Fermentation and gas bubble formation in psychrophilic yeasts

by

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This dissertation is dedicated to my father, Paul Louis Saaiman, mother, Martha Catherina Saaiman, sister, Anna Elizabetha Hendrina Saaiman and brother, Paul Louis Saaiman.

I, Susanna Elizabeth Saaiman, declare that the Master's Degree research dissertation or interrelated, publishable manuscripts/publishable articles, or coursework Master's Degree mini-dissertation that I herewith submit for the Master's Degree qualification at the University of the Free State is my independent work, and that I have not previously submitted it for a qualification at another institution of higher education.

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Contents

Chapter 1

Literature Review

Chapter 2

Fermentation by psychrophilic yeasts

Chapter 3

Exposing gas bubbles in psychrophilic yeasts

Chapter 4

Summary

Chapter 1

Literature Review

1.1 Motivation

Yeasts have been used for thousands of years in processes ranging from primitive to industrial fermentation biotechnology for the production of bread, beer, wine, champagne and bioethanol (Van Maris *et al*., 2001). During ethanol fermentation carbon dioxide (CO_2) is produced as a byproduct in the cytoplasm of yeasts and excreted into the cell environment. Due to the large quantities of CO_2 produced in these yeast cells, it is expected that CO_2 gas will build up in the cytoplasm of the yeast as gas bubbles (Swart *et al*., 2012). However, the presence of intracellular gas bubbles has not been reported before 2012. Hemmingsen and co-workers report in several articles on the techniques used to induce gas bubble formation in living cells without any success (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.,* 1985; Hemmingsen *et al*., 1990; Ryan & Hemmingsen, 1988). In 2012, Swart and co-workers discovered gas bubbles formed during ethanol fermentation in the yeast *Saccharomyces*, using Light Microscopy (LM), Transmission Electron Microscopy (TEM) and Nano Scanning Auger Microscopy (NanoSAM) (Swart *et al*., 2012). The discovery of gas bubbles lead to several research questions: Are these gas bubbles conserved in *Saccharomyces*? Are these gas bubbles conserved in yeast? What is the effect of these gas bubbles on living cells? Are gas bubbles only present in fermenting cells? Since 2012, gas bubbles have been found in 15 yeasts and 1 fungus during ethanol fermentation at 30°C. This lead to more questions which are also the aims of this project: Are gas bubbles produced at temperatures lower than 30°C? What are the fermentation abilities of psychrophilic yeasts?

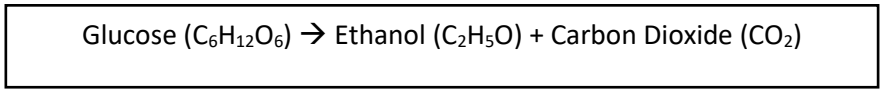
1.2 Fermentation and gas bubbles

Yeasts are widely used in the industry for the production of fermentation products such as bread, beer, wine, champagne and bioethanol (Van Maris *et al*., 2001). The model organism, *Saccharomyces cerevisiae*, is widely used industrially for its fermentation abilities, however several yeasts are able to ferment sugars producing ethanol and carbon dioxide (CO_2) as products.

All these yeast cells can catabolise glucose via aerobic respiration and some via aerobic or anaerobic fermentation (Pronk *et al*., 1996; Reynders *et al*., 1997; Van Maris *et al*., 2001) and

can thus be divided into three categories regarding fermentation: 1) obligate fermenters, 2) facultative fermenters and 3) non-fermenters (Visser *et al*., 1990). In *S. cerevisiae* respiration yields approximately 16 ATP molecules and fermentation yields 2 ATP molecules per molecule of glucose (Van Maris *et al*., 2001; Pfeiffer & Morley, 2014). Theoretically, fermentation supplies sufficient energy for growth, however the oxygen requirements of the yeast have an influence on the ability of the yeast to ferment (Visser *et al*., 1990). Oxygen presence and absence plays a major role in fermentation, as can be seen in the Pasteur Effect, Custers Effect, Kluyver Effect and Crabtree Effect (Fugelsang, 2007; Pronk *et al*., 1996; Visser *et al*., 1990).

During ethanol fermentation, a sugar is catabolised via the fermentation pathway to ethanol and CO_2 as can be seen by the Gay-Lussac equation (Fugelsang, 2007; Pronk *et al*., 1996). Due to the CO_2 production during fermentation, there is the expectation that fermenting yeast cells should contain intracellular gas bubbles which have not been released into the environment. The first report of intracellular gas bubbles was made by Swart and co-workers in 2012.



The lack of information on gas bubble formation before 2012 is not due to a lack of research. Hemmingsen and co-workers did extensive research on the formation of gas bubbles in living cells (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.,* 1985; Hemmingsen *et al*., 1990; Ryan & Hemmingsen, 1988). A wide range of experiments were conducted, subjecting unicellular organisms to hyperbaric pressure followed by decompression, but gas bubble formation was not observed. The resistance of the cell to form intracellular gas bubbles at high gas supersaturation was ascribed to the lack of sufficient amounts of intracellular water present in the cell (Ryan & Hemmingsen, 1988). Gas bubbles were observed in *Tetrahymena* sp. (Hemmingsen & Hemmingsen, 1983), a ciliate Protozoa (Elliott, 1973), when these cells contained food vacuoles which acted as nucleation sites at supersaturation levels (Hemmingsen & Hemmingsen, 1983).

