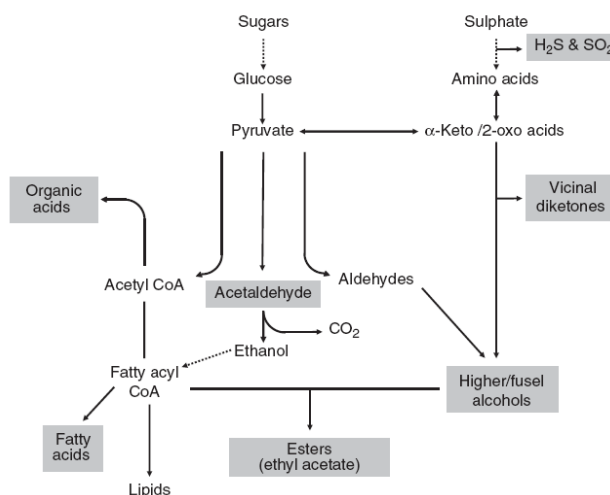


Figure 1.14. Interrelationships between the main metabolic pathways contributing to flavor-active compounds in beer (Lodolo et al. 2008)

***Saccharomyces pastorianus* (lager yeast)**

Lager yeast, *S. pastorianus*, is distinct from *S. cerevisiae* in both physiological and genetic characteristics and is proposed to have originated from the selective pressures applied during cold brewing conditions. According to the nomenclature *S. pastorianus* has previously been known as *S. carlsbergensis* and *S. uvarum*. A lower optimal growth temperature indicates that the genetic makeup of lager brewing yeast may be different than that of ale brewing yeast. Southern hybridization analysis of various lager brewing genes (*BAP2* [chr. II], *HIS4* [chr. III], *LEU2* [chr. III], *MAT* [chr. III], *HML* [chr. III], ect...) have almost all reached a similar finding. In most cases, the researchers found two divergent types of the gene in question. One allele exhibited a restriction and hybridization pattern identical or almost identical to that found in the corresponding *S. cerevisiae* gene, and another gene type that showed divergent patterns (Kodama et al. 2005). The first type of genes are referred to as *S. cerevisiae*-like (Sc-) and the divergent genes are referred to as *S. pastorianus*-, lager-, non-*cerevisiae*-, or *S. carlsbergensis*-specific (non-SC-). The finding of two types of genes is consistent with the possibility that lager brewing strains contain two types of chromosomes, Sc- and non-SC-like chromosomes (Kodama et al. 2005). Southern blot analysis and the use of various DNA probes have confirmed that the chromosomes of both *S. bayanus* and *S. cerevisiae* are present in bottom-fermenting lager yeast (Table 1.8) (Tamai et al. 1998).

Table 1.8. DNA relatedness of bottom fermenting yeasts (Tamai et al. 1998)

Strain	DNA relatedness (%) with:		
	<i>S. pastorianus</i> IFO 1167	<i>S. bayanus</i> IFO 1127	<i>S. cerevisiae</i> IFO 10217
BFY 070	117	41	59
BFY 023	111	59	80
IFO 1167	100	59	78
IFO 1127	73	100	62
IFO 10217	56	53	100
IFO 10609	29	34	48

*BFY 070 & 023 were lab strains in Tamai's experiments

Rainieri et al, (2006) evaluated 35 different yeast strains composed of *S. bayanus*, *S. pastorianus*, and *S. cerevisiae* (Table 1.9) by PCR-restriction fragment length polymorphism (PCR/RLFP) analysis. *S. bayanus* was subdivided into two groups for the experiment: *S. bayanus* var. *bayanus*, which contains a miscellany of hybrid cultures, and *S. bayanus* var. *uvarum*, which is referred to as *S. uvarum* and contains strains of non-hybrid origin. Rainieri's results indicated that there were pure strains of *S. bayanus* and *S. uvarum*, as well as hybrid lines that contain an unidentified lager genome. Group 1 hybrids (*S. cerevisiae*/*S. bayanus*/Lager Genome) represented lager brewing strains *S. pastorianus* strain CBS 1538, *S. carlsbergensis* strain CBS 1513, *S. monacensis* strain CBS 1503 and lager strains CBS 1486 and NBRC 2003. Chromosomes I and X were the same for all 5 strains found in Group 1. *S. cerevisiae* fragments were amplified from the 5 strains but not recovered in the same location. Group 2 hybrids (*S. cerevisiae*/*S. uvarum*/*S. bayanus*/Lager) included only two strains, CBS 1462 and CBS 8614. Both *S. bayanus* and/or *S. uvarum* patterns were detected in both strains at the same time for the Group 2 hybrids. Group 3 hybrids (*S. bayanus*/*S. uvarum*/Lager) was composed of *S. bayanus* type strain CBS 380, *S. globosus* strain CBS 424 and strain NBRC 2031, which is a Pilsner brewing strain that contains no *S. cerevisiae* genomic content. These strains typically had only a single genome represented at the analyzed sites. An interesting note on the study was that it suggested that the complexity of nomenclature for *S. uvarum*, *S. bayanus* and *S. pastorianus* is difficult and that the *S. pastorianus* name be used for multiple genetic lines that contain the *S. cerevisiae* genome (Rainieri et al. 2006).

Table 1.9. List of strains and PCR/RFLP data obtained in a preliminary study (Rainieri et al. 2006)

Species ^a	Old epithet	CBS ^b	NBRC ^c	Remarks	PCR/RFLP analysis		
					<i>FUN14</i>	<i>RIP1</i>	<i>HIS3</i>
<i>S. bayanus</i> *	<i>S. bayanus</i>	380	11022	Type strain	Su+Sb	Su	Sb
<i>S. bayanus</i>	<i>S. uvarum</i>	395	11025	Type strain	Su	Su	Su
<i>S. bayanus</i> *	<i>S. globosus</i>	424	10557	Type strain	Sb	Su	Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		1174		Brewing strain (Saaz)	Sc+Sb	Sc+Sb	Sb
<i>S. pastorianus</i> *		1462	250	Brewing strain	Sc+Su	Sc+Su	Su
<i>S. bayanus</i> or <i>S. pastorianus</i> †		1483		Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		1484		Isolated from cloudy beer	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. pastorianus</i> *		1486	1961	Brewing strain (Saaz)	Sc+Sb	Sc+Sb	Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		1502		Brewing strain	Su	Su	Su+Sb
<i>S. pastorianus</i> *	<i>S. monacensis</i>	1503	10610	Type strain	Sc+Sb	Sb	Sc+Sb
<i>S. pastorianus</i> *	<i>S. carlsbergensis</i>	1513	11023	Type strain	Sc+Sb	Sc+Sb	Sb
<i>S. pastorianus</i> *	<i>S. pastorianus</i>	1538	11024	Type strain	Sc+Sb	Sc+Sb	Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		1542			Su+Sb	Su+Sb	Su+Sb
<i>S. bayanus</i>		1545	1344	Brewing strain	Su+Sb	Su+Sb	Su+Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		2156		Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		2165		Brewing strain	Sc	Sc	Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		2440		Brewing strain (Saaz)	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		2443		Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i>		2946	573		Su	Su	Su
<i>S. bayanus</i>		3008	10558	Wine strain	Sb	Su	Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		5184	1343	Brewing strain	Su+Sb	Su+Sb	Su
<i>S. bayanus</i> or <i>S. pastorianus</i> †		5792		Brewing strain	Su	Su	Su
<i>S. bayanus</i> or <i>S. pastorianus</i> †		5832		Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> or <i>S. pastorianus</i> †		6903		Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> *	<i>S. abuliensis</i>	7001		<i>S. uvarum</i> representative	Su	Su	Su
<i>S. bayanus</i> or <i>S. pastorianus</i> *†		8614		Cider strain	Sc+Su	Sc+Su	Sc+Su
<i>S. bayanus</i>			251		Su+Sb	Su+Sb	Sb
<i>S. bayanus</i>			213		Su+Sb	Su+Sb	Sb
<i>S. bayanus</i>			539		Sb	Sb	Sb
<i>S. bayanus</i>			1048		Su+Sb	Sb	Su+Sb
<i>S. bayanus</i> *			1948	Brewing contaminant	Sb	Sb	Sb
<i>S. pastorianus</i> *			2003	Brewing strain	Sc+Sb	Sc+Sb	Sc+Sb
<i>S. bayanus</i> *			2031	Brewing strain (Pilsner)	Sb	Sb	Su+Sb
<i>S. bayanus</i>			10158	Brewing strain	Su	Su	Su
<i>S. bayanus</i>			10551		Su	Su	Su

^a *, strains selected for further study; †, species as indicated in the CBS database at the time of purchase.
^b CBS (Centraal Bureau voor Schimmelcultures, Utrecht, The Netherlands) strain.
^c NBRC (NITE Biological Resource Center, Department of Biotechnology, National Institute for Technology and Evaluation, Chiba, Japan) strain.

The minimum total size of the lager brewing yeast genome was found to be 23.2 Mbp, which is approximately twice the size of the *S. cerevisiae* genome. As in other studies the data presented two groups: 1-those with a DNA identity to *S. cerevisiae* of more than 98% (Sc-type), 2-those with identities around 85% (non-SC-type). *S. cerevisiae* and *S. uvarum* appear to be closely related within the total *Saccharomyces sensu stricto* group, with highly similar genomes of 16 chromosomes, and differing in five reciprocal translocations and three inversions (Kellis et al. 2003). One of the taxonomic markers to distinguish *S. pastorianus* and *S. bayanus* from other *Saccharomyces* species is the gene for the symporter to actively transport fructose. *S. bayanus* is generally isolated from enological environments, rich in fructose, which is not a major sugar in brewing, but the gene seems to be retained in lager brewing yeast. In the nuclear genome, the

mitochondrial DNAs (mtDNA) of *S. cerevisiae* and *S. uvarum* are quite different in both size and gene orders. *S. uvarum* was found to possess a smaller mtDNA than *S. cerevisiae* (57 kb vs. 70-85 kb), likely due to a lower number of ori/rep like sequence (4 vs. 8) and GC-rich clusters (50-60 in *S. uvarum* compared to 200 clusters in *S. cerevisiae*). Mitochondrial genome size differences are due to the presence/absence of optional introns and to the length of intergenic regions (Cardazzo et al. 1998). Differential expression of homoeologues, or genes that do not recombine regularly with *S. cerevisiae* genes, in the lager brewing yeast genome have been reported for the *BAP2* gene (encoding a branched-chain amino acid permease) [Kodama et al. 2001], *MET 2* (encoding homoserine O-acetyl transferase), and *MET 14* (encoding adenosylphosphosulphate kinase) [Johannesen et al. 2002]. This insinuates that lager brewing yeast is not merely a polyploid with two divergent, but similarly functioning, genome parts, but is a unique organism with a biological complexity greater than the species cited for its formation (Kodama et al. 2005).

Pope et al, (2007) took a metabolics approach to clarify the chemotaxonomy of brewing yeasts with the goal being able to discriminate yeast used to make ale and lager beers. This approach is considered to be a more functional level at which to study biological systems, as it represents the final downstream product of gene expression. Nine yeasts (Table 1.10) were chosen to be analyzed by CGH, PCR-RFLP, and a PCR-based genotyping method, called AFLP. The study identified a number of genes that are associated with carbohydrate metabolism in brewing, such as *ADH7*, *AAD3*, *AAD15*, and *AAD16* (alcohol metabolism), *ALD2* (aldehyde metabolism), *HXT15* and *HXT16* (glucose metabolism), *MAL33* (maltose metabolism) and *GDB1* and *GPH1* (glycogen metabolism). A number of asparaginases were also identified (*ASP3-1*, *ASP3-2*, *ASP3-3*, and *ASP3-4*) which may be useful for discrimination purposes as asparagine is a key amino acid in wort. A variable cocktail of metabolites were also detected including various amino acids and organic acids, carbohydrates, lipids, alcohols and phosphorylated compounds. The ale strains in this study showed divergence at the genotypic level despite conservation at the phenotypic level, suggesting that genetic divergence does not always lead to metabolic divergence (Pope et al. 2007). This study shows genetic differences between *S. cerevisiae* and lager brewing strains which may lead to quicker detection methods to differentiate between lager and ale yeast.

Table 1.10. Strains used in Pope et al. (2007) study

NCYC strain	Description	Classification method		
		Historical	PCR-RFLP	Growth at 37°C
505	Type strain	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
1187	Ale	<i>S. cerevisiae</i>	Hybrid	<i>S. cerevisiae</i>
1332	Ale	<i>S. cerevisiae</i>	Hybrid	<i>S. cerevisiae</i>
453	Lager	<i>S. pastorianus</i>	Hybrid	<i>S. pastorianus</i> or <i>S. bayanus</i>
530	Lager	<i>S. pastorianus</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>
680	Lager	<i>S. pastorianus</i>	Hybrid	<i>S. pastorianus</i> or <i>S. bayanus</i>
1056	Lager	<i>S. pastorianus</i>	Hybrid	<i>S. pastorianus</i> or <i>S. bayanus</i>
1324	Lager	<i>S. bayanus</i>	Hybrid	<i>S. pastorianus</i> or <i>S. bayanus</i>
2340	Lager	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>	<i>S. cerevisiae</i>

Historical classification was based on traditional chemotaxonomy and represents how the strains were originally accessioned into the NCYC. The hybrid nature of strains was demonstrated by PCR-RFLP analysis, the term 'hybrid' indicating that the strain contained a mixture of *S. cerevisiae*, *S. bayanus* and the as-yet unidentified 'lager' genomes. Classification based on ability to grow at 37°C differentiated between *S. cerevisiae*, which could grow at this temperature, and *S. pastorianus* and *S. bayanus*, which could not.

“Instantaneous speciation” due to an interspecific hybridization event that occurred at the cold, selective growth conditions is the current proposed mechanism for the allopolyploidy found in lager yeast (Dunn et al. 2008). Allopolyploids are polyploids, organisms with two or more sets of homologous chromosomes, with chromosomes derived from different species. It has been shown that different brewing yeast isolates can contain different combinations of genomes (or partial genomes) of *S. cerevisiae*, *S. bayanus*, and *S. bayanus* var. *uvarum*. The chromosomes derived from lager brewing yeast were found to be of three types (Kodama et al. 2005):

- I- Homologous chromosomes, which recombined normally with *S. cerevisiae* counterparts
- II- Homoeologous chromosomes, which rarely combined with *S. cerevisiae* counterparts
- III- Mosaic chromosomes that were composed of homologous and homoeologous segments

Sato et al, (2002) mated four different *S. cerevisiae* strains (DM338, W197, W177, and W192) with the cryophilic yeast *S. bayanus* (strain YM84). The results suggested that hybridization with *S. bayanus* would be useful for improving the low-temperature fermentability of the top fermenting *S. cerevisiae*. This suggests that the cryophilic performance of bottom-fermenting yeasts is due to the presence of *S. bayanus* (Sato et al. 2002). This evidence implies that the presence of the *S. bayanus* and *S. bayanus* var. *uvarum* genomes in the hybrid *S. pastorianus* give the yeast its observed ability to carry out cold fermentation better than *S. cerevisiae* genes alone (Dunn et al. 2008).

Dunn et al, (2008) used microarray analysis to study the genomic differences between 17 lager strains of *S. pastorianus*. Dunn used 60-mer oligonucleotide microarrays to detect and distinguish between sequences from both the *S. cerevisiae* and *S. bayanus* var. *uvarum* genomes (CGH- Comparative Genome Hybridization method). While the lager yeast studied only contained *S. cerevisiae* and *S. bayanus* genomic moieties, the *S. bayanus* sequences hybridized quite well with most of the *S. bayanus* var. *uvarum* probes and negligibly to the *S. cerevisiae* microarray probes. Genomic DNA from each of the lager strains was applied to the arrays and hierarchical clustering of the data (using both *S. cerevisiae* and *S. bayanus* genomic portions) revealed that the strains fell into two distinct groups. Group 1 had lost a significant portion of the *S. cerevisiae* genome through whole-chromosome or segmental aneuploidy, but retained virtually all of the *S. bayanus* genome. Group 2 retained virtually all genomic content of both *S. cerevisiae* and *S. bayanus* genomes (Appendix A&B). The two groups correlate with two types of lager yeast identified by the brewing industry as Saaz type (Group 1) and Frohberg type (Group 2). The 17 lager strains represent a variety of collection dates, from 1883 to 1976, as well as a variety of geographical locations and beer types (Appendix C). It was noted that the two groups had geographical/brewery groupings, with Group 1 representing both Saaz-type beers (Czech Republic beers) as well as Carlsberg brewery strains (Denmark), while Group 2 contained strains from the Netherlands (Heineken) and from non-Carlsberg Danish breweries and two North American breweries (Dunn et al. 2008). Group 1 strains, that are missing a significant portion of the *S. cerevisiae* genome, will be denoted as Saaz-type. Group 2 strains, which contain the genomic content of both *S. cerevisiae* and *S. bayanus*, will be referred to as Frohberg-type.