Even though gas vesicles are not similar to gas bubbles, the presence of gas vesicles in Cyanobacteria provided an idea of what gas in a cell would look like using microscopy. Walsby (1994) did an extensive review on gas vesicle formation. Gas vacuoles in Cyanobacteria are packed with gas vesicles. These gas vesicles are described as cylindrical vesicles with conical ends which are 1 μm in length and 75 nm in diameter with a single wall of 2 nm. Gas vesicles are gas permeable and gas can freely diffuse into the vesicle. These gas vesicles play a major role in the buoyancy of aquatic Cyanobacteria in order to enable Cyanobacteria to reach the surface of the water to obtain light for photosynthetic purposes. Gas vesicles also play a role in aerophilic bacteria, to enable these bacteria to reach the surface of the water where the water is more oxygenated. Walsby described the gas present in Cyanobacteria when viewed with light microscopy (LM) as glistening refractile areas (Walsby, 1985). Klebahn found that these gas vesicles could be identified with a light microscope due their disappearance when pressure is applied to the cell (Klebahn, 1895). If the pressure on the cell is increased above atmospheric pressure, the gas vesicles collapse, resulting in a decrease in glistening refractile areas (Fig. 1.1).

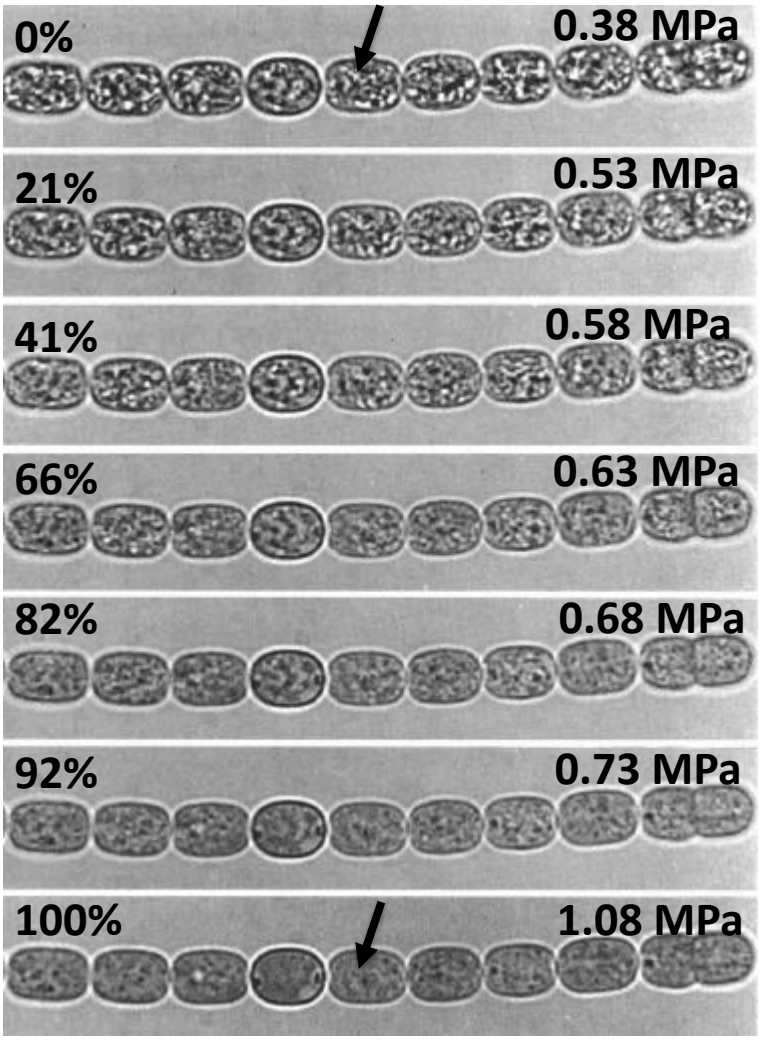


Figure 1.1: The internal pressure of the cell is 0.38 MPa. As there is an increase in pressure, the gas vesicles collapse, and the amount of glistening refractile areas decrease (Walsby, 1994).

In 2012, Swart and co-workers reported the presence of gas bubbles in *S. cerevisiae* and *S. pastorianus* with LM as light scattering granules, somewhat similar in appearance to gas vesicles in Cyanobacteria. To search for gas bubbles in *S. cerevisiae and S. pastorianus*, the yeast was cultivated in fermentable Yeast Malt (YM) media to promote gas production via fermentation, and in non-fermentable Yeast Peptone Glycerol (YPGlycerol) media, as a respiring control. When subjecting these cells to LM, light scattering granules were present in the yeast cells cultivated in fermentable media and absent in the yeast cells cultivated in non-fermentable media (Fig. 1.2). The light microscope examination was the first step in the search for gas bubbles in yeast (Swart *et al*., 2012).

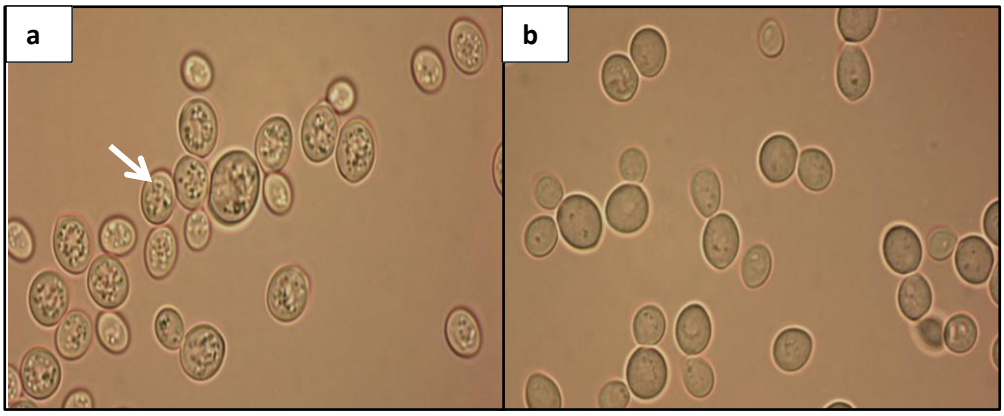


Figure 1.2: a) *Saccharomyces pastorianus* cells cultivated in fermentable media. Light scattering granules were present which indicated the presence of gas bubbles in the cells formed during fermentation. b) *Saccharomyces pastorianus* cells cultivated in non-fermentable media. No light scattering granules were observed which indicated the absence of gas bubbles (Swart *et al*., 2012).

Transmission Electron Microscopy (TEM) was used by Swart and co-workers in order to view the two dimensional (2-D) ultrastructure of the yeast cells to confirm whether the light scattering granules observed with LM were indeed gas bubbles (Swart *et al*., 2012). During TEM preparation, the samples are cut as 60 nm sections and stained with metal stains uranyl acetate and lead citrate. When the samples are bombarded with electrons, the electrons are scattered by the surrounding stained electron dense cell material but the bubbles appear electron lucent with little electron scattering. Swart and co-workers observed the presence of electron transparent structures within the cells cultivated in fermentable media (Fig. 1.3a). A few small electron lucent structures were observed in the cells cultivated in non-fermentable media (Fig. 1.3b). This was ascribed to the small amount of CO_2 formed during respiration. Even though fermentation yields less CO_2 than respiration, the fermentation process is more rapid, therefore it is expected that there should be more gas build up in the cells fermenting than respiring. With increased magnification, it was observed that the electron transparent structures contained no envelope, therefore they concluded that these structures are not membrane-bound cell organelles and thus most likely gas bubbles. The observation of non-enveloped electron transparent structures by TEM was reported as the second step in the search for gas bubbles in yeast.

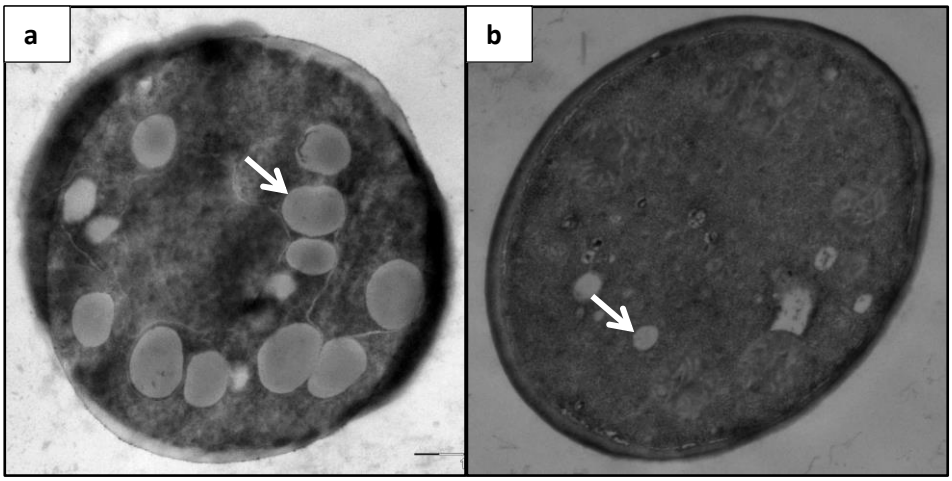


Figure 1.3: a) The two dimensional ultrastructure of *S. pastorianus* cells cultivated in fermentable media. Several non-enveloped electron transparent structures were present in the cells, which indicated that these structures might be gas bubbles. b) The two dimensional ultrastructure of *S. pastorianus* cells cultivated in non-fermentable media. A few small electron transparent structures were present due to CO_2 produced during respiration that occurred in these cells (Swart *et al*., 2012).

Nano Scanning Auger Microscopy (NanoSAM) was used to view the three dimensional (3-D) ultrastructure of the yeast cells in order to confirm the results obtained by LM and TEM (Swart *et al*., 2012). The NanoSAM etches the cell to a depth of 20 nm min^{-1} followed by the use of the NanoSAM Scanning Electron Microscopy (SEM) function to obtain an image of the cell. When etching into the cell, several bubble-like holes were observed in the cells cultivated in fermentable media (Fig. 1.4a), indicating that the structures observed with LM and TEM were indeed gas bubbles. Only a few small bubble like holes were observed in the cells cultivated in non-fermentable media (Fig. 1.4b), which was ascribed to the small amount of CO_2 produced during respiration. The NanoSAM results confirmed the presence of gas bubbles in fermenting *S. pastorianus* cells appearing as empty structures.