Dunn and Sherlock indicated that the average total DNA in a given hybrid lager strain for Group 1 was much lower than that of the Group 2 strains (Table 1.11). The results show that in Frohberg-type lager yeast there is an average of approximately two to three times more *S. cerevisiae* DNA relative to *S. bayanus* DNA within the genome, indicated by a group average of 2.53. This means that the Frohberg-type lager yeast has an average of 2.53 times more genomic content from *S. cerevisiae* than from *S. bayanus*. Among the non-Saaz Group 1 strains there was an equal amount of DNA contributed by *S. cerevisiae* and *S. bayanus*, indicated by a group average of 1.04. It is probable that there may be two or more relatively complete *S. cerevisiae* genomes present in the Group 2 strains (Frohberg), but only one genome each of *S. cerevisiae* and *S. bayanus* in the non-Saaz group 1 strains. For the Saaz strains in Group 1 it seems that there is

proportionately more *S. bayanus* DNA sequences relative to *S. cerevisiae* sequences (group average 0.63), due to *S. cerevisiae* chromosomal, or chromosomal portion, loss from the Saaz-like lager strains (Dunn et al. 2008).

Table 1.11. Ratios of *S. cerevisiae*: *S. bayanus* array intensities (Dunn et al. 2008)

Group average ^a	Sc:Sb ^b	Strain
0.63	0.59	GSY509
	0.65	GSY133
	0.66	GSY501
1.04	1.02	GSY131
	1.01	GSY137
	1.20	GSY129
	0.93	GSY134
2.53	2.42	GSY132
	2.74	GSY138
	3.98	GSY139
	2.96	GSY135
	3.36	GSY136
	2.24	GSY516
	2.19	GSY515
	2.24	GSY503
	1.65	GSY504
	2.24	GSY508

Each group, as described by microarray clustering (see text), is shown here (in top-to-bottom order): Group 1–Saaz (three members), Group 1–Non-Saaz (four members), and Group 2 (10 members).

^aThe average Sc:Sb ratio within each group is given, and is the geometric mean of the ratios (within that group) that are shown in the middle column.

^bThe within-strain ratio of *S. cerevisiae*:*S. bayanus* DNA (calculated by taking the average red-only intensity of all *S. cerevisiae* probes to the average red-only intensity of *S. bayanus* probes, as described in Methods); this is called the Sc:Sb ratio.

Yeast Dehydration

The basic quantitative component of the cell is water, which performs a number of functions. Microbial biomass contains 70-90% water. Water is the structural component of both, biopolymers and biomembranes (Beker et al. 1987). Free water is a medium and substrate for biochemical reactions, while bound water determines the structure of biological macromolecules and the interaction of various molecules. Cell dehydration under certain conditions may cause transition of living organisms into an anabiotic state. At present anabiosis is understood as a temporary reversible suspension of metabolic processes or their considerable delay (Beker et al. 1984). Transition into the anabiotic state can occur in a number of ways:

- 1- Anhydrobiosis- a state, caused by significant losses of water through evaporation
- 2- Cryobiosis- taking place by freezing organisms
- 3- Osmobiosis- a state, brought about by extraction of water from organisms by various solutions with a high osmotic pressure
- 4- Anoxybiosis- caused by a decrease of oxygen concentration in the gas phase below critical limits for aerobic metabolism (Beker et al. 1987)

In microorganisms the state of anabiosis can be expressed in two ways. Under unfavorable conditions, many species are known to display a series of special reactions, to change their metabolism to a considerable degree and to produce spores. Most often the sporulation is due to a nutritional deficiency. Drying does not usually stimulate spore formation. Under these conditions it is the vegetative cells of microorganisms that pass into the state of anabiosis. In nature the microorganisms in air, on plant surface, or in the soil can very often be subjected to dehydration, remain in anabiosis, rehydrate and revitalize to normal activity. Dried organisms are less affected by temperature extremes than organisms in an active state, which is ideal for storage and maintenance of starter cultures (Beker et al. 1987).

Resistance to drying is a specific characteristic of microorganisms and depends on the taxonomical and physiological properties that determine the chemical composition of the species or strain. The viability of a given organism is affected by various detrimental factors depending on structural and physiological resistance. Physical (structural) resistance of biological objects is expressed by a total absence of viability. In that case, the complete suspension of metabolic activity is dependent on the integrity and physiological resistance (durability) of biological structures. Physiological resistance is the resistance of a functional biological system connected with adaptive and reparative processes. It is important that the repair systems of the organism are intact, in order to recover from damage to cell ultrastructures or macromolecules (Beker et al. 1987).

As mentioned before, biopolymers and membranes are dispersed in a water medium, and life takes place only in the presence of water. Bound water is a part of intracellular water, which combines directly with proteins, nucleic acids, membranes or other substances and which is responsible for maintaining structural organization. Yeast biomass contains 15-20% bound water,

cell membranes (cytoplasmic) about 25% on a wet weight basis. Convective drying of yeast biomass consists of two processes:

- 1- Water from the surface of the outer layers of biomass granules evaporates into the atmosphere (intergranular or free water)
- 2- Within the granules water displaces by way of diffusion (intragranular translocation)

During the period of constant drying the intercellular and a part of the intracellular (free) water is evaporated. When the bound water starts to evaporate, the drying rate decreases, this takes place in yeast at around 20% moisture. At 20% moisture content the physiological behavior of cells drastically changes. In the range between 20% and 10% moisture content, together with dissolved substances, form a gel and can no longer act as a medium for biochemical reactions. Water (2.5-4.0%) is preserved in yeast during desiccation by changing membrane protein conformation, which, in turn increases the permeability of intracellular membranes (Beker et al. 1987).

Cultivation conditions can greatly influence a microorganism's resistance to drying. The resistance of the same strain may vary within very wide limits, depending on the composition of nutrient medium used for cultivation. Viability of *S. cerevisiae*, grown on a rich molasses medium may reach 90%, while that of the same strain grown on a synthetic medium may be 20-40%. High viability of dried yeast can be obtained by growth on concentrated molasses media with a limited nitrogen source (Harrison, 1963). Yeast sampled at the exponential growth phase are considerably more labile against drying than those sampled from the stationary growth phase (Beker, 1981).

Dehydration causes an anabiotic state in yeast in which there is an intensive folding of the cytoplasmic membrane. Yeast organisms transfer to the state of anabiosis due to cell dehydration at a residual moisture of 8-12%. *S. cerevisiae* can be brought down to 15-20% moisture without much loss of viability. However, considerable inactivation will occur during storage. Yeast with 12-13% water content are not suitable for storage either, while a 8-12% moisture content will allow minimal losses in viability over a long period of time (Beker et al. 1987). Dehydration also causes damage to the nuclear membrane rigidity, which is manifested by a considerable widening of nuclear pores upon rehydration of dehydrated yeasts. Upon rehydration, dehydrated yeasts cells may lose up to 30% of various intracellular compounds, which testifies to a considerably increased permeability of the cytoplasmic membrane.

Among substances leaking from the cell there are amino acids, vitamins, nucleotides, inorganic compounds and others. Figure 1.15 represents a normal cell at the lag phase of culture growth. Figure 1.16 represents a living cell, reactivated after dehydration. The nucleus of these cells is altered in form, there is division of the mitochondria and a considerable number of endoplasmic reticular membranes and one or several large vacuoles are present. Figure 1.17 represents a cell that has died during the dehydration-rehydration process. These dead cells are characterized by marked transformations of their ultrastructural organization, namely, a manifest plasmolysis, deep invaginations of the cytoplasmic membrane and ruptures in separate parts, a marked widening of nuclear pores and the swelling of the nuclear and mitochondrial membranes. A considerable number of vesicles appear in these cells due to the disintegration of membranes of the endoplasmic reticulum. Ultrastructural changes of intracellular membranes in dehydrated organisms (cytoplasmic membrane invaginations, a damaged rigidity of the nuclear membrane and others) in viable cells are repaired during their reactivation from anabiosis. Yeasts are transformed to the anabiotic state as a result of leakage from cells of all the free and a part of the bound water. The extraction of the bound water from the cytoplasmic membrane elements causes a decrease in hydrophobic interactions and an increase in electrostatic interaction. The transfer to the anabiotic state very possibly destroys the internal organization of phospholipid molecules and may in turn cause an increase in membrane permeability (Beker et al. 1984).

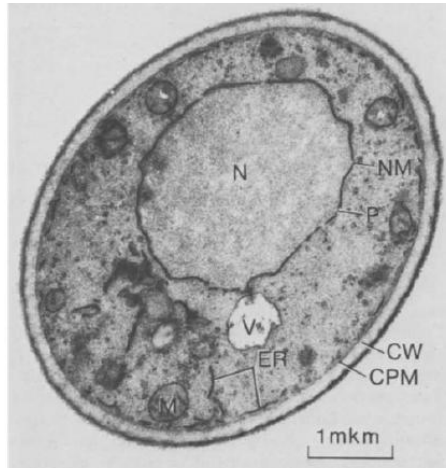


Figure 1.15. Electron microscopy of intact yeast *Saccharomyces cerevisiae*, taken from the stationary phase of culture growth. N, nucleus; NM, nuclear membrane; P, pores of nuclear membrane; M, mitochondria; ER, endoplasmic reticulum; V, vacuole; CPM, cytoplasmic membrane; CW, cell wall (Beker et al. 1984)

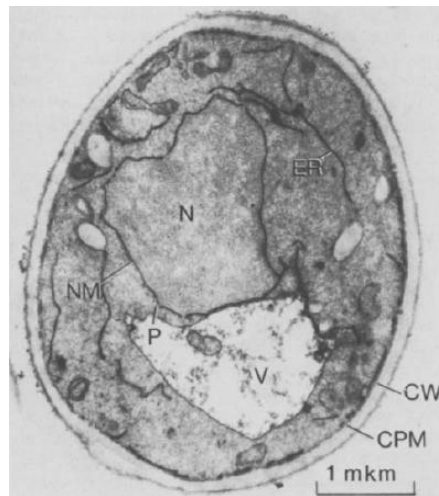


Figure 1.16. Electron microscopy of living *Saccharomyces cerevisiae* cells, reactivated after dehydration for 1.5 hr. Captions as in Fig. 15 (Beker et al. 1984)

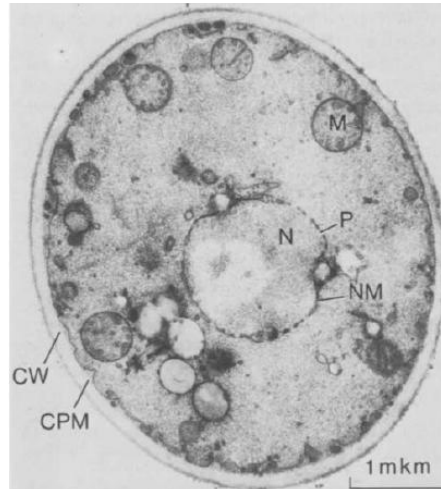


Figure 1.17. Electron microscopy of yeast *Saccharomyces cerevisiae* cells which died during the processes of dehydration-rehydration. Captions as in Fig. 15 (Beker et al. 1984)

A scheme of several structural transformations in a yeast cell during dehydration and subsequent rehydration can be seen in Figure 1.18. Part A represents the initial cell, while parts B-D show the formation of the cytoplasmic membrane invagination during dehydration, plasmolysis, chromatin condensation and separation of chromosome containing part of the nucleus. Parts E-F illustrate the moistening of cells during reactivation, a further degradation of the part of the nucleus to be separated and its elimination by phagosomes. The illustration, part G, shows the division of a reactivated cell. In the majority of cases only cells that maintain their native morphology (oval cells) preserve their viability, while cells that become elongated are non-viable. Cells inactivated by dehydration differ from those in anabiosis by plasmolysis, showing many ruptures in nuclear and cytoplasmic membranes (Figure 1.17). Furthermore, damaged membranes of the endoplasmic reticulum and a drastic decrease in size of the nucleus are typical (Beker et al. 1981).

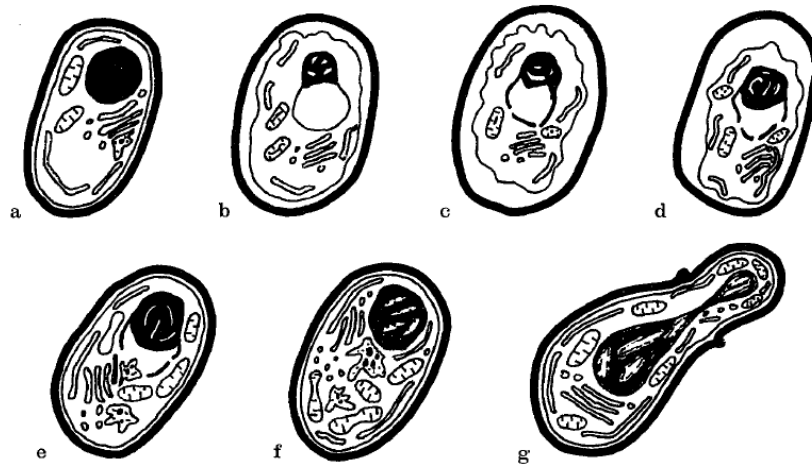


Figure 1.18. Scheme of several structural transformation in a yeast cell during dehydration and reactivation (Beker et al. 1987)

Slow and gradual rehydration of dried cells in water vapor diminishes membrane damages as compared to a rapid moistening, by just immersing dried yeasts into liquid medium. The viability of rehydrated populations increases as well, indicating the significance of the rehydration process in preserving the structural integrity of the cell. During rehydration the permeability of the dried yeast cytoplasmic membrane increases sharply. As mentioned earlier the total losses of cell components, in addition to the water, may amount to 20-30% of the dry weight. A considerable leakage of free amino acids (Table 1.12) has been observed after rehydration, whereas only traces of amino acids were released from intact cells. Considerable increase of the free amino acid pool in cells in a complex medium during reactivation indicates that the change of permeability also accounts for reverse processes, such as substances entering into the cell. However, a 1.5 hour reactivation/rehydration time can sharply decrease the permeability of the cytoplasmic membrane and bring it close to the cytoplasmic membrane of intact organisms (Beker et al. 1981; Beker et al. 1987)

Table 1.12. Release of free amino acids from dehydrated *S. cerevisiae* into water (Beker et al. 1987)

Amino acid	Free amino acid content mkg g ⁻¹	Release of amino acids into water	
		mkg g ⁻¹	% of content
Lysine	63.8	5.8	9.1
Hystidine	23.8	7.1	30.0
Arginine	33.2	3.4	10.4
Asparaginic acid	43.2	22.2	51.5
Methionine	3.0	3.1	100.0
Threonine	39.0	22.7	58.0
Serine	40.9	11.2	27.3
Glutamic acid	344.6	110.6	32.1
Proline	24.3	25.0	100.0
Glycine	7.6	8.0	100.0
Alanine	171.0	68.5	40.1
Cystine	47.8	14.1	29.5
Valine	61.5	17.1	27.8
Isoleucine	24.1	5.9	24.6
Leucine	21.8	4.8	22.0
Tyrosine	12.5	13.0	100.0
Phenylalanine	11.1	4.0	36.0

Lipids and their fatty acid composition help determine the expression of the main functional properties of intracellular membranes. Fatty acid composition affects the activity of the yeast cytoplasmic membrane transport proteins by altering membrane lipid fluidity as the amount of unsaturated fatty acids is varied. Depending on whether cytoplasmic membrane phospholipids contain more or less phosphatidylethanolamine and phosphatidylcholine, the sensitivity of yeast cells to surface active compounds, osmotic shock, increased temperatures and other extreme effects changes (Beker et al. 1987). Changes of intracellular yeast membranes during dehydration and rehydration are, again, related to water. Water plays a special role in biological membranes, the very appearance of the biomolecular lipid layer, with strictly oriented phospholipid molecules, is possible only in a water medium due to hydrophobic interactions between molecules. Membranes are made up of 30-50% water and 20-30% of that water is bound. Upon rehydration of dried organisms, the membrane systems restore their normal configuration with 20-30% relative moisture of the cells. During dehydration, at 8-10% moisture content, transformations in the cell membrane occur. The loss of the water hydrate layer causes disorientation of phospholipid molecules in some membrane parts (Figure 1.19 a and b). Under anabiotic conditions the

membrane lipids may change their configuration from lamellar to hexagonal, whereas the orientation of the membrane is determined by electrostatic powers (Beker et al. 1977; Beker, 1977).

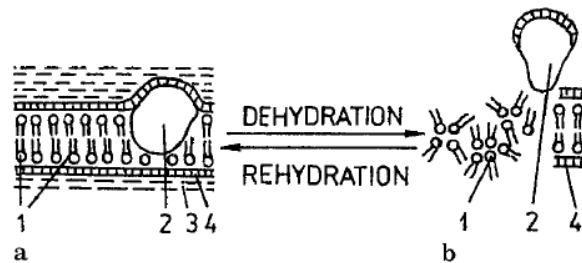


Figure 1.19 a and b. A hypothetical model of transformations in a part of the yeast cytoplasmic membrane during dehydration and rehydration; a- native membrane; b- a part of membrane after dehydration. 1- Lipid Molecules; 2- Membrane Protein; 3- Free Water; 4- Bound Water (Beker et al. 1977)

During dehydration the bulk of intracellular compounds undergo certain changes of the multivariate character. Some are aimed at preserving cell viability under extreme conditions of dehydration, others may have little to no effect on the state of the dried organisms. Nucleic acids are one of the basic intracellular compounds of interest during dehydration. Changes of water content affect the structural organization of nucleic acid molecules. Water, surrounding the DNA molecule, enhances the stability of its conformation and dehydration may bring about a collapse of the structure of macromolecules, and cause anomalous biological properties of DNA. Drying may also cause the rupture of DNA strands. RNA may also be disrupted or destroyed by desiccation. RNA degradation takes place mainly during the initial stages of drying, when moisture content decreases to ~20%. This may link the degradation of RNA to enzymatic processes (Beker et al. 1987).

Carbohydrate content is also an intracellular component of interest during desiccation. The total amount of carbohydrates in intact yeast cells depends on the cultivation conditions, growth phase and some other factors, and it may reach 50% of cell dry matter. The basic cell wall carbohydrate components, mannane and glucane, are mostly not affected by dehydration. However, one carbohydrate, trehalose, has been directly correlated to the quality of dried yeast. Some yeast can accumulate amounts of trehalose, as high as 10-15%. Trehalose, does not take part in reactions between the reduced groups of sugars and free amino acid groups of dried proteins,

rather it inhibits the reactions by forming a shell around the proteins. A high level of trehalose in yeast cells has been associated with increased osmotolerance, thermotolerance, and ethanol tolerance. Trehalose formation is stimulated by increased (37°C) temperature and diminished aeration at the final stage of yeast growth. Accumulation of trehalose is also enhanced by 1 hour of oxygen starvation about 2-3 hours prior to the end of growth (Majara et al. 1996; Beker et al. 1987).

Rehydration is a critical step in the recovery of dried microorganisms. Cells that are subjected to injury may not be able to repair the damage that has occurred if they are rehydrated under inappropriate conditions. The various manifestations of damage in cells include alteration in the membrane permeability, an increased sensitivity to many selective agents, an extended lag phase of growth, and an increased nutritional need. Injury itself may not lead directly to death because, in a suitable environment, damaged cells may repair and regain normal functions. The temperature of rehydration can influence cell recovery after drying. Rehydration at 15 -25°C has been shown to produce the highest number of recovered cells (*Salmonella anatum*), compared to 35°C and 45°C where the cell recovery was lower but the growth was more rapid (Ray et al. 1971). When reactivating yeast from an anabiotic state, three processes can be distinguished (Beker et al. 1987):

- 1- Moistening of the yeast (Rehydration)
- 2- Reparation of the damaged cell structures and macromolecules, and re-synthesis of a great variety of compounds, to replace those degraded during the dehydration & rehydration processes (Reactivation)
- 3- Restoration of the former number of cells within the population by multiplication of viable cells (Cell Restoration)

Rehydration is a purely physical process, when saturation of the cell with water takes place. The time required for the return of water into the cell is not long and it is assumed that during rehydration a part of the reversibly damaged cell elements revert to their native expression. Rehydration should be a gradual process, initially preformed in an atmosphere saturated with water vapor (0.01-0.1mg water mg yeast⁻¹ second⁻¹). The temperature of rehydration is also of importance. Loss of intracellular components decreases if the rehydration is carried out at higher

temperatures. Higher temperatures (up to 43°C) improve conditions for phospholipid hydration, since phospholipid hydration is difficult at temperatures lower than that of phase-transition (Beker et al. 1987).

Complex rehydration media may play a role in promoting repair of damaged cells by providing additional nutrients and essential cell components required for injured cells (Morgan et al. 2006). Costa et al, (2000) showed that complex media such as 10% non-fat skimmed milk and PTM (1.5% peptone, 1% tryptone, 0.5% meat extract) as well as a 10% sucrose solution were found to produce a significantly higher cell recovery than media such as a phosphate buffer, sodium glutamate and water. High recoveries obtained by complex rehydration media might be related to the rate of hydration of the samples. An environment of high osmotic pressure may control the rate of rehydration and avoid osmotic shock. Sugars, especially disaccharides, seem to play a role in the stabilization of dry biological membranes by hydrogen bonding to the polar head of the phospholipid membrane (Costa et al. 2000). Calcium ions and glucose also show a stabilizing effect on membranes at rehydration. Binding themselves to the polar head groups of two neighboring phospholipids, calcium ions form salt bridges, thus increasing the rigidity of membrane structure. Glucose may permeate into the cell and stimulate the formation of protein gels, which prevents diffusion of components from the cell (Beker et al. 1987).

After the rehydration of yeast, the resources of energetic and constructive metabolism provides for the reactivation of the yeast. The duration of the reactivation period depends on the character and level of intracellular damage. Since a part of the cells becomes irreversibly inactivated when dried, after reactivation a certain time is needed for the multiplication of the viable individuals and restoration of the number within the population. This requires a rich nutrient medium and the optimal cultivation techniques. After reactivation, with a prolonged lag phase, the exponential growth phase sets in.

The following process may take place upon reactivation (Beker et al. 1987):

- 1- Biosynthesis of new molecules (micro and macromolecules) to replace damaged ones
- 2- Compensation for the function of damaged structures is facilitated with the help of reserve mechanisms
- 3- Destruction and isolation of partly damaged structures

4- Reparation of reversibly altered structure

Drying Technology

Dehydration is an important process for food and microbial preservation. The reduced weight and bulk of dehydrated products and their dry shelf stability reduces product storage and distribution costs. Dehydrated foods are preserved because water activity is at a level where no microbiological activity can occur and where deteriorative chemical and biochemical reaction rates are reduced to a minimum. Water activity (a_w) is measured as the equilibrium relative humidity (ERH), the percent relative humidity (RH) of an atmosphere in contact with a product at the equilibrium water content. Water activity is also the ratio of the partial pressure of water in the headspace of a product (P) to the vapor pressure of pure water (P^0) at the same temperature. Reducing a_w below 0.7 prevents microbial growth but in order to prevent other deteriorative reactions the water activity should be reduced to 0.3 (Toledo, 2007).

A consequence for cells undergoing a reduction in water activity is an increase in osmotic pressure, which interferes with nutrient, water, and waste transport between a cell and its surroundings. The osmotic pressure is related to the activity coefficient in Equation 1.1:

$$\pi = (-RT/V) \ln x\gamma$$

Equation 1.1: Osmotic pressure equation

Osmotic pressure = π (atm); R= gas constant (82.06mL * atm/gmole * K); γ = activity coefficient; V= molar volume = 18 mL/gmole for water; x = mole fraction of water; and T = absolute temperature. As water activity approaches its highest point of 1.0, the osmotic pressure approaches its lowest point of 0.0 (Toledo, 2007).

Freeze drying or spray drying methods are used in the brewing industry for the preservation of microorganisms for long term storage with a concentration of cells at or greater than 10^8 cells per mL. High cell concentrations are used on the premise that a large portion of the viable cells will die in long term storage. Freeze drying is the preferred method for culture collections worldwide, including the American Type Culture Collection (ATCC) and the National Collection of Type Cultures (NCTC). The generic drying process is as follows in Figure 1.20.

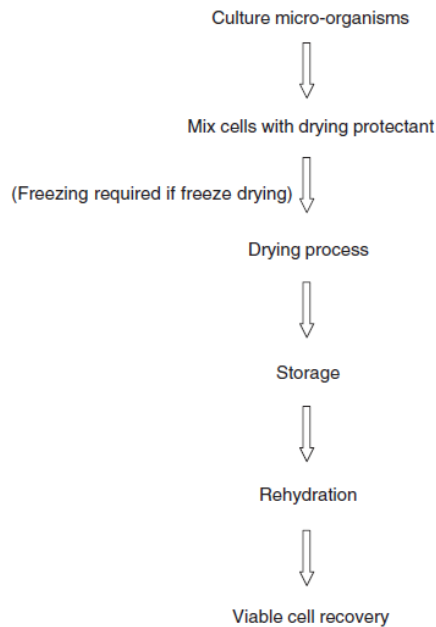


Figure 1.20. The generic drying process layout (Morgan et al. 2006)

The growth of most microorganisms in batch culture has four distinct phases: lag, log, stationary and death phases. The stationary phase corresponds to specific physiological states within the cells, normally due to the exhaustion one or more essential nutrients that triggers a stress response within the cell population. Stress during growth of an organism induces a number of survival strategies that afford protection to the microorganism upon drying. Microorganisms undergoing drying are faced with an increasing osmotic stress as water activity decreases. One way organisms counteract the osmotic stresses encountered during the spray drying process is to accumulate compatible solutes to maintain the osmotic balance between the highly concentrated extracellular environment and the more dilute intracellular environment. These solutes can also help to stabilize proteins and the cell membrane during osmotic stress conditions induced by low water activity during drying processes. Another way cells counter the stress of drying is the accumulation of stress response proteins. The stress response proteins are crucial to maintaining cell viability during the recovery stage after drying (Morgan et al. 2006). Dried yeasts inoculated directly into a nutrient medium may have a longer lag phase (by 1-2 hours) as compared to fresh

yeast. Depending on the character and level of damages, the specific growth rate of yeast cells previously dried may also be lower than that of the initial fresh yeast (Beker et al. 1987).

Spray drying is the predominant process used in the dairy industry to dry large amounts of ingredients relatively inexpensively. Spray-dried powders can then be transported at a lower cost and can be stored in a stable form for prolonged periods (Silva et al. 2002). Generally spray dried products in the food industry are products such as milk, whey and corn. Spray drying produces dry granulated powders from a slurry solution, by atomizing the wet product at a high velocity within a chamber. Essentially a liquid droplet is rapidly dried as it comes in contact with a stream of hot air. The atomized droplets are therefore dried into granules before they hit the side of the chamber. The spray drying process injects high velocity air at high temperatures, up to 200°C, which then blasts the slurry from a nozzle into granules. Alternatively, atomization could be produced by a high pressure liquid pump while the hot air could enter separately to dry at a relatively low pressure. The outlet air is usually at equilibrium with the temperature of the product as it leaves the spray drying chamber, which is relative to the holding time of the granules within the chamber (Morgan et al. 2006).

The small size of the liquid droplets allows for rapid drying and the residence time of the material inside the spray drier is in the order of seconds. The dried material is separated from the outlet air in a cyclone separator. The dried material is continuously withdrawn and cooled. If the product maintains contact with the high temperature of the drying air for prolonged periods of time, heat damage could occur. As the droplet is drying, the temperature remains at the wet bulb temperature of the drying air until a critical moisture content is reached. For this reason, very high temperatures of the drying air can be tolerated in a drier with a minimum of damage to heat sensitive components as long as the moisture content does not fall too low (Toledo, 2007). A diagram of a model spray drier is illustrated in Figure 1.21.

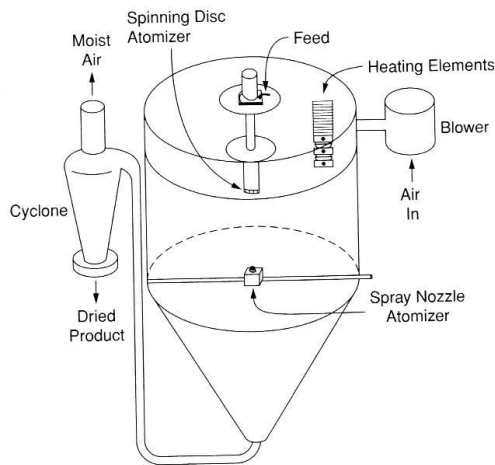


Figure 1.21. Schematic diagram of a spray drier (Toledo, 2007)

A major requirement for a successful spray drying process is the reduction of the moisture content of the particle to a dryness level that would prevent the particle from sticking as the particle collides with a solid surface. The rate of drying along with the trajectory and velocity of the droplets from the atomizer must be such that from the time the droplet leaves the atomizer to the time just before collision with the surface, the particle is dry. The rate of drying and the time required to dry are dependent upon the temperature of the air, the heat transfer coefficient between the air and the droplet, and the diameter of the droplet being dried (Toledo, 2007).

In the spray drying process there are two drying stages characterized by a constant rate and a falling rate. As wet droplets leave the atomizer, their surfaces rapidly lose water and suspended solids and solidified solutes rapidly form a solid crust on the surface of each particle. The diameter of the particle usually decreases as drying proceeds. The formation of the solid crust constitutes the end of the constant rate stage of drying and occurs at what is referred to as the critical moisture content. The rate of diffusion of water to the surface becomes rate limiting and the drying rate decreases. As water is trapped inside the particle by the outer crust the temperature of the particle increases and the liquid trapped in the interior of the particle vaporizes and generates pressure. Eventually, a portion of the crust breaks and the vapor is released. Spray dried particles consist of hollow spheres or fragments of spheres. This shape of the particles is responsible for the excellent rehydration properties of spray dried powders (Toledo, 2007).

Spray-dried powders containing high levels of viable microorganisms provide a convenient form of these cultures for storage purposes and applications in functional food developments. The aim of preserving cells during drying is to enable storage at ambient temperatures. Due to the inherent expense of chilled or frozen storage, reducing the storage temperature is not always a financially plausible option to increase long term storage. The major limitation of spray drying cultures is the loss of viability which occurs during processing and storage of the dry powders. The survival rate of the culture during spray drying and subsequent storage depends upon a number of factors, including the species and strain of the culture, the drying conditions, the medium used, pre-adaptation of the culture to acquire resistance to processing conditions and the use of protective agents. *Lactobacillus paracasei*, a common probiotic culture, was found to have 100% viability after one week of storage at both 4°C and 15°C. However, there was a 20-80% drop in cell viability after four weeks at both temperatures. These results were from cultures that had a high initial bacterial load (7×10^7 CFU mL⁻¹), the enumeration of these high cell concentrations gave results with up to 20% variation. The variable survival rate indicates that cell injury can also occur during storage. Silva et al, (2002) showed that the storage temperature was a critical parameter affecting the survival of micro-organisms. In all experiments, survival rates were higher at the lower temperature of storage. To achieve long term storage of spray dried powders, possible improvements in the storage conditions should be investigated, such as the optimal storage temperature, the relative humidity of the storage environment and the presence of oxygen. Packaging methods such as storage under vacuum or in nitrogen may be a more cost effective way of regulating the storage environment. Along with the storage environment, the outlet temperature of the air during spray drying can have a profound affect on the viability of spray-dried cultures. Analysis of probiotic powders showed that, as the outlet temperature of spray drying increased, the moisture content of the spray-dried powders decreased (Desmond et al. 2002). Relatively small changes in the outlet temperature were also found to have significant effects on *Lactobacillus* strains by Silva et al (2002). This indicates that spray drying processing conditions (inlet and outlet temperature) need to be individually tailored for every different culture.