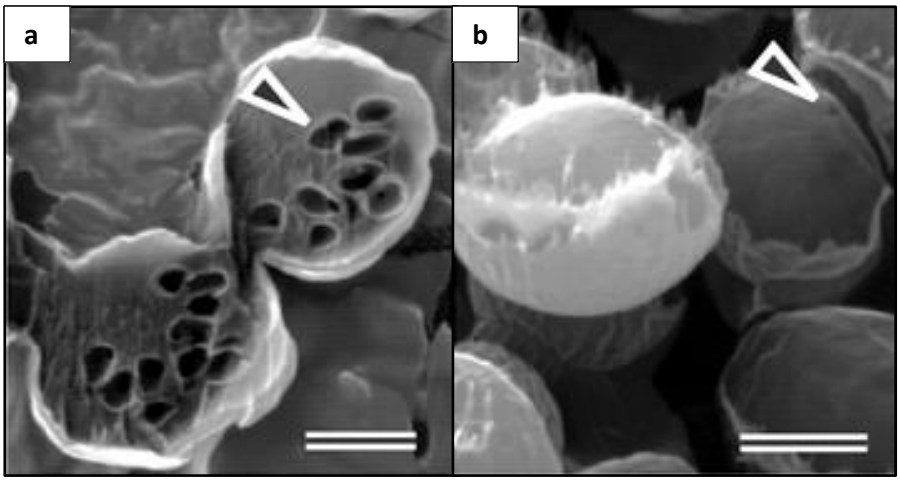


Figure 1.4: a) The three dimensional ultrastructure of *S. pastorianus* cells cultivated in fermentable media. Bubble-like holes were present in the cell, confirming the results obtained by LM and TEM. b) The three dimensional ultrastructure of *S. pastorianus* cells cultivated in non-fermentable media. Only a few small bubbles are present in the image (Swart *et al*., 2012).

The gas bubbles were also traced in the cells with NanoSAM using the metal salt $ZnSO_4.7H_2O$ to verify that these gas bubbles do contain CO_2 (Swart *et al*., 2012). The authors expected these gas bubbles to be surrounded by carbonic acid (H_2CO_3) due to the reaction of CO_2 with H_2O from the cytoplasm of the cell to form H_2CO_3. The addition of the metal salt to the medium caused the gas bubbles to appear 'galvanized' due to the zinc reacting with carbonic acid surrounding the gas bubbles (Fig. 1.5). Swart and co-workers also used Auger-architectomics to map the three-dimensional structure of the cell and to determine the element composition of the gas bubbles (Swart *et al*., 2013). They found a C:O ratio of 1:2, which indicated the structures most likely contained CO_2 thus confirmed as gas bubbles.

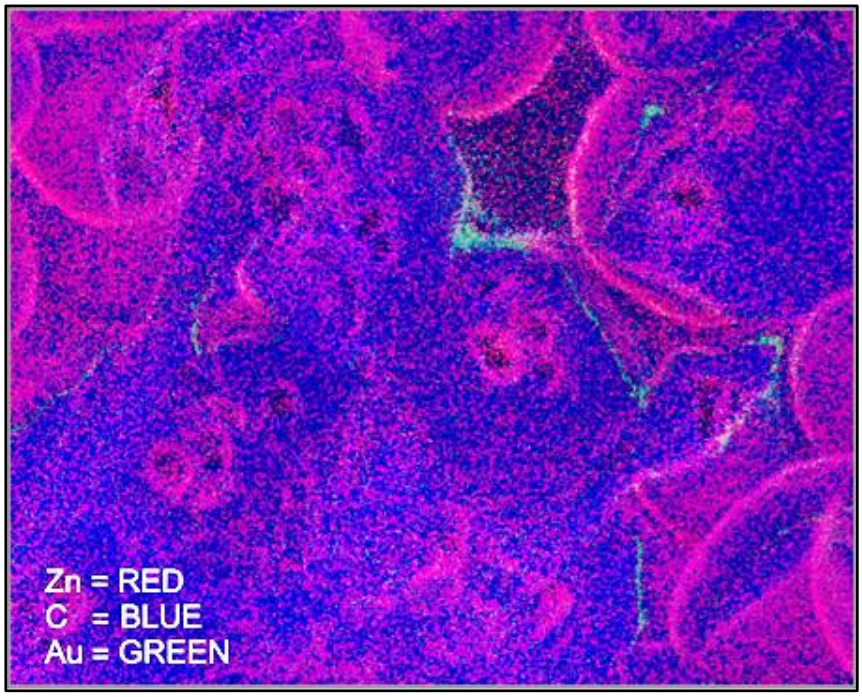


Figure 1.5: NanoSAM image of the element analysis of *S. pastorianus* cultivated in the presence of $ZnSO_{4.7}H_2O$. The metal salt reacted with carbonic acid surrounding the gas bubble, causing the bubbles to appear "galvanized' (Swart *et al*., 2012).

These gas bubbles have also been shown to cause indentations in cell organelles when the bubbles and the membrane of the cell organelles are in contact (Swart *et al*., 2013). The authors concluded that the indentations were due to direct and indirect compression exerted by the gas bubbles on the cell organelles.

The discovery of gas bubbles in yeast cells formed during fermentation in *S. cerevisiae* and *S. pastorianus* leads to the question whether these gas bubbles are present in all fermenting yeast cells. Several projects were dedicated to explore the hypothesis that gas bubbles are present in all fermenting cells. Since 2012, gas bubbles have been found in several organisms during fermentations at 30°C (Table 1.1). These gas bubbles were present during fermentation and absent or present in low numbers during respiration, similar to what was observed in *S. cerevisiae* and *S. pastorianus.*

Table 1.1: Organisms tested for gas bubble formation with their strain numbers. All of these experiments were conducted at 30°C.