Freeze drying is the preferred method of drying in the microbiological industry; however it is not without problems such as loss of viability and cross-contamination. One of the main causes of cell viability loss is the need to freeze the sample in order for it to be dried. The process of freezing and especially the rate of freezing can be detrimental to the viability of a cell. Another

drawback is the risk of cross-contamination and volume limitations within a freeze drying container. Cross contamination within a freeze dryer is proportionately higher, with increased concentrations of microorganisms within a product. Distribution of contaminants within the dryer is especially common during collapse of a product. A collapsed product is effectively entering into a liquid phase and boiling, this process is highly likely to produce aerosols within the chamber, which can distribute micro-organisms randomly throughout the dryer (Morgan et al. 2006).

There are two ways to freeze a sample before drying, either within the freeze dryer chamber, through cooled shelves, or the product can be frozen prior to loading onto the freeze dryer. The drying cycle after freezing has occurred is comprised of two main parts, primary and secondary drying. Primary drying is where frozen moisture is removed by sublimation of the ice crystals to water vapor. This is accomplished by reducing the chamber pressure below the vapor pressure of the ice within the product (Morgan et al. 2006). A successful freeze drying process must maintain an absolute pressure in the drying chamber of at least 620 Pa (Toledo, 2007). While there is frozen water present within the product, collapse and melting are possible, if product temperatures increase above the temperature where water molecules can re-enter into a highly mobile liquid phase. Secondary drying commences once all frozen water has been sublimed and only bound water remains. Bound water is trapped within the solid matrix and can take extended periods of time to be removed. The end point of secondary drying is usually determined by analysis of the residual moisture remaining within the freeze dried product after its removal from the chamber (Morgan et al. 2006). Analysis of freeze drying is different from that of conventional drying in that drying proceeds from the exposed surfaces toward the interior. The outer layers are completely dry as the ice core recedes. Vaporization of water occurs at the surface of the ice core. Heat of sublimation is conducted to the surface of the ice core through the dried outer layer. Vaporized water diffuses through the pores of the dried outer layer before it leaves the solid and goes to the atmosphere in the drying chamber. Figure 1.22 shows a common freeze drying setup (Toledo, 2007).

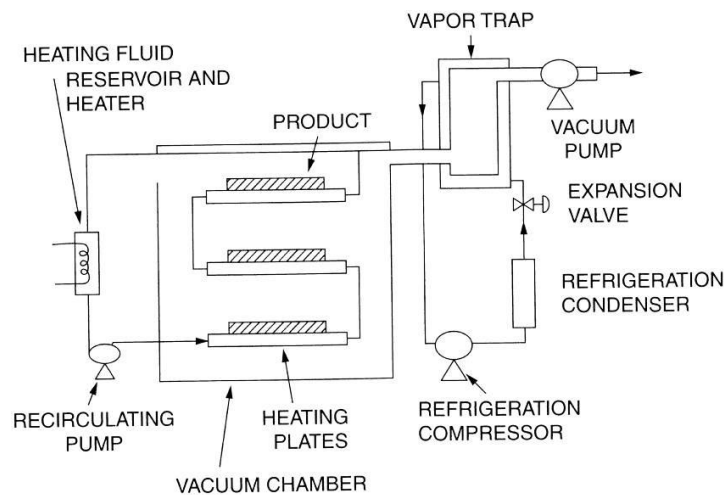


Figure 1.22. Schematic diagram of a freeze drier showing a vapor trap external to the vacuum chamber (Toledo, 2007)

Spray drying can produce stable powders of certain bacterial and fungal species, however, with the high temperatures involved in this process, the species require a certain level of thermotolerance. This drying method is also not suitable for precise bacterial preservation, as the cell dispersion within the process is reliant on atomization and therefore only produces an approximate cell load per ml of powder. Freeze drying is more suitable for precise numbers of bacteria being preserved as accurate aliquots of frozen material can be preserved within individual containers such as ampoules, or glass vials. The disadvantages of freeze drying are the effects of freezing on cell viability. In addition the cost for large outputs, unlike spray drying, freeze drying can take days to complete for large product loads due to the slow energy transfer needed to dry the material (Morgan et al. 2006).

Once a culture is dried it must be packaged in such a way as to maintain the product in a suitable state for re-use. This may involve careful selection of the packaging material to control gas exchange, prevent the loss or gain of moisture and avoid contamination of the product (Costa et al. 2002). As with most spoilable products the most common reactives to avoid are; oxygen, moisture, light, microbial contamination and elevated temperatures. In general, freeze-dried products are stored within ampoules, or glass vials. For spray dried products high barrier bags or

blister packs are an option (Morgan et al. 2006). Low barrier packages are more permeable to oxygen and water than high barrier bags or glass vials, so dried cells could be partly rehydrated and could begin respiration and other degradation processes. After drying microorganisms do not become totally inert and respiration does not completely cease. As the storage temperature increases, mortality also increases and storage time is reduced. Low temperatures (0-5°C) maintain metabolic activity at a low level and increase storage stability (Costa et al. 2002).

Hypothesis Introduction

S. pastorianus has been identified as a hybrid organism with genomic content from *S. cerevisiae*, *S. bayanus*, and *S. bayanus* var. *uvarum* that can be divided into two distinct groups based on genomic DNA. One group (Saaz type) had lost a significant portion of the *S. cerevisiae* genome, but retained virtually all of the *S. bayanus* genome. The other group (Frohberg type) retained virtually all genomic content of both *S. cerevisiae* and *S. bayanus* genomes (Dunn et al. 2008). The hypothesis of this study is that these two hybrid types of *S. pastorianus* may differ in desiccation tolerance and subsequent fermentation performance as a result of injuries suffered during the drying process. If true, the selection of the appropriate strains could help reduce the variability in ADLY performance.

MATERIALS AND METHODS

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Yeasts

Three lager strains were used in this study: *S. bayanus*, *S. pastorianus* var. *pastorianus* CBS 1486 (Saaz Type) and CBS 1260 (Frohberg Type) (CBS Fungal Biodiversity Center, Utrecht, Netherlands). One ale strain was used: *S. cerevisiae* CATI #226 (Lallemand Ethanol Technologies, WI, U.S.A). Fresh cultures of the strains were maintained on plates of YM (3% Yeast Extract, 3% Malt Extract, 5% Peptone, 10% Dextrose, 15% Agarose) media at 4°C until needed. Mother cultures were maintained on glycerol stocks at -80°C.

Wort Media Production

Yeast cultures were propagated on wort media. The wort media was produced with 100 L of carbon filtered water, 50 g CaCl₂, and 28 kg of Pale American Two-Row Malt Barley (2.25 Lovibond). The production facilities were a 2.5 BBL mash tun and boil kettle (Diversified Metal Engineering Ltd., P.E.I., Canada). The process for the production of the wort is illustrated in Figure 2.1. The barley malt was milled using a roller mill (Apollo Machine & Products Ltd., Saskatoon, Canada) and added to 70 L of 48°C water. Light agitation/stirring was manually applied to the mixture over the entire mashing process. The heating/mashing schedule was 48°C (20 min), 52°C (20 min), a slow rise to 72°C over 10 min, 72°C (10 min). After heating the wort was drained into the boil kettle and 30 L of water was added for a final volume of 100 L. Prior to use as the fermentation medium, the wort was boiled (60 min) and stored at 4°C until needed. The wort (pH 4.6) was diluted to 12.6° Brix (13.1° Plato) and boiled for 30 minutes. Part of the wort was filtered through a 0.45 µm filter for use in fermentations. The non-filtered portion was used for yeast propagation and spray drying experiments.

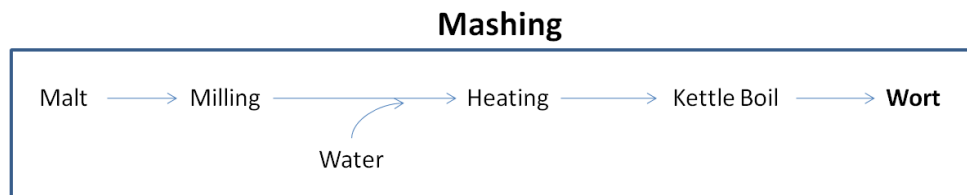


Figure 2.1. An overview of the brewing process used to produce wort for experimentation

Propagation and Fermentation

Laboratory-scale yeast propagation was carried out in 1 L baffled Erlenmeyer flasks (Pyrex, NY, U.S.A.) with 500 mL of wort (13.1° Plato) in an incubator (New Brunswick Scientific-I26, NJ, U.S.A.). The propagation was carried out at 30° C with shaking at 150 rpm for 48 hours.

Laboratory-scale fermentations took place in 250 mL wort (13.1° Plato) at 25° C and 0 rpm. Before dehydration (BD), strains were pitched at an O.D. at 600 nm of 0.25 and correlated with hemacytometer counting with methylene blue staining at 1×10^6 viable cells mL^{-1} . For *S. bayanus*, which is cryophilic and filamentous, the strain was pitched based on dry biomass weight.

Fermentations were monitored over a 72 hour period in which samples were taken for analysis.

Viable cell counts, plating, and O.D. readings were performed immediately after sampling. HPLC samples taken over the duration of the fermentation were filtered through a 0.45 μm filter and frozen at -20° C until analyzed.

Dehydration and Rehydration

1 L propagated yeast was centrifuged at 3,000 rpm for 20 minutes and resuspended in spent media to 450 mL. The concentrated yeast was dehydrated in a laboratory scale spray-dryer (LabPlant™SD-Basic, Chelmsford, U.K.) (Figure 2.2) at an inlet temperature of 140° C and an outlet temperature of 65-70° C. Dehydrated samples were rehydrated in 150 mL of phosphate buffer (5.7% Na_2HPO_4 , 4.1% KH_2PO_4) (pH 7.0) for 30 minutes at 25° C with shaking at 50 rpm. After dehydration and rehydration (AD), samples were immediately pitched at 1×10^6 viable cells mL^{-1} (by methylene blue staining) for fermentation.



Figure 2.2. Laboratory scale spray dryer used for experimentation

Viability Measurements

Viability was measured after propagation, dehydration/rehydration, and during fermentation. Real time viability methods were: hemacytometer counting (Fisher Scientific, GA, U.S.A.) with 1% methylene blue dye, CASY® Cell Counter Model TTC (Innovatis, PA, U.S.A.), and a capacitance probe/yeast monitor model 220 (Aber Instruments Ltd., Wales, U.K.). Cells were also plated onto YM media (described above) by a Whitley automatic spiral plater (DW Scientific, West Yorkshire, U.K.) and enumerated after aerobic incubation (30° C for 48 hours) by ProtoCOL software (Synbiosis Ltd., Cambridge, U.K.). The production of ethanol and utilization of the brewing carbohydrates was tracked by using Breeze-2 High Performance Liquid Chromatography (HPLC) (Waters Corp., MA, U.S.A.) with a IC-Pak Ion Exclusion column (Waters Corp., MA, U.S.A.).

The number of cells in a population was determined by a direct microscopic count using a hemacytometer. In a hemacytometer, a grid is marked on the surface of the glass slide, with squares of known area. Over each square on the grid is a volume of known amount. The number of cells per unit of area of grid can be counted under the microscope, giving a measure of the number of cells per volume (Madigan et al. 2006). When a hemacytometer is paired with a

methylene blue stain the number of organisms present in the sample and their viability can be determined.

Methylene blue staining is a brightfield staining procedure that involves the apparent exclusion of methylene blue dye by viable cells. Methylene blue stains dead cells blue while “live” yeast cells remain colorless. This method has been adapted as a common assay for yeast viability in the brewing industry (Smart et al. 1999). The hemacytometer procedure used was to mix 1 mL of sample with 2 μL of methylene blue. After thorough mixing, 10 μL of sample would then be placed on the hemacytometer and covered with a glass slip. Under the microscope the four largest outer squares would be counted for both viable and non-viable cells. The number of cells per milliliter of suspension was calculated by equation 2.1.

Cell concentration per milliliter = Total cell count in 4 outer squares X 2500 X dilution factor

Equation 2.1. Hemacytometer cells per milliliter conversion equation

A sample taken straight from the fermentation is considered undiluted. 100 μL of undiluted sample would be added to 900 μL DI water to make a 1:10 dilution. If a 1:10 dilution was suitable for counting there would be between 100-500 cells in each of the outer squares of the hemacytometer. A sample total cells mL^{-1} calculation for the hemacytometer would be $(120+150+130+140) \times 2500 \times 1 \times 10^1$, which would equate to 1.4×10^7 total cells mL^{-1} . With the methylene blue stain there may be a dead cells, the total dead cells mL^{-1} are calculated exactly as the total cells mL^{-1} . As an example if there were a sum of 10 dead cells in the 4 outer squares it would equate to 2.5×10^5 dead cells mL^{-1} . The difference between total cells mL^{-1} and total dead cells mL^{-1} is viable cells mL^{-1} . Following the example if there were 1.4×10^7 total cells mL^{-1} and 2.5×10^5 dead cells mL^{-1} , then there are ultimately 1.3×10^7 viable cells mL^{-1} . Viability is calculated by equation 2.2. If as mentioned earlier there are 1.3×10^7 viable cells mL^{-1} and 1.4×10^7 total cells mL^{-1} then the percentage viability is 98.15.

Percent viability (%) = (Viable Cells mL^{-1} / Total Cells mL^{-1}) X 100

Equation 2.2. Percentage viability equation

The CASY® cell counter and analyzer system (Figure 2.3) utilizes pulsed area analysis to deliver a wide variety of results. Using the CASY® counter, the amount of cell debris, viable cells,

dead cells and cell aggregates in a sample can be determined simultaneously with one measurement. In addition to the cell and particle concentration, all information about the diameter and volume of the fractions tested can be viewed. The CASY® counter combines a particle measurement technique, referred to as the resistance measurement principle, with a modern method of signal evaluation, pulse area analysis. Measurement is performed by suspending the desired cells in CASY®ton, an electrolyte developed specifically for counting and aspirating cells through a precision measuring pore of defined geometry at a constant flow speed. The precision measuring pore is designed as a hole within an artificial precious stone, which is cast inside the capillary body. During the measurement process, a pulsed low voltage field with 1 MHz is applied to the measuring pore by two platinum electrodes. The electrolyte-filled measuring pore represents a defined electrical resistance. During their passage through the measuring pore, cells displace a quantity of electrolyte corresponding to their volume. Since intact cells can be considered isolators, an increased level of resistance is achieved over the measuring pore. This resistance is used to measure the volume of the cells. By contrast, dead cells whose membrane no longer acts as an electrical barrier are recorded by the size of their nucleus. It is thus important to make appropriate dilutions so that cells pass through the measuring pore individually.

The measuring signal is scanned by CASY® at high frequency. As well as the amplitude of the measuring signal, CASY® also records the shape of the signal as a whole. CASY® calculates the integral of the measuring signal from the individual measurements. The calculated signal areas are evaluated by a standardized method and accumulated in a multi-channel analyzer (pulse area analysis). A diameter-linear size distribution with a resolution of 1024 display channels is calculated from a linear-volume, original distribution with a resolution of 524,288 measuring channels. This distribution forms the basis for the calculation of all other measuring parameters. The overall principle behind the CASY® counter is similar to that of a capacitance probe.

To utilize the CASY® counter, the capillary would be rinsed 3 times with yeast CASY®ton electrolyte. Next a series of dilutions from 1×10^{-1} to 1×10^{-4} would be made from the original, undiluted sample. After that, 100 μL of the lowest dilution, accepted by the CASY® cell counter, would be mixed with 4.9 mL of yeast CASY®ton and placed under the capillary. After the readings were generated the debris and dead cell bars would be adjusted to best fit the cell count based on the distribution displayed as mentioned above.