Organism	Strain number	Property
Saccharomyces cerevisiae	CBS-1171 (Swart *et al*., 2012)	
	UOFS Y-2169 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
Saccharomyces pastorianus	WS 34-70 (Swart *et al*., 2012)	
	UOFS Y-1494 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
	UOFS Y-1496 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
Schizosaccharomyces pombe	UOFS Y-2714 (Kgotle, unpublished)	Distantly related to *S. cerevisiae*, fission yeast
Rhizopus oryzae	UOFS Y-2807 (Saaiman, unpublished)	Fermenting fungus
Torulaspora globosa	UOFS Y-0847 (Kgotle, unpublished)	Crabtree positive
Zygosaccharomyces bailli	UOFS Y-1535 (Kgotle, unpublished)	Crabtree positive
Kluyveromyces marxianus	UOFS Y-1191 (Kgotle, unpublished)	Crabtree negative
Debaryomyces hansenii	UOFS Y-219 (Kgotle, unpublished)	Crabtree negative
Lipomyces starkeyi	UOFS Y-1999 (Kgotle, unpublished)	Strictly respire
Pichia membranifaciens	UOFS Y-823 (Kgotle, unpublished)	Strictly respire
Yarrowia lipolytica	UOFS Y-1138 (Kgotle, unpublished)	Strictly respire
Rhodotorula glutinis	UOFS Y-0519 (Kgotle, unpublished)	Strictly respire
Saccharomyces bayanus var. *uvarum*	UOFS Y-1480 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
	UOFS Y-1484 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
	UOFS Y-1485 (du Plooy, unpublished, 2015)	Commercial strain of the genus *Saccharomyces*

	UOFS Y-1487 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
	UOFS Y-1495 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
	UOFS Y-1521 (du Plooy, unpublished)	Non-commercial strain of the genus *Saccharomyces*
	UOFS Y-1632 (du Plooy, unpublished)	Commercial strain of the genus *Saccharomyces*
Saccharomyces kudriavzevii	UOFS Y-2567 (du Plooy, unpublished)	Non-commercial strain of the genus *Saccharomyces*
Saccharomyces mikatae	UOFS Y-2569 (du Plooy, unpublished)	Non-commercial strain of the genus *Saccharomyces*
Saccharomyces paradoxus	UOFS Y-1687 (du Plooy, unpublished)	Part of the genus *Saccharomyces*

1.3 Cryosphere

A large area of the world (Antarctica, Arctic area, mountain regions, deep seas, polar region, alpine, marine water, fresh water, cold deserts, glacial habitats as well as polar and alpine soils) is exposed to low temperatures (Feller, 2003; Hamid *et al*., 2014; Margesin & Miteva, 2011; Raspor & Zupan, 2006; Tsuji *et al*., 2014) and most ecosystems, about 85% of the biosphere, are permanently or periodically exposed to temperatures below 5°C (Hamid *et al*., 2014; Margesin & Miteva, 2011; Margesin *et al*., 2007; Raspor & Zupan, 2006; Tsuji *et al*., 2013a). Thirty-five percent of the Earth's land mass is permanently or periodically covered in snow (Margesin & Miteva, 2011), 24% is covered in permafrost, 10% consist of glaciers and 13% of the Earth's surface is covered in sea ice (Margesin & Miteva, 2011). Antarctica is considered the driest and coldest terrestrial area on Earth due to 98-99% of this continent being covered in snow and ice (Buzzini *et al*., 2012; Hamid *et al*., 2014; Raspor & Zupan, 2006). The temperature in the coastal regions of Antarctica range from 5°C to -35°C and the temperature on the plateau range from -25°C in the summer to -70°C in the winter (Buzzini *et al*., 2012; Tsuji *et al*., 2013a).

These cold environments contain a wide variety of psychrophilic microorganisms including bacteria, archaea, filamentous fungi, yeasts and algae (D'Amigo *et al*., 2006; Margesin &

Miteva, 2011; Margesin *et al*., 2007). Recent reports mostly focus on bacteria and archaea while little research has been done on psychrophilic yeasts (Hamid *et al*., 2014).

1.4 Psychrophilic yeasts

Psychrophiles are cold loving microorganisms (Hamid *et al*., 2014; Vishniac, 2006) and can be divided in two groups: Obligate psychrophiles and facultative psychrophiles (Buzzini *et al*., 2012; Hamid *et al*., 2014; Turchetti *et al*., 2008). Obligate psychrophiles have an optimum growth temperature of 15°C, a maximum growth temperature of 20°C or lower and a minimum growth temperature of 0°C or lower (Buzzini *et al*., 2012; Gerday *et al*., 1997; Hamid *et al*., 2014; Margesin *et al*., 2007; Martinez *et al*., 2016; Raspor & Zupan, 2006; Turchetti *et al*., 2008). Facultative psychrophiles/psychrotolerant organisms have a minimum growth temperature of 0°C or lower and a maximum growth temperature of 20°C-30°C (Buzzini *et al*., 2012; Gerday *et al*., 1997; Hamid *et al*., 2014; Raspor & Zupan, 2006; Turchetti *et al*., 2008).

All life on Earth requires liquid water and the freezing point of cellular water limits the lowest temperature at which life is expected to thrive (Feller & Gerday, 1997; Margesin *et al*., 2007). At low temperatures, ice formation occurs, which limits the amount of water present for biological uses, therefore low water activity due to low temperatures is an important factor which controls growth of microorganisms at low temperatures (Raspor & Zupan, 2006). Although the freezing point of pure water is 0°C, the temperature permitting life can decrease below 0°C due to the presence of sodium chloride and small solutes in the cell (Feller & Gerday, 1997; Margesin *et al*., 2007). The current low temperature limit for psychrophilic reproduction is -12°C and for metabolism is -20°C (D'Amigo *et al*., 2006; Margesin & Miteva, 2011; Margesin *et al*., 2007).

The first report of yeasts isolated from Antarctica was made by Di Menna in 1960 (Di Menna, 1960). Since 1960, seven hundred fungal species (Tsuji *et al*., 2013a) and 70 yeast species have been isolated from this continent (Buzzini *et al*., 2012). In the Arctic area, 46 yeast species have been isolated and 41 yeast species have been isolated from European glaciers: the Alps, Pyrenees and Apennines. Forty-one yeast species have also been isolated from Patagonia, Argentina and some psychrophilic yeasts have also been isolated from the Asian cryosphere.

The most dominant psychrophilic yeasts isolated from these cold environments are *Mrakia*, *Cryptococcus*, *Candida* and *Rhodotorula* species (Buzzini *et al*., 2012; Hamid *et al*., 2014).

Di Menna (1996) found the yeast abundance in Antarctica to be between 5-10^5 CFU g^{-1} and reported that *Mrakia* species to be the dominant yeast in the soil of Antarctica, representing 24% of the yeast species isolated in the study. Other research showed that 35% of culturable fungi isolated from lake sediment and soil from East Antarctica were *Mrakia* species and stated that they are the dominant culturable fungi in East Antarctica (Tsuji *et al*., 2013a; 2014). Eight species of *Mrakia* are currently recognized: *Mrakia frigida*, *M. gelida*, *M. stokesii*, *M. nivalis*, *M. curviuscula*, *M. psychrophila*, *M. robertii* and *M. blollopis* (Tsuji *et al*., 2013b; 2014). Seven of the 8 species are able to ferment glucose and sucrose, with *Mrakia curviuscula* lacking this ability (Bab'eva *et al*., 2002; Thomas-Hall *et al*., 2010; Tsuji *et al*., 2013b; 2014). All the species from the genus *Mrakia* have a maximum growth temperature of 20°C or lower except *Mrakia curviuscula*. This suggests that *Mrakia curviuscula* may not belong to the *Mrakia* genus (Thomas-Hall *et al*., 2010).

Twenty-five percent of the yeasts isolated from Antarctica, 33% of the yeasts isolated from the Arctic, 39% of the yeasts isolated from European glaciers and 33% of the yeasts isolated from Argentina consist of *Cryptococcus* species (Buzzini *et al*., 2012). *Candida* species are also commonly found in Antarctica, but their presence is not as dominant as *Cryptococcus* species (Shivaji & Prasad, 2009).

1.5 Adaptions to low temperature

Microorganisms isolated from cold environments have mainly been studied to investigate their ability to survive these conditions (Turchetti *et al*., 2008). Temperature has a major influence on microorganisms such as: increase in the viscosity of the medium, reduced growth rates, reduced membrane fluidity, decreased nutrient availability, altered protein conformation, reduced enzymatic reaction rates and decreased water activity (Buzzini *et al*., 2012; Margesin & Miteva, 2011; Margesin *et al*., 2007; Padtare & Inouye, 2008). To combat this, these microorganisms have adapted in several ways.

They have increased membrane fluidity at low temperatures by changing the composition of the fatty acids (Buzzini *et al*., 2012; D'Amigo *et al*., 2006; Margesin & Miteva, 2011; Rossi *et al*., 2009). There is a correlation between the increase in fatty acid unsaturation and the decrease in growth temperature in psychrophilic yeasts (Raspor & Zupan, 2006). At low temperatures, these organisms show an increase in fatty acid unsaturation which allows for continuous membrane fluidity even at temperatures lower than 0°C (Vishniac, 2006). At extremely low temperatures, the percentage unsaturated fatty acids increase from 50% to 90% in the membranes. There is also a decrease in the fatty acid chain length and a decrease in sterol/phospholipid ratio (D'Amigo *et al*., 2006; Margesin *et al*., 2007; Margesin & Miteva, 2011). At low temperatures, linoleic and linolenic acids are the dominant fatty acids in *Mrakia*, *Leucosporidium* and *Rhodotorula* species, and at higher temperatures closer to the maximum growth temperature, oleic and linoleic acids are the dominant fatty acids in these organisms (Buzzini *et al*., 2012; Rossi *et al*., 2009).

Another adaption is the synthesis of protecting proteins as a response to thermal stress (Buzzini *et al*., 2012). When the environmental temperature changes rapidly, Prokaryotic and Eukaryotic organisms have cold shock responses and produce cold shock proteins to combat the harmful effects of cold shock on the cell (Buzzini *et al*., 2012; D'Amigo *et al*., 2006; Gerday *et al*., 1997; Padtare & Inouye, 2008). The cold shock response in psychrophiles differ in two ways from the cold shock response in mesophiles: 1) housekeeping proteins are still produced during cold shock and 2) the number of cold shock proteins increase as the cold shock increases (Margesin & Miteva, 2011). Cold shock proteins have been observed in mesophilic yeasts such as *Saccharomyces cerevisiae* since the 1990's, but only a small number of reports are available on the production of cold-shock proteins by psychrophilic yeasts (D'Amigo *et al*., 2006; Padtare & Inouye, 2008). Psychrophilic yeasts also produce heat shock proteins to protect the cell from temperature increases (Buzzini *et al*., 2012; D'Amigo *et al*., 2006; Deegenaars & Watson, 1997).

Some psychrophilic yeasts secrete glycosylated ice-binding proteins which are also called antifreeze proteins (Buzzini *et al.,* 2012; D'Amigo *et al*., 2006; Kawahara, 2008; Lee *et al*., 2010). These antifreeze proteins act as cryoprotectant molecules by exhibiting 1) thermal hysteresis activity which lowers the freezing point of the water without changing the melting point and 2) recrystallization inhibition activity where these proteins prevent the formation

of large ice crystals (Gerday *et al*., 1997; Lee *et al*., 2010; Margesin & Miteva, 2011). Psychrophilic yeasts also synthesize cryoprotectant molecules to reduce ice crystals present in the cytoplasm and to prevent damage occurring to the cytoplasmic membrane due to a low freezing rate (Lee *et al*., 2010). In the natural habitats of these yeasts, the freezing rate is low therefore these macromolecules are very important to prevent cell damage (Buzzini *et al*., 2012). One of these cryoprotectant macromolecules is trehalose (D'Amigo *et al*., 2006; Kawahara, 2008; Padtare & Inouye, 2008). The trehalose biosynthesis pathway is widespread in nature and also protects the cell against osmotic stress. In *Mrakia frigida* a high concentration of trehalose was observed in the cytoplasm, possibly as a strategy to decrease the freezing point of the cytoplasm.