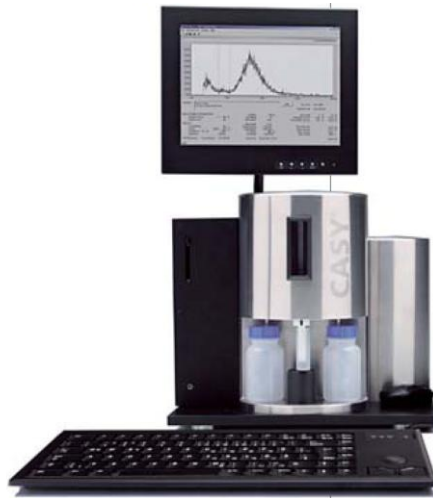


Figure 2.3. CASY® cell counter and analyzer system (Innovatis AG)

A capacitance probe (Figure 2.4) is a common tool used for online measurements of viable yeast cells in a brewery. Capacitance measurements can also be used to assess the concentration of viable microbial cells in fermentation media. Under the influence of an electric field, living microorganisms act as tiny capacitors, due to the presence of the intact plasma membrane which isolates the cell content from the surrounding medium (Mas et al. 2001). The output signal from a capacitance probe is directly proportional to the membrane-enclosed volume fraction of the microbial culture and this volume is dependent upon the concentration of cells, their viability and the cell radius (Austin et al. 1994). For viability, capacitance measurements were taken by submerging the capacitance probe into 30 mL wort (before dehydration) and blanking the probe. The capacitance probe then measured the capacitance of the yeast sample. After dehydration and rehydration, the probe was blanked in the rehydration buffer and the capacitance of the cells was measured again.



Figure 2.4. Capacitance probe (Aber Instruments Ltd.)

Agar pour plate procedures are routinely used for the quantitative determination of microorganisms. In the traditional method, an unknown sample is diluted many times and a known sample of each dilution is spread over an agar plate. After incubation, that plate which has between 30 and 300 colonies is counted, and the resultant count is multiplied by the appropriate dilution to obtain the microbial concentration in the sample. The spiral plating method uses a machine which deposits a known volume of sample on a rotating agar plate in an ever decreasing amount in the form of an Archimedes spiral. After the sample is incubated, different colony densities are apparent on the surface of the plate. A modified counting grid relates the area of the plate to the volume of the sample. By counting the appropriate area of the plate, the number of microorganisms in the sample is estimated (Gilchrist et al. 1973). Samples were diluted to 1×10^{-1} and 1×10^{-5} and then plated on YM agar plates (Fischer, G.A., U.S.A.) using the spiral plating method. After plating, the samples were incubated for 48 hours at 30°C . After incubation the plates were enumerated using an automated computer-based counter with Protocol software (described earlier). The plate counts were used to calculate the specific growth rate (μ) and doubling time (t_d) of the various yeast strains (Equation 2.3, 2.4).

$$\mu = (\ln N_2 - \ln N_1)/(t_2 - t_1)$$

Equation 2.3. Specific growth rate equation. μ (hr^{-1}) is the specific growth rate; N (CFU/mL) is the number of cells; t (hrs) is the time.

$$t_d = (\ln 2) / \mu$$

Equation 2.4. Doubling time equation. Doubling time (t_d) in hrs; μ (hr^{-1}) is the specific growth rate.

After the colony forming units mL^{-1} have been determined for specific times during the fermentation sample equations 2.3 and 2.4 can be utilized. As an example, a sample starts its log phase 4 hours into the fermentation and is at 1×10^6 CFU mL^{-1} , the sample then grows exponentially and just before it begins stationary phase at 12 hours it is at 1×10^7 CFU mL^{-1} . The specific growth rate would be calculated as $(\text{LN } 1 \times 10^7 \text{ CFU } \text{mL}^{-1} - \text{LN } 1 \times 10^6 \text{ CFU } \text{mL}^{-1}) / (12 \text{ hrs} - 4 \text{ hrs})$. This would reduce to $\mu = 2.3 / 8$, $\mu = 0.29 \text{ hr}^{-1}$. The doubling time would then be calculated as $\text{LN } (2) / 0.29$, $t_d = 2.41 \text{ hrs}$.

HPLC is used in order to separate out chemical compounds based on their affinity for a stationary and/or mobile phase. It is widely used for the analysis of small molecules and ions, such as sugars, vitamins, and amino acids, and is applied to the separation and purification of macromolecules, such as proteins, nucleic acids, and polysaccharides. A basic HPLC system consists of a pump, injector, column(s), detector and recorder/integrator/data system. The pump delivers mobile phase through the system. An injector injects the sample into the flowing mobile phase for introduction onto the column. The HPLC column, connected between injector and detector, consists of stainless steel hardware filled with a packing material. Column packing materials can be classified as silica-based (porous silica, bonded phases) or polymeric (microporous, macroporous). Detectors used in the HPLC included UV-Vis absorption, fluorescence, refractive index, electrochemical and several other types. Electronic integrators and computer-controlled systems offer data handling capabilities to record the results of the chromatographic separation (Nielsen, 2003).

HPLC was used in this experiment to provide information about the critical components of the fermentation (ethanol generation and sugar utilization). A Breeze system was used which included a 1515 HPLC pump, 717plus autosampler, external column heating module, 2414 RI detector, and Breeze software. The column used was a 7.8150 mm IC-Pak™ Ion Exclusion column (WAT010295), the pre-column was 6.0X50 mm SH-1011P(WAT034243) at a column temp of 75°C , a flow rate of 1.0 mL min^{-1} with a mobile phase of 0.5 mM sulfuric acid and an

injection volume of 5 μL . The RI sensitivity was 32 with a RI time constant of 0.2 sec, a sampling rate of 5 pts sec^{-1} and an RI detection temperature of 30°C.

The exhaustion of glucose (from HPLC data) during the log phase was used to calculate the glucose consumption rate (GCR) (Equation 2.5). As an example, an organism begins its log phase at 5 hours and ends log phase at 25 hours. At the beginning of the log phase it had 10 g L^{-1} of glucose available for consumption, at the end of the log phase it had 0.0 g L^{-1} of glucose available. The GCR could be calculated as $(10 \text{ g L}^{-1} \text{ Glu} - 0.0 \text{ g L}^{-1} \text{ Glu}) / (25 - 5)$, which can be reduced to 10 / 20. The GCR is thus 0.5, which means that the organism consumes 0.5 g L^{-1} of glucose per hour during its logarithmic growth phase.

$$\text{GCR} = (\text{GLU } T_1 - \text{GLU } T_2) / (T_2 - T_1)$$

Equation 2.5. Glucose consumption rate (GCR) equation. GLU is glucose concentration in g L^{-1} , T is time in hours.

Polymerase Chain Reaction (PCR)

PCR is a procedure for amplifying DNA *in vitro* and employs a heat-stable DNA polymerase from thermophilic prokaryotes. Heat is used to denature the DNA into two single-stranded molecules, each of which is copied by the polymerase. After each cycle, the newly formed double strands are separated by heat again, and a new round of copying begins. At each cycle, the amount of target DNA doubles (Madgin et. al 2006). In this experiment PCR was used for sequencing purposes because the genes of interest (*POP4*, *ATH1*, *ECM29*) were amplified from known flanking sequences. The genes of interest are strongly implicated in dehydration tolerance. The deletion of vacuolar trehalase (*ATH1*), has been demonstrated to convey a significant survival advantage during short-term dehydration storage (Kim et al. 1996). The deletion of ribonuclease MRP activity and ribonuclease P activity (*POP4*) as well as protein binding (*ECM29*), have also been indicated in improving the resistance to stress conditions, giving these yeast a survival advantage (D'Elia et al. 2005).

DNA was isolated with a MasterPure™ DNA Purification Kit (AT. No. MPY80200, EpiCentre® Biotechnologies) following the manufacture's procedure. Primers for each of the genes were from Eurofins MWG Operon. Primers are listed in bold in the gene sequences below.

Gene: POP4

LOCUS: Z36126 1634 bp DNA linear PLN 11-MAR-1998

DEFINITION *S. cerevisiae* chromosome II reading frame ORF YBR257w.

ACCESSION Z36126 Y13134

VERSION Z36126.1 GI:536680

ORIGIN:

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Gene: ATH1

LOCUS: X84156 3883 bp DNA linear PLN 13-NOV-1995

DEFINITION *S. cerevisiae* ATH1 gene.

ACCESSION X84156

VERSION X84156.1 GI:1061283

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ATH1R: 5' TTCCTCCAATCTGTGTTCCC 3'

Gene: ECM29

LOCUS: NC_001140 5607 bp DNA linear PLN 16-JUN-2008

DEFINITION Saccharomyces cerevisiae chromosome VIII, complete sequence.

ACCESSION [NC_001140](#) REGION: 40082..45688

VERSION NC_001140.5 GI:82795252

ORIGIN/CDS:

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ECM29R: 5' TTCTGAATGATCAGGCCACC 3'

PCR was run on an Eppendorf AG Thermocycler (No. 5345) using TempAssure 0.2mL thinwall PCR tubes (USA Scientific). Gels were 1% Agarose (SeaKem LE) and were run at 100V and stained with GelStar® nucleic acid gel stain (Lonza, ME, USA). Gels were analyzed with a Bio-Rad Labs (Milan, Italy) Universal Hood and Quantity One (Version 4.4.0) Software.

RESULTS

Johnathon Blake Layfield

Department of Food, Bioprocessing, and Nutrition Sciences

North Carolina State University

Fermentation Profile Before and After Dehydration and Rehydration Experiments

The four yeast strains (*S. cerevisiae*, *S. pastorianus*-Frohberg, *S. pastorianus*-Saaz, *S. bayanus*) were characterized by their fermentation performance and desiccation tolerance. The fermentation profiles of each strain were tracked by optical density (O.D.) at 600nm, hemacytometer counting (methylene blue staining), spiral plating, and HPLC. Viability and fermentation performance were used as indicators of desiccation tolerance. Viability was measured by hemacytometer counting (methylene blue staining), CASY® cell counter, capacitance probe, and spiral plating. For cells that had undergone desiccation and rehydration, the final viability of the cells was a percentage of the initial viable cells before dehydration.

Before dehydration three strains had similar growth profiles, with one strain deviating (Figure 3.1). The starting O.D. (600nm) was 0.25, which was roughly equal to 1×10^6 viable cells mL^{-1} (by hemacytometer staining), for each of the strains. *S. cerevisiae* had the fastest growth ($\mu = 0.19 \text{ hr}^{-1}$; $t_d = 3.68 \text{ hrs}$), followed by *S. pastorianus*-Saaz ($\mu = 0.17 \text{ hr}^{-1}$; $t_d = 4.12 \text{ hrs}$) and *S. pastorianus*-Frohberg ($\mu = 0.15 \text{ hr}^{-1}$; $t_d = 4.62 \text{ hrs}$). Lastly, *S. bayanus* which had the slowest growth ($\mu = 0.06 \text{ hr}^{-1}$; $t_d = 12.59 \text{ hrs}$) (Table 3.1). After dehydration and rehydration the growth profile of the three strains differed not only between the strains but also in each strain before and after dehydration (Figure 3.2). *S. cerevisiae* and *S. pastorianus*-Saaz had a 16H lag phase whereas *S. pastorianus*-Frohberg had a 30H lag phase. The logarithmic growth of *S. pastorianus*-Frohberg was also slower and longer log phase compared to *S. cerevisiae* and *S. pastorianus*-Saaz. *S. pastorianus*-Frohberg ($\mu = 0.23 \text{ hr}^{-1}$; $t_d = 3.06 \text{ hrs}$) had a 23H log phase whereas *S. cerevisiae* ($\mu = 0.31 \text{ hr}^{-1}$; $t_d = 2.23 \text{ hrs}$) and *S. pastorianus*-Saaz ($\mu = 0.26 \text{ hr}^{-1}$; $t_d = 2.63 \text{ hrs}$) had a 12H log phase.

Table 3.1. Tabulated data of the fermentation profile of *S. cerevisiae*, *S. pastorianus*-Frohberg, *S. pastorianus*- Saaz and *S. bayanus*. BD= Before Dehydration, AD= After Dehydration/Rehydration

Sample	Lag Time (hrs)	Log Time (hrs)	Doubling Time (hrs)	Total Fermentation Time (hrs)	Initial Cell Density (CFU/mL)	Max Cell Density (CFU/mL)
<i>S. cerevisiae</i> BD	6	16	3.68	32	3.0×10^9	1.4×10^{10}
<i>S. cerevisiae</i> AD	16	12	2.23	41	2.8×10^6	1.3×10^8
<i>S. pastorianus</i> - <i>Frohberg</i> BD	6	16	4.62	32	4.0×10^9	1.3×10^{10}
<i>S. pastorianus</i> - <i>Frohberg</i> AD	30	23	3.06	72	3.5×10^4	1.3×10^8
<i>S. pastorianus</i> - <i>Saaz</i> BD	6	16	4.12	32	1.4×10^9	1.4×10^{10}
<i>S. pastorianus</i> - <i>Saaz</i> AD	16	12	2.63	41	9.8×10^5	1.0×10^8
<i>S. bayanus</i> BD	24	22	12.59	72	6.3×10^9	1.0×10^{10}

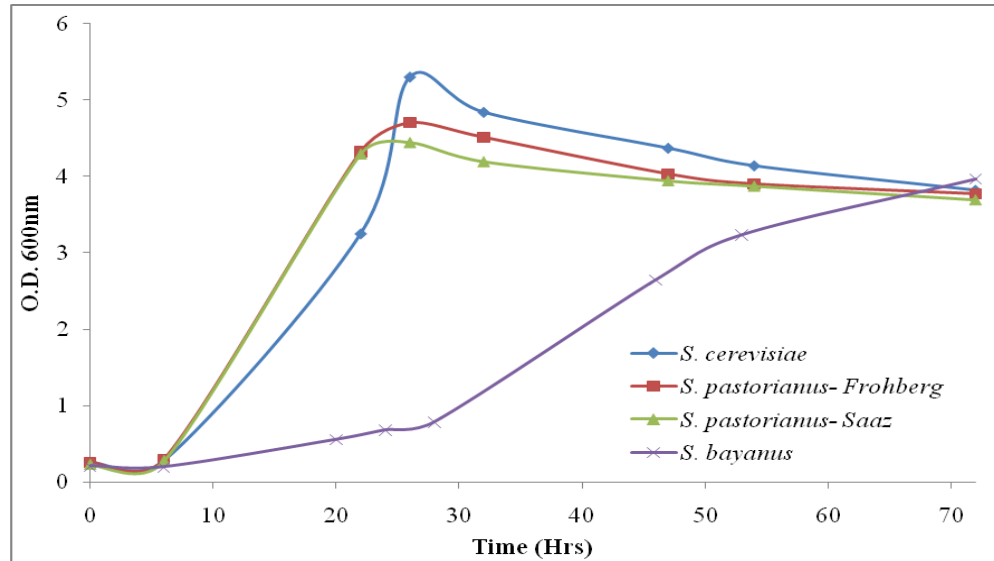


Figure 3.1. Growth curves for *S. cerevisiae*, *S. pastorianus-Frohberg*, *S. pastorianus-Saaz* and *S. bayanus* before dehydration/rehydration at O.D. (600nm) over 72H

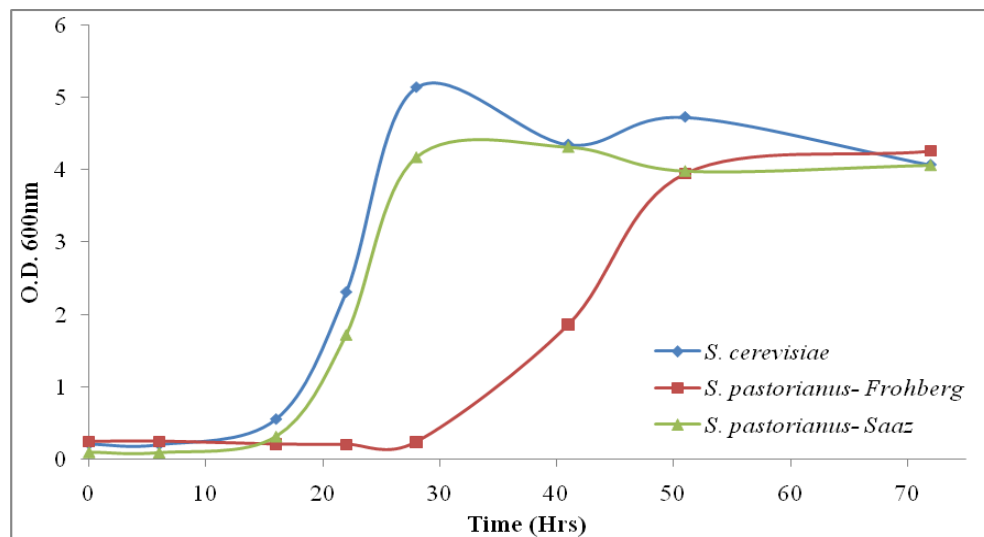


Figure 3.2. Growth curves for *S. cerevisiae*, *S. pastorianus-Frohberg*, and *S. pastorianus-Saaz* after dehydration/rehydration at O.D. (600nm) over 72H