Psychrophilic yeasts undergo metabolic changes due to low temperatures (Buzzini *et al*., 2012; Xin & Zhou, 2000) where metabolic enzymes have high activity and weak thermal stability (Buzzini *et al*., 2012). In mesophilic yeasts transcription and translation enzymes' activity and protein folding activity decrease at low temperatures (D'Amigo *et al*., 2006). In psychrophilic yeasts enzymes active in these processes are optimized to work at low temperatures. Frozen basidiomycetous yeasts isolated from Antarctica showed an active metabolism, however the temperature influences the growth rate of the yeasts as metabolic activity is directed at survival and maintenance, not growth (Amato *et al*., 2009; Rossi *et al*., 2009). These cells might also be capable of repairing macromolecular cell damage caused by being trapped in ice for long periods (Amato *et al*., 2009).

Syntheses of cold active enzymes by psychrophiles are the most studied adaption of psychrophilic yeasts (Buzzini *et al*., 2012). These enzymes have the ability to be up to 10 times more active at low temperatures than their mesophilic counterparts (D'Amigo *et al*., 2006; Margesin & Miteva, 2011; Struvay & Feller, 2012). Cold active enzymes function at low and moderate temperatures with a high specific activity and have increased catalytic activity at lower temperatures which is based on their improved structural flexibility which lowers the activation energy of the enzymes (D'Amigo *et al*., 2006; Gerday *et al*., 1997; Hamid *et al*., 2014; Margesin *et al*., 2007; Shivaji & Prasad, 2009; Struvay & Feller, 2012). The increased flexibility of these enzymes leads to their weak thermal stability and cause them to be easily heat inactivated (Feller, 2012; Feller & Gerday, 1997; Margesin *et al*., 2007; Struvay & Feller,

2012). These properties make cold active enzymes industrially important as will be discussed in the next section.

At low temperatures the solubility of gasses and therefore the concentration of reactive oxygen species (ROS) increase significantly (D'Amigo *et al*., 2006; De Maayer *et al*., 2014). For the survival of microorganisms at low temperatures they must be protected against ROS (D'Amigo *et al*., 2006; De Maayer *et al*., 2014; Margesin & Miteva, 2011). To combat the effect of ROS on the cell, certain psychrophiles have enhanced antioxidant capacity and several genes encode for catalases and superoxide dismutases.

1.6 Biotechnological applications

Microorganisms adapted to cold environments have major biotechnological potential (Raspor & Zupan, 2006). Although literature on cold adapted yeasts is limited (Birgisson *et al.,* 2003; Tsuji *et al*., 2014), the research showed that these yeasts have applications in the health, agriculture, detergent, medical, pharmaceutical, food, textile, domestic and fine chemical synthesis industries (Hamid *et al*., 2014; Tsuji *et al*., 2013a; 2014). They have advantages over mesophilic microorganisms, including ecological and economic advantages (Hamid *et al*., 2014; Margesin *et al*., 2005). By example biotechnological applications of psychrophilic yeasts include low temperature fermentations, the production of cold active enzymes, cold-shock proteins, cold-acclimation proteins and pigments (Hamid *et al*., 2014; Shivaji & Prasad, 2009; Thomas-Hall *et al*., 2010).

Cold active enzymes

Cold active enzymes have major potential in the biotechnological industry (Buzzini & Margesin, 2013; Buzzini *et al*., 2012; Hamid *et al*., 2014; Raspor & Zupan, 2006) due to their high activity at low temperatures, easy inactivation at moderate temperatures, their ability to survive unfavorable industrial conditions, the fact that they can be used to transform heat-sensitive products and through energy savings in biocatalysis (Buzzini *et al*., 2012; Gerday *et al*., 1997; Hamid *et al*., 2014; Hua *et al*., 2010; Raspor & Zupan, 2006). Processes catalysed by cold active enzymes have two economic advantages: 1) the process is protected from contamination and 2) the process saves energy (Birgisson *et al*., 2003; Hamid *et al*., 2014;

Margesin *et al*., 2007; Nakagawa *et al*., 2004; Xin & Zhou, 2007). Four thousand enzymes produced by psychrophilic yeasts are known, from which approximately 200 enzymes are used industrially (Hamid *et al*., 2014). These enzymes are more stable than similar enzymes produced in bacteria, plants and animals and the production is easier and safer than the alternative due to the smaller production scale compared to plants and animals and the enzymes can be produced with no risk of infection to employees (Gerday *et al*., 1997; Hamid *et al*., 2014). Psychrophilic yeasts are screened for the production of the following cold active enzymes for industrial use: amylase, beta-glucosidases, cellulase, glycoamylases, inulase, invertase, lipase, pectinase, phytase, protease and xylanase (Buzzini & Margesin, 2013; Hamid *et al*., 2014; Hua *et al*., 2010; Martinez *et al*., 2016).

Lipases are hydrolytic enzymes which catalyse the hydrolysis of long chain fatty acids (Hamid *et al*., 2014). Lipases produced by psychrophilic yeasts are more desirable since these enzymes can function at lower temperatures and are more stable, safer and easier to produce than similar lipases produced by plants, animals, bacteria or fungi (Hamid *et al*., 2014). Cold active lipases have biotechnological applications in the following industries: medical, pharmaceutical, fine chemical synthesis, domestic, food as well as in the environment (Gerday *et al*., 1997; Hamid *et al*., 2014; Struvay & Feller, 2012). *Mrakiella aquatica* produces cold active lipase A and lipase B (Hamid *et al*., 2014; Struvay & Feller, 2012; Xin & Zhou, 2007). Lipase A has very high thermal stability when compared to other lipases as it is active at temperatures higher than 90°C (De Maria *et al*., 2005). Lipase B is a patented enzyme and is used to produce optically active alcohols (Hamid *et al*., 2014).

Beta-galactosidase is also a hydrolytic enzyme which hydrolyses lactose into glucose and galactose (Struvay & Feller, 2012). Cold active β-galactosidase has the potential to break down lactose in milk at low temperature, where commercially available β-galactosidase requires the milk to be heated to become active, thus reducing the milk quality, taste, texture and requiring energy.

Cellulases are hydrolytic enzymes hydrolyzing the β-1,4 glucosidic bonds in cellulose, the renewable and polymeric compound in plants (Caf *et al*., 2014; Carrasco *et al*., 2016). Cellulases are used in the biofuel, textile, animal feed and food industry for the breakdown of cellulose. Cold active cellulases have the potential to be used industrially since the cellulases

currently used in the industry have an optimum temperature of 50°C, thus using a large amount of energy and producing undesirable byproducts.

Xylanases are also hydrolytic enzymes which hydrolyze β-1,4-xylan, breaking down hemicellulose in plant cell walls (Struvay & Feller, 2012). Cold active xylanases might have an application in waste digestion of sewage, agricultural and industrial wastes (Shivaji & Prasad, 2009). Xylanases are also used in the bread industry to improve the bread quality (Struvay & Feller, 2012) and may be used industrially to save energy in the bread making process.

Pectic substances are commonly found in the plant kingdom and pectinolytic enzymes or pectinases are enzymes targeting these pectic substances by hydrolyzing the glucosidic bonds of the substance (Hamid *et al*., 2014; Margesin *et al*., 2005). Pectinases include pectate lyase, polygalacturonase and pectin esterase (Hamid *et al*., 2014). Pectate lyases are important industrial enzymes which have various biotechnological applications in the textile processing industry, cotton bioscouring industry, in the degumming of plant fibers and in the pretreatment of wastewater originating from fruit and vegetable processing (Hamid *et al*., 2014; Margesin *et al*., 2005). The main source of industrial pectinases is from *Aspergillus niger* as it produces high amounts of pectinases and has GRAS status (Birgisson *et al*., 2003). Cold active pectate lyases are industrially desirable to degrade pectic substances at low temperatures for cost-effective processing (Margesin *et al*., 2005). *Mrakia frigida* produces cold active pectate lyases and have the ability to grow on pectin as the sole carbon source (Hamid *et al*., 2014; Margesin *et al*., 2005; Xin & Zhou, 2007).

Other biotechnological uses

Psychrophilic yeasts also have the ability to degrade a wide range of petroleum hydrocarbons and phenol-related compounds at low temperatures (Buzzini *et al*., 2012). Petroleum hydrocarbons are widespread contaminants of the environment and since petroleum reserves occur in Antarctica and the Arctic and some of these sites are contaminated, there is a need for psychrophilic organisms to treat these contaminated areas (Margesin *et al*., 2007).

It has been suggested that certain psychrophiles produce carotenoid pigments in their cell membrane to combat low temperatures or temperature fluxes by acting as fluidity modulators (Amaretti *et al*., 2014; D'Amigo *et al*., 2006; De Maayer *et al*., 2014). Carotenoid

pigments have major biotechnological applications as vitamin A precursors, pigments, antioxidants and photoprotectants (Amaretti *et al*., 2014; Liu & Wu, 2007; Liu *et al*., 2006; Yang *et al*., 2011). *Xanthophyllomyces dendrorhous* is a psychrophilic yeast which produces a carotenoid pigment called astaxanthin and can be used in the commercial production of astaxanthin using fermentation technology (Liu & Wu, 2007; Liu *et al*., 2006; Reynders *et al*., 1997; Yang *et al*., 2011). Astaxanthin (a carotenoid) is a naturally occurring pigment (orange-red) that is found in birds, crustaceans and salmon and is used in the aquaculture industry as a food pigment (Liu & Wu, 2007; Reynders *et al*., 1997; Yang *et al*., 2011).

Psychrophilic yeasts can also be used in low temperature fermentations (Kourkoutas *et al*., 2002; Xin & Zhou, 2007). However, most of the psychrophilic yeasts are basidiomycetous yeast (Shivaji & Prasad, 2009) and little is known about the fermentation ability of basidiomycetous yeasts (Tsuji *et al*., 2013b; 2014). The use of these yeasts in low temperature fermentation might have desirable commercial applications (Kourkoutas *et al*., 2002). Ethanol fermentation at low temperatures has been used to make wine and sake (Tsuji *et al*., 2014).

1.7 Conclusions

After extensive research on the formation of gas bubbles, Hemmingsen and co-workers concluded that intracellular gas bubbles are not formed in living cells (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al*., 1985; Hemmingsen *et al*., 1990; Ryan & Hemmingsen, 1988). However, in 2012 Swart and co-workers discovered gas bubbles in the fermenting yeast *Saccharomyces*. These gas bubbles were present in *Saccharomyces* during fermentation and only present in small numbers when the yeast was respiring. Swart and co-workers