Before dehydration the strains exhausted the main brewing carbohydrates (maltose and glucose) and produced the maximal ethanol yield after 32 hours (Figure 3.3, 3.4, 3.5). *S. bayanus* slowly utilized glucose and barely utilized maltose (Figure 3.6). There is no significant difference in the GCR of *S. cerevisiae* (GCR= 0.62), *S. pastorianus-Frohberg* (GCR= 0.61) and *S.*

pastorianus- Saaz (GCR= 0.61). Once *S. bayanus* (GCR= 0.48) began to grow it utilized glucose at a slower pace than that of the brewing strains. As *S. bayanus* is cryophilic yeast strain, fermentation at 25°C may have contributed to the slow growth and poor utilization of the brewing carbohydrates. However, its poor performance at the given conditions before dehydration disqualified it as an ADLY brewing strain. Thus it was not included in the after dehydration testing. After dehydration and rehydration *S. cerevisiae* and *S. pastorianus*-Saaz exhausted the main brewing carbohydrates (maltose and glucose) and produced the maximal ethanol yield after 41H (Figure 3.3, 3.5). *S. pastorianus*- Frohberg completed its fermentation at 72H (Figure 3.4), and the main brewing carbohydrates were not utilized by the Frohberg type strain after dehydration and rehydration until 28H after pitching. *S. pastorianus*- Frohberg had the slowest rate of glucose consumption (GCR= 0.49), while *S. cerevisiae* (GCR= 0.64) and *S. pastorianus*- Saaz (0.75) consumed it faster. The utilization of the carbohydrates once the yeast began to metabolize the substrates took 25H for *S. cerevisiae* and *S. pastorianus*-Saaz, whereas *S. pastorianus*-Frohberg took 44H. Compared to their fresh yeast counterparts the dehydrated/rehydrated cultures of *S. cerevisiae* and *S. pastorianus*-Saaz had a 28% longer total fermentation time, which is equivalent to 9H. *S. pastorianus*-Frohberg type had a 125% longer total fermentation time compared to its fresh yeast counterpart, which is equal to 40H.

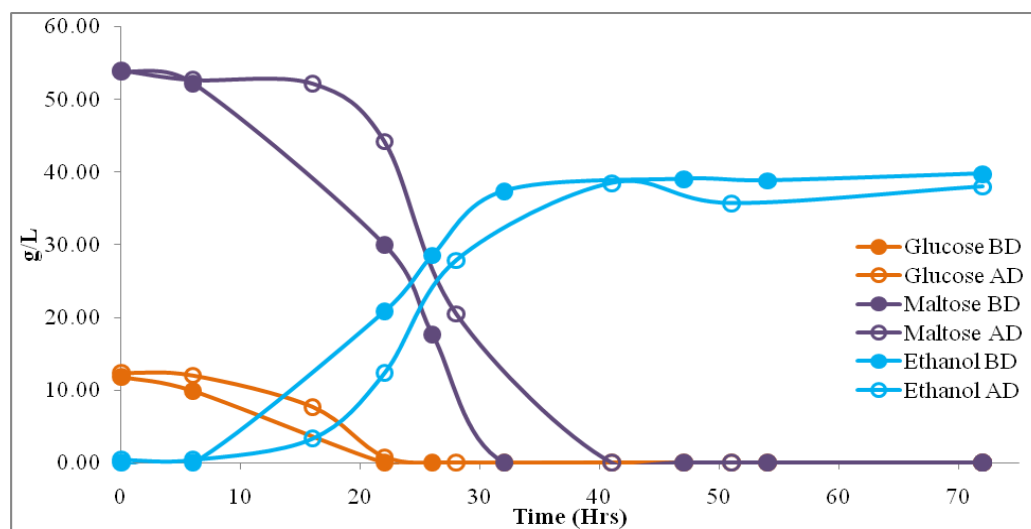


Figure 3.3. Substrate consumption and end product formation for *S. cerevisiae* before dehydration (BD) and after dehydration and rehydration (AD)

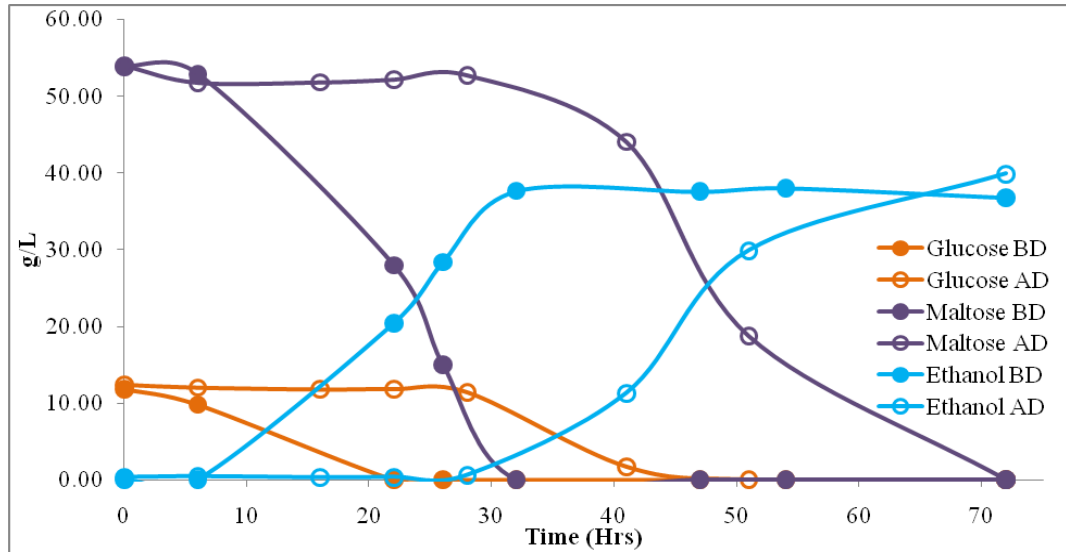


Figure 3.4. Substrate consumption and end product formation for *S. pastorianus*- Frohberg before dehydration (BD) and after dehydration and rehydration (AD)

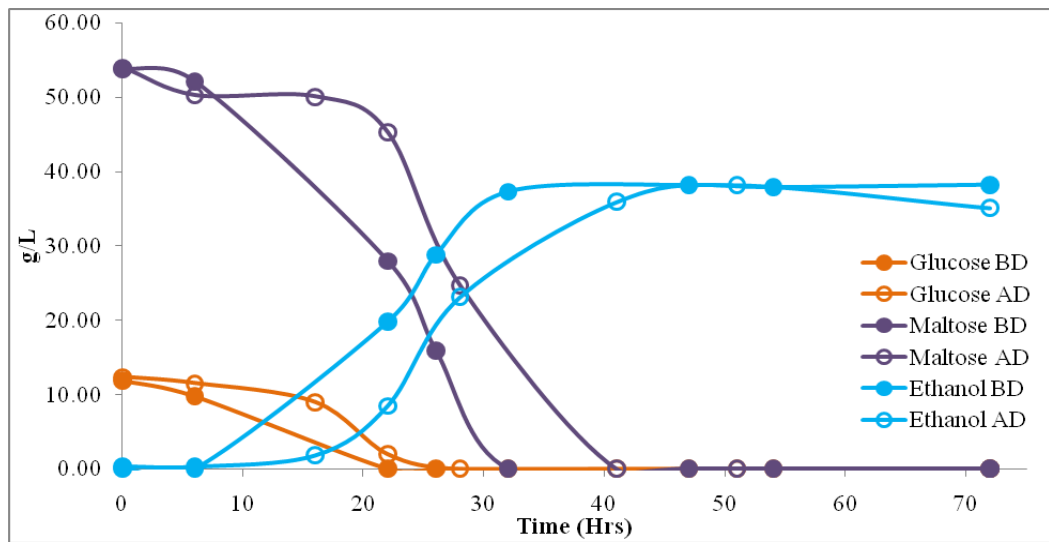


Figure 3.5. Substrate consumption and end product formation for *S. pastorianus*- Saaz before dehydration (BD) and after dehydration and rehydration (AD)

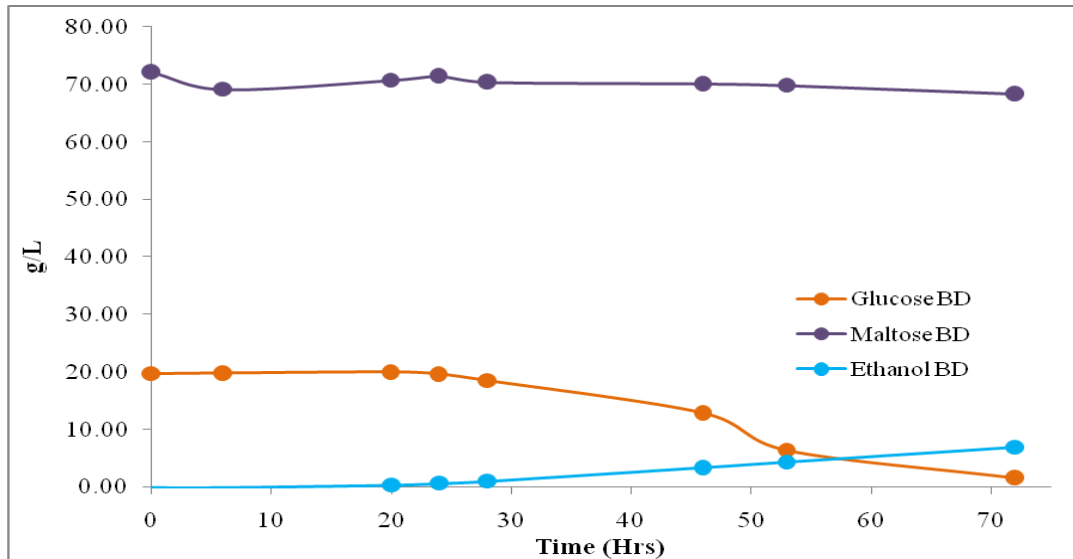


Figure 3.6. Substrate consumption and end product formation for *S. bayanus* before dehydration (BD)

Cell Viability During Fermentation

The viability of the strains, before dehydration, by staining was greater than 99% throughout the fermentation. Each of the strains grew approximately 1 log above the initial CFU/mL, based on spiral plating data, over the duration of the fermentation. The viability of the dehydrated and rehydrated strains improved gradually throughout the fermentation (Figure 3.7). Each of the strains had an initial reduction in viability upon pitching. *S. cerevisiae* and *S. pastorianus*-Saaz had a rapid recovery rate and reached viabilities around 90% after 22H. *S. pastorianus*-Frohberg recovered very slowly and did not reach viabilities greater than 90% until 51 hours into the fermentation. The average viability over the whole fermentation was 69% for *S. pastorianus*-Frohberg, 84% for *S. cerevisiae* and 87% for *S. pastorianus*- Saaz.

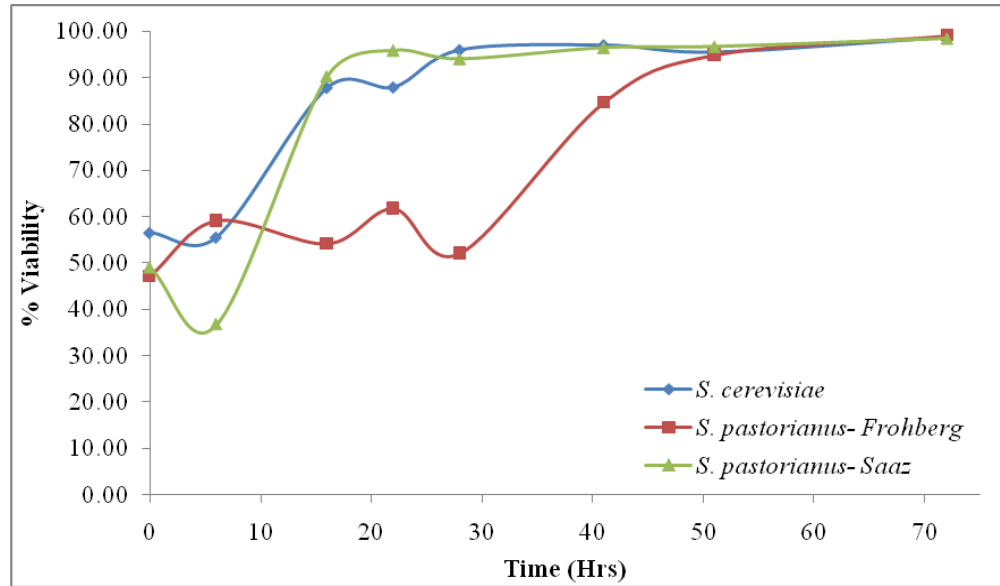


Figure 3.7. Viability, based upon methylene blue staining, of 3 experimental strains over 72 H of fermentation after dehydration/rehydration

Effects of Dehydration and Rehydration on Cell Viability

The average final viability of cells, after dehydration/rehydration, was measured as a percentage of the initial viability before dehydration/rehydration (Figure 3.8). Cell viability was measured in real time by the hemacytometer (with a methylene blue staining), CASY® cell counter, and the capacitance probe. The hemacytometer data indicates that *S. pastorianus-Frohberg* is slightly less affected than *S. cerevisiae* and *S. pastorianus-Saaz* to by dessication and rehydration. The CASY® cell counter conflicts with the hemacytometer data and shows *S. cerevisiae* and *S. pastorianus-Saaz* performing better than *S. pastorianus-Frohberg*. The hemacytometer and CASY® cell counter data show no statistically significant differences between strains ($p < 0.05$). However, the capacitance probe shows significant differences between strains. *S.*

cerevisiae and *S. pastorianus*-Saaz are significantly different ($p < 0.05$) from *S. pastorianus*-Frohberg. *S. cerevisiae* is not significantly different ($p < 0.05$) from *S. pastorianus*-Saaz type. The three real-time measurements of cell viability (methylene blue staining, CASY® cell counter, capacitance) all show cellular injury. The injury shown by the real time methods accounts for the difference in fermentation performance between fresh cells (BD) and dehydrated and rehydrated (AD) cells. The fermentation profile is altered from BD to AD due to the cells recovering from the effects of desiccation. Cell viability was also measured by spiral plating, which allows cells to recover on a nutrient rich agar plate over 48H. There are significant differences ($p < 0.05$) between all strains after dehydration and rehydration. *S. cerevisiae* has the highest recovery rate ($91 \pm 4\%$), followed by *S. pastorianus*-Saaz ($65 \pm 6\%$), and lastly *S. pastorianus*-Frohberg ($20 \pm 3\%$) when compared to their initial viability. The stark differences between the real time assays and the plating data can be attributed to injured cells recovering over time in an ideal environment.

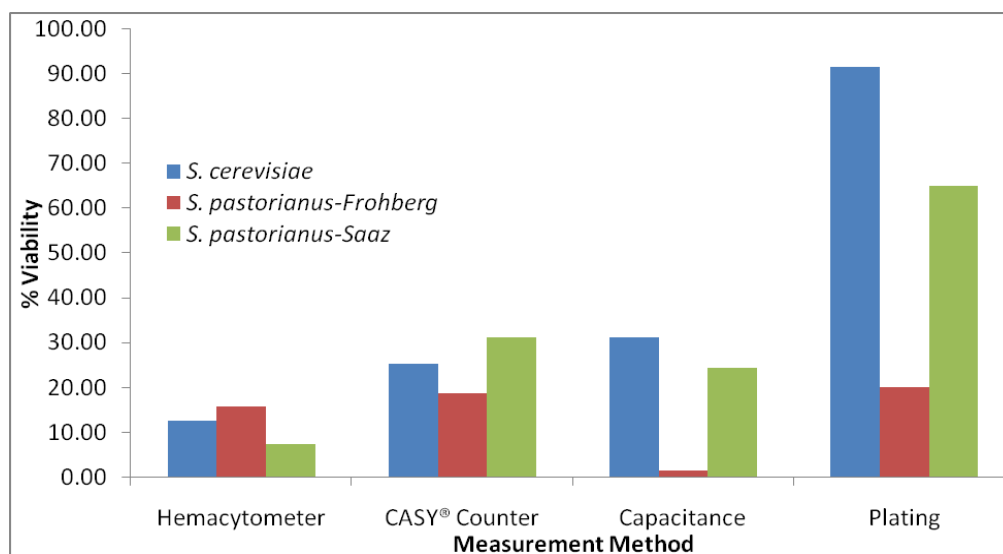


Figure 3.8. Average final viability as a percentage of initial viability before dehydration and rehydration

Effects of Heat on Cell Viability

Cells that undergo spray drying could be affected by the effects of both heat and desiccation. The outlet temperature of a spray dryer is the maximum temperature that the cells are subjected to. The effect of the outlet temperature on the viability of the cells, based on methylene blue staining, seems to be minimal (Figure 3.9). The effect of heating over a short period of time,

has little effect on any of the strains. Long periods of heating illustrate slight differences in thermal tolerances between the strains. Since the spray drying process does not hold cells in the atomizer for more than 30 seconds it can be assumed that the majority of the damage is due to the effects of dehydration.

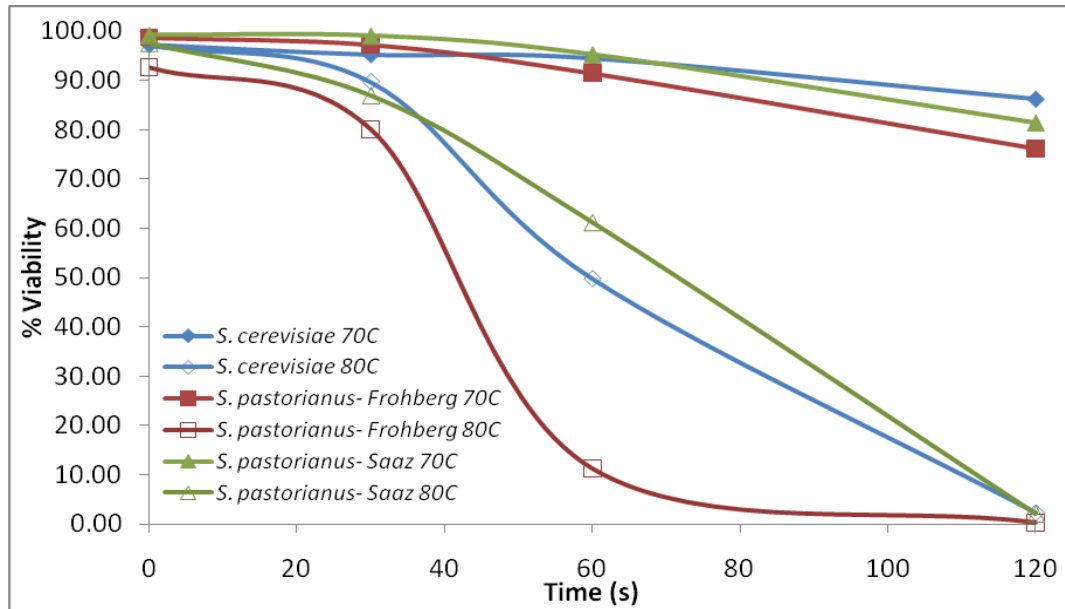


Figure 3.9. Percentage viability of ale and lager strains after heating at 70 and 80°C

Genetic Indicators for Desiccation Tolerance

The *POP4* gene (1634 bp) which has ribonuclease MRP activity (cleaves mitochondrial replication primers) and ribonuclease P activity (cleaves tRNA) is found in *S. cerevisiae* and *S. pastorianus*-Frohberg and it may be found in *S. bayanus*, but it is not in *S. pastorianus*-Saaz (Figure 3.10). The vacuolar trehalase or *ATH1* gene (3883 bp), leads to the metabolism or stored trehalose. The *ATH1* gene is found in *S. cerevisiae*, and may be in *S. pastorianus*-Frohberg, and *S. pastorianus*-Saaz, but not in *S. bayanus* (Figure 3.11). The gene *ECM29* (5607 bp) is a major component of the proteasome that tethers the proteasome core particle to the regulatory particle, enhancing the stability of the proteasome. The *ECM29* gene is found in *S. cerevisiae* and may be in *S. pastorianus*-Frohberg, however it is not in *S. pastorianus*-Saaz or *S. bayanus* (Figure 3.12).

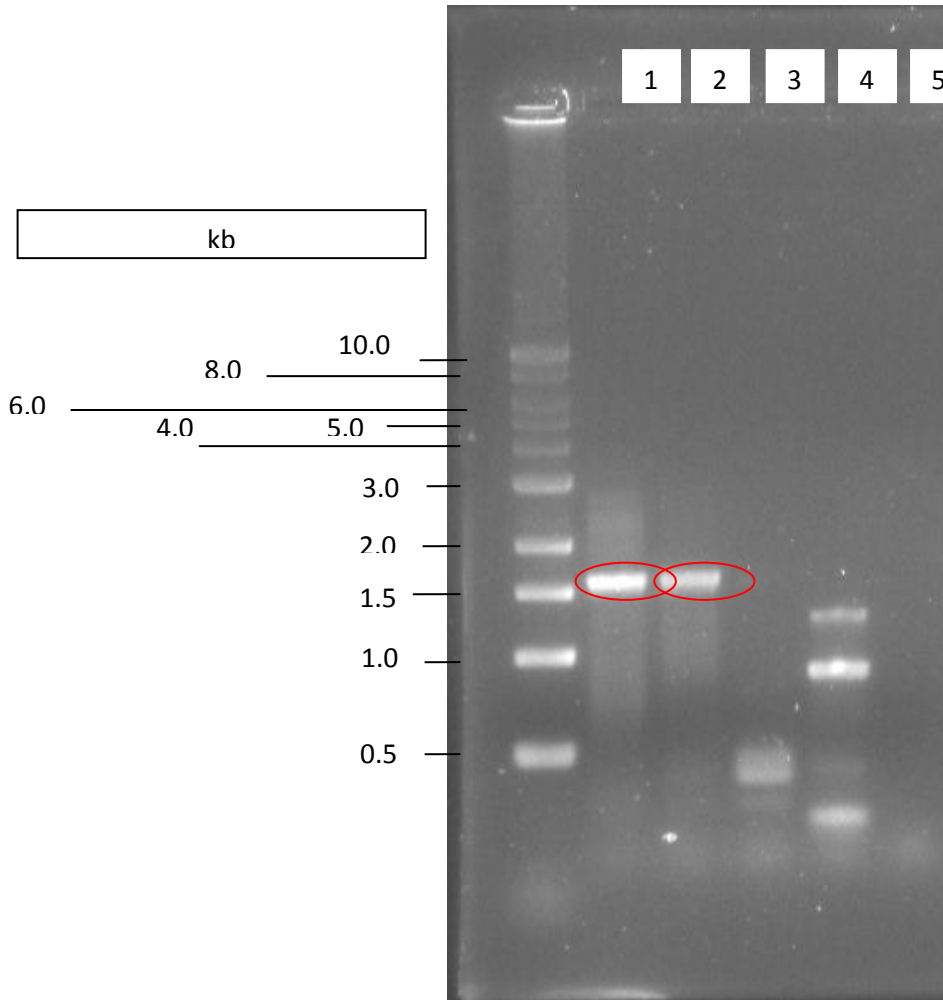


Figure 3.10. PCR gel for the *POP4* gene in *S. cerevisiae* (1), *S. pastorianus*- Frohberg (2), *S. pastorianus*- Saaz (3) and *S. bayanus* (4). Lane 5 is a water blank.

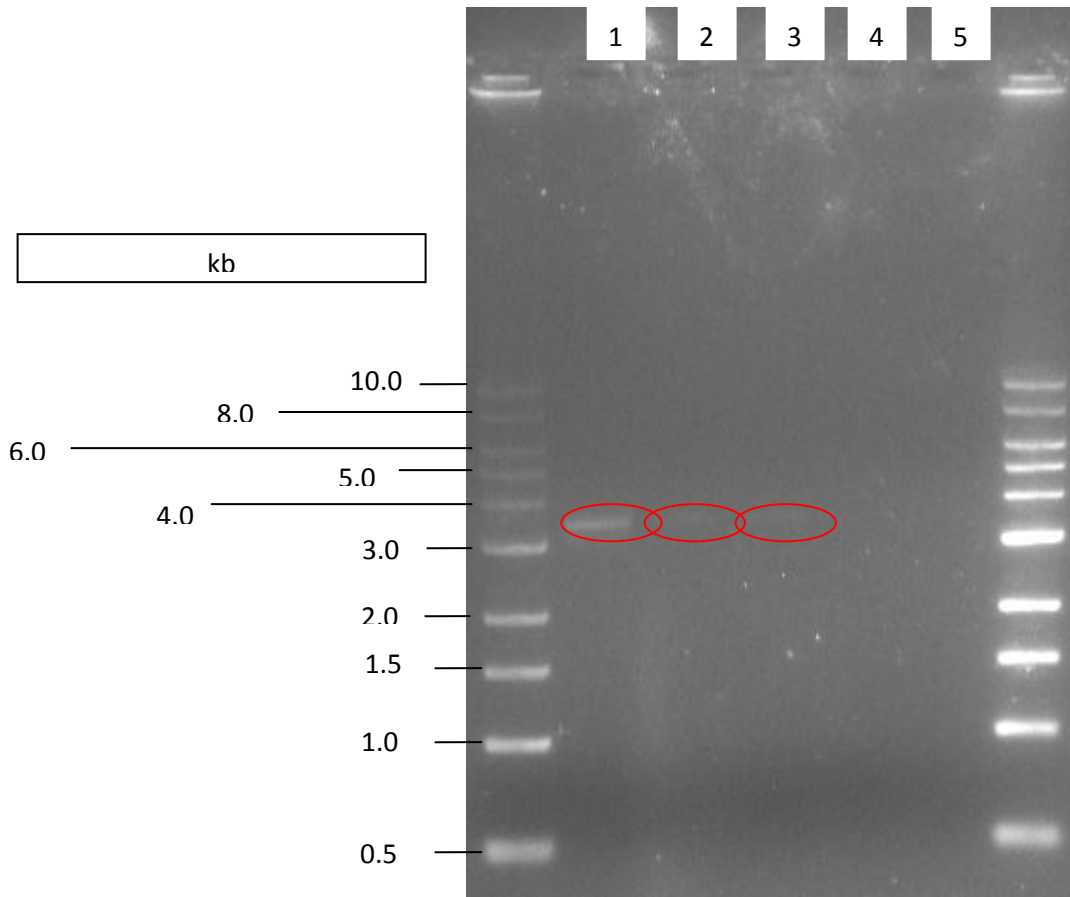


Figure 3.11. PCR gel for the *ATH1* gene in *S. cerevisiae* (1), *S. pastorianus*- Frohberg (2), *S. pastorianus*- Saaz (3) and *S. bayanus* (4). Lane 5 is a water blank.

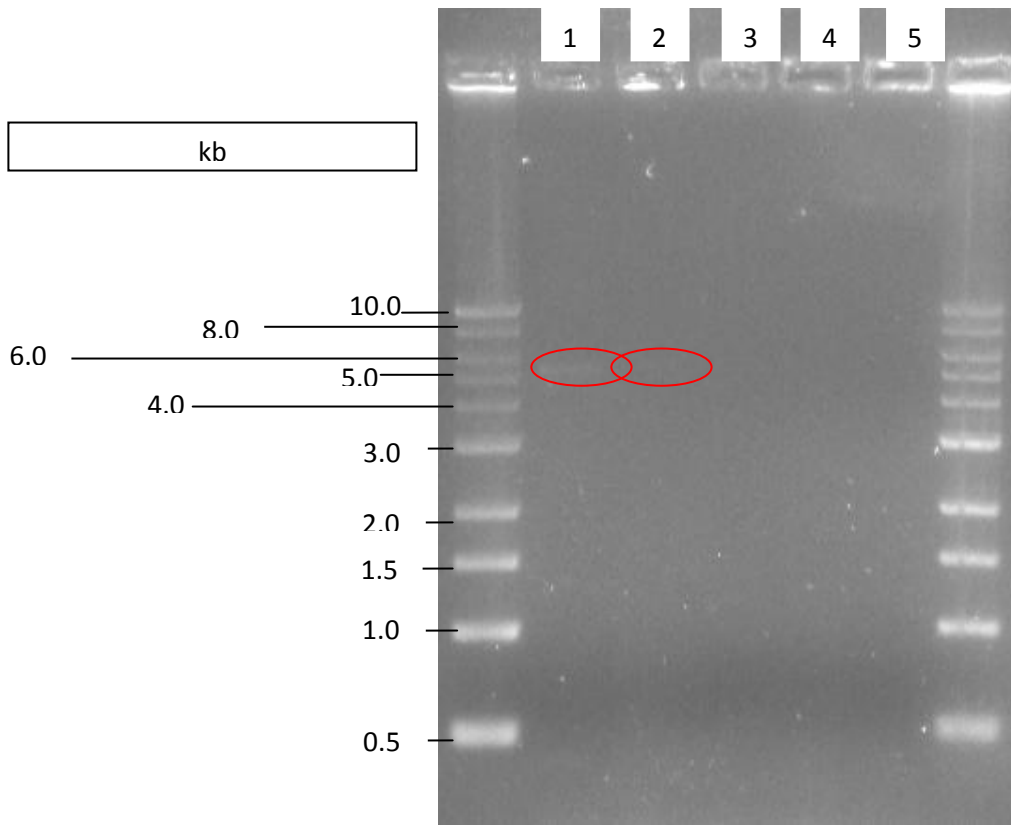


Figure 3.12. PCR gel for the *ECM29* gene in *S. cerevisiae* (1), *S. pastorianus*-Frohberg (2), *S. pastorianus*-Saaz (3) and *S. bayanus* (4). Lane 5 is a water blank.

DISCUSSION

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Dunn et al, (2008) proposed that lager yeast (*S. pastorianus*) can be divided into two distinct groups based on genomic DNA. One group (Saaz type) had lost a significant portion of the *S. cerevisiae* genome, but retained virtually all of the *S. bayanus* genome. The other group (Frohberg type) retained virtually all genomic content of both *S. cerevisiae* and *S. bayanus* genomes. The average total DNA in a given Saaz type strain was much lower than that of the Frohberg type strains. The Frohberg-type lager yeast have, on average, approximately two to three times more *S. cerevisiae* DNA relative to *S. bayanus* DNA within the genome. The retention of multiple *S. cerevisiae* chromosomes would suggest that the Frohberg type strains would behave similarly to *S. cerevisiae*. Saaz type strains have proportionately more *S. bayanus* DNA sequences relative to *S. cerevisiae* sequences. This would suggest that Saaz type strains would behave more like *S. bayanus* due to loss of *S. cerevisiae* chromosomes, or chromosomal portions. However, the data presented show that *S. bayanus* was not an acceptable brewing strain due to its fermentative ability. Therefore, the retained *S. cerevisiae* genetic portions, found in *S. pastorianus*, are most likely are allocated to fermentative characteristics. The retained *S. bayanus* genomic content in *S. pastorianus* may impact the maturation or lagering phase of lager beer production.

ADY (*S. cerevisiae*) and ADLY (*S. pastorianus*) are popular within the brewing industry and are utilized in hundreds of breweries around the world (Lallemand, 2009). Cry et al, (2007) showed that along with a lower viability, compared to ADY, ADLY is highly variable in terms of viability and vitality of commercially produced ADLY. Based on the findings of Dunn et al, (2008) it was hypothesized that the large variability in yeast viability and activity was related to the use of one or both types of *S. pastorianus* hybrids for the production of ADLY. The quality of dry yeast can be described in terms of “viability” and “vitality”. Yeast viability describes the ability of cells to grow, reproduce, and interact with their environment. Yeast vitality is described as a measure of activity, fermentation performance, or the capacity to overcome and recover from physiological stress. In order to ascertain yeast viability or vitality many cellular parameters can be examined such as replication capacity, levels of cellular components, and metabolic activity. Unfortunately, each assessment technique for viability or vitality is based on one parameter or aspect of metabolic activity meaning that one technique is limited in usefulness for predicting all of the attributes that contribute to fermentation performance. The viability of yeast cells was assessed in this work by multiple techniques including methylene blue staining, two forms of capacitance measurements, and plating. Some of these methods (plating, methylene blue staining, and

capacitance) are used extensively in the brewing industry to monitor yeast viability or activity (Smart et al. 1999).

Each of the methods used to ascertain the viability or activity of yeast in this study used a different parameter for ascertaining viability and activity. Yeast activity, or vitality, was primarily measured by the HPLC which showed the utilization of carbohydrates and the production of end products. The HPLC is a fairly fast and accurate way to measure vitality however, the equipment is costly. Yeast activity was also indicated by certain strains ability to recover and overcome the stresses imposed by dehydration. This recovery can be seen when comparing the real-time viability measurements (capacitance and methylene blue staining) to plating. The real-time viability measurements indicated low yeast viabilities, for all strains, immediately after desiccation and rehydration. However, when given time to recover on a nutrient rich agar plate *S. cerevisiae* CAT#1 and *S. pastorianus*-Saaz recovered significant amounts of viability. The recovery seen on the plates was also seen during the fermentations where *S. pastorianus*-Frohberg seemed to be either more injured, or have less activity than *S. cerevisiae* CAT#1 and *S. pastorianus*- Saaz.

The viability of yeast was measured by four methods (methylene blue staining, two types of capacitance measurements, and plating). Methylene blue staining is a quick, easy, and inexpensive way to quantify total, dead and viable yeast cells. However, the method is considered to be less than accurate at low viabilities. The CASY® cell counter is a very fast, easy method for ascertaining total, dead, and viable yeast cells as well as cell size. This method is however expensive and draws its viabilities based on subjective selection of cell debris after the cell counter analyses the sample. The capacitance probe which generates a constant electrical field, as opposed to the pulsed field cell counter, is relatively fast, easy and inexpensive method for determining viable cells. The capacitance probe can only identify viable cells and it must be correlated with some type of cell count in order to quantify the amount of cells in a system. The sample around the probe must also be representative of the system as a whole and in larger fermentations it may be difficult to get a homogeneous sample for accurate capacitance readings, especially after flocculation. The last method, plating, is a good method to measure both yeast viability (i.e. the cells ability to grow) and yeast vitality (i.e. capacity to overcome and recover from physiological stress). The only drawback to plating is that the method is slow (i.e. 24-48 hrs) which may lead to problems if not combined with a real-time method for establishing viability. Based on the results

of this study the combination of HPLC, Aber® capacitance probe, and methylene blue staining make an excellent combined method to measure both viability and activity.

The identification of genes that are essential for survival over long periods of dormancy and those that hinder the return to logarithmic growth are crucial for developing dry yeast that perform up to the necessary commercial standards. The genetic analysis of the 4 yeast strains shows that *S. pastorianus*- Froberg has the *POP4* gene while *S. pastorianus*- Saaz is missing the *POP4* gene. The deletion of this gene from other yeast strains has been shown to give a significant survival advantage for at least 6 months of dehydration (D'Elia et al. 2005). D' elia et al, (2005) showed that strains that had the *POP4* gene deleted were deficient in ribonuclease MRP activity (cleaves mitochondrial replication primers) and ribonuclease P activity (cleaves tRNA), and showed a 67-fold survival advantage over the same strain with the *POP4* gene.

The *ATH1* gene (vacuolar trehalose), is responsible for the metabolism of stored trehalose. Deletion of this gene has been shown to convey a significant, short-term, survival advantage to dehydrated yeast. *S. pastorianus*- Froberg, *S. pastorianus*- Saaz, and *S. cerevisiae* CAT#1 were found to contain this gene. *S. cerevisiae* had high amplification of the gene while both *S. pastorianus*- Froberg and *S. pastorianus*- Saaz had low amplification. This may indicate that the copy number of this gene is low in the hybrid strains. D' elia et al, (2005) indicated that over long periods of storage (i.e. 6 months) the deletion of *ATH1* conveys no significant survival advantage to desiccated yeast. The deletion of *ECM29* (protein binding) has also been indicated to convey a survival advantage during dehydration (D'Elia et al. 2005). The *ECM29* gene was found in *S. cerevisiae*, and may be in *S. pastorianus*-Froberg, but was absent in both *S. pastorianus* strains and also in *S. bayanus*.

Before dehydration each of the three strains of yeast behaved in a similar manner in terms of lag, log, and total fermentation time, with the exception of *S. bayanus*. The pattern of substrate utilization and max cell density was also similar among all strains. This suggests that the retention or deletion of *S. cerevisiae* genes has little effect on the vitality or fermentative capability of hybrid *S. pastorianus* strains before dehydration. After dehydration and rehydration the results illustrate a distinct difference between hybrid strains of *S. pastorianus*. Saaz and Froberg type lager strains can be clearly differentiated by lag, log, and total fermentation times. The results indicate that after dehydration and rehydration *S. pastorianus*- Saaz behaves more like *S. cerevisiae* than *S.*

pastorianus- Frohberg, despite the loss of portions of the *S. cerevisiae* chromosome. As mentioned earlier the deletion of specific genes with known or unknown functions may aid in desiccation tolerance and subsequent fermentation performance.

Fermentation performance is a compilation of viability and activity. The hybrid strains of *S. pastorianus* had significant differences in fermentation performance, likely due to the loss of viability, caused by the effects of spray drying. Heat and dehydration are the two primary stresses imposed on spray dried yeast. The results illustrate that the effect of heating, at the given exhaust temperature (70°C), are minimal and negligible until almost 2 minutes when real time loss of viability can be seen. The main loss of viability is then, most likely, the effect of desiccation on the cells. When compared with other reports of viability (by plating) the results of this study correlate well. The viability of ADY are reported to be around 80±7% whereas commercial ADLY are reported to have 64±7% (Quain and Boulton, 2006). Cry et al (2007) reported that the viability of commercial ADLY is more variable with one strain having a viability of 25.2±2% and another having a viability of 71.1±2%. The results of this study show an ADY viability (by plating) of 91±4%, and an ADLY viability of 65±6% (Saaz type), and 20±3% (Frohberg type). The ADY and ADLY (Saaz) viabilities are comparable to those obtained by Quain and Boulton (2006), the ADLY viabilities for Saaz and Frohberg are also similar to Cry et al (2007) publication. The identification of Saaz and Frohberg type hybrids of *S. pastorianus* may explain the variability in commercial ADLY seen by Cry et al (2007).

The high loss of viability, due to the spray drying process, in the *S. pastorianus*- Frohberg type strain resulted in an extended lag phase and ultimately an extended fermentation time. Although the *S. pastorianus*- Frohberg type strain retained much of the genomic content of *S. cerevisiae*, its performance after desiccation was not similar. The performance of *S. pastorianus*-Saaz, which is missing various portions of the *S. cerevisiae* chromosome, did perform similarly to *S. cerevisiae* after desiccation. Large viability loss due to desiccation was observed for the *S. pastorianus*- Frohberg type strain while only moderate losses were observed for the *S. pastorianus*-Saaz type strain. The ultimate reason for this phenomenon is unknown, however, it may be that *S. pastorianus*- Saaz type strains has a similar membrane makeup/construction, or a particular stress protein that allows it to survive and thrive after desiccation similar to that of *S. cerevisiae*. The absence of specific genes such as *POP4* may also shed light on the survivability of Saaz versus Frohberg. The lack of expression of a stress protein or a difference in membrane build or function

may also account for the poor desiccation performance of *S. pastorianus*- Frohberg, in relation to *S. cerevisiae*. Ultimately, based on the results, *S. pastorianus*- Saaz is more suitable for use in ADLY than *S. pastorianus*- Frohberg. The use of *S. pastorianus*- Saaz type strains may reduce the variability in the viability of ADLY yeast seen in the Cry et al, (2007) paper. Use of Saaz based ADLY may reduce the total fermentation time or chance of a stuck or contaminated fermentation (due to long lag times) when utilizing ADLY.

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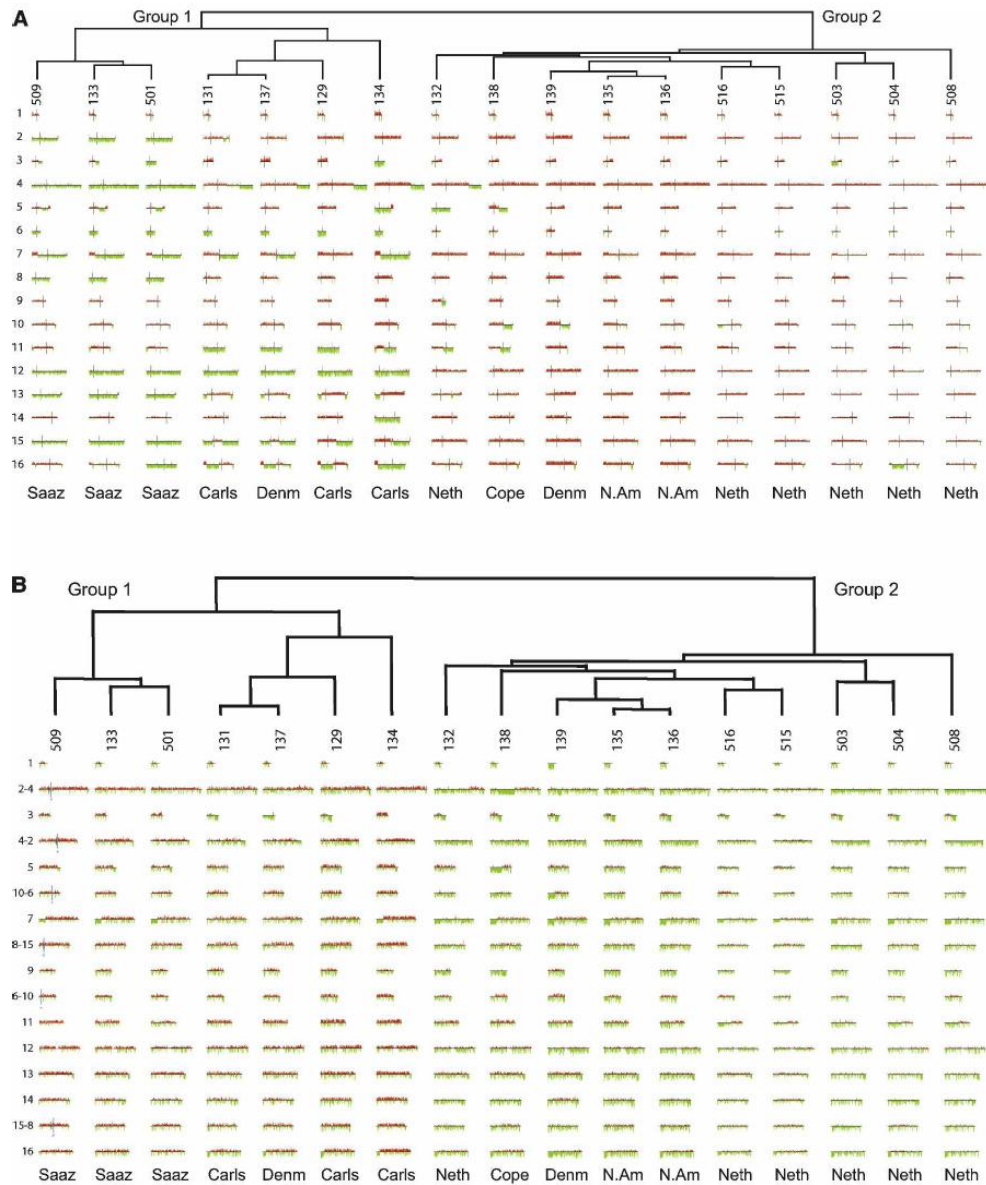
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APPENDIX



- A) Array CGH of the *S. cerevisiae* genomes. Array CGH data for the *S. cerevisiae* chromosomes of the *S. pastorianus* strains are shown in numerical order with chromosome I at the top and chromosome XVI at the bottom. Regions that are strongly green represent loss of that portion of the *S. cerevisiae* genome; regions that are strongly red represent regions of the *S. cerevisiae* genome that are amplified; and regions that are neither strongly green nor strongly red have a normal complement of the *S. cerevisiae* genomic content for that region.
- B) Array CGH of the *S. bayanus* genomes. Array CGH data for the *S. bayanus* chromosomes of the examined strains are shown in a manner identical to that described in A; note that the three pairs of *S. bayanus* chromosomes that have experienced reciprocal translocations

relative to the *S. cerevisiae* genome are shown as they exist in *S. bayanus*, that is, in their translocated form. For these six chromosomes, the translocation breakpoint is shown (for GSY509 only, but are the same for all strains) as a blue vertical line. In every case for the translocated chromosomes, the number of the chromosome is such that the chromosome to the left of the breakpoint is listed first in its name (e.g., chromosome 2-4 has the chromosome homologous to the *S. cerevisiae* chromosome II to the left side of the blue vertical line, and has the homologous chromosome IV to the right side of the vertical line). For the translocated chromosomes, centromeres are shown as shorter black vertical lines; for all nontranslocated chromosomes, the centromeres are in the same location as for the *S. cerevisiae* chromosomes in A.

Appendix A and B. Array CGH of the *S. cerevisiae* and *S. bayanus* genome portions of 17 *S. pastorianus* strains (Dunn et al. 2008)

	Strain	Culture collection aliases ^a			Earliest collection entry date	Other information	Collection locale
		CBS	DBVPG ^b	NCYC			
<i>S. pastorianus</i> strains	Group 1						
	GSY509	2440		398	June 1952		Brewery-Saaz type beer; bottom yeast
	GSY133	1486	6258 ¹	397	June 1935		Brewery-Saaz type beer
	GSY501	1174			June 1931		Brewery-Saaz type beer
	GSY131	1538	6047 ¹	392	October 1935 (described by Hansen in 1904)	<i>S. pastorianus</i> -type strain	Carlsberg Brewery
	GSY137		6284			AJL248	Alfred Jorgensen's Laboratorium (now Danbrew)
	GSY129	1513	6033 ¹	396	October 1947 (original culture 1883, Hansen)	<i>S. carlsbergensis</i> -type strain	Carlsberg Brewery; bottom yeast no. I
	GSY134	1503	6261		(original culture 1908, Hansen)	<i>S. monacensis</i> -type strain	Carlsberg Brewery ; bottom yeast no. II
	Group 2	GSY132	1260	6257	400	March 1937	Frohberg-type bottom yeast, Netherlands
		GSY138		6285 ²			Copenhagen
		GSY139		6560 ²		1962	Denmark
		GSY135		6282 ²		1962	Labatt Brewery, Canada; bottom-fermenting
		GSY136		6283 ²		1969	Rainier Brewery, WA; bottom-fermenting
		GSY516	6903			September 1976	Brewery, Netherlands
	GSY515	5832			December 1967	Brewery, Netherlands	
	GSY503	1483			July 1927	Brewery-Heineken, Netherlands; bottom yeast	
	GSY504	1484			February 1925	Cloudy beer—Oranjeboom, Netherlands; bottom yeast	
	GSY508	2156		457	June 1955	Brewery, Netherlands	
<i>S. cerevisiae</i> strains	Ale strains						
	GSY161					Wyeast1388	Belgian Strong Ale; probable origin Duvel
	GSY708					Wyeast1056	American Ale Yeast; probable origin Sierra Nevada and/or Ballantine breweries
	GSY934					Leinenkugel Ale	Miller brewery collection, Leinenkugel ale, WI

Boldface indicates the collection from which we obtained the isolate.

^a(CBS) Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands; (DBVPG) Dipartimento Biologia Vegetale Perugia, Yeast Industrial Collection, Perugia, Italy; (NCYC) National Collection of Yeast Cultures, Norwich, UK.

^bSuperscript numbers 1 and 2 indicate strains in Liti et al. (2005) that were placed into two groups based on repeat sequences.

Note that most strains were most likely serially propagated at breweries until the time of collection, at which point they were "evolutionarily arrested" by being kept in a frozen state; this may not be true for the oldest cultures, however, due to the lack of freezers until the mid-century.

Appendix C. *S. pastorianus* and *S. cerevisiae* strains, and the culture collection aliases (Dunn et. al 2008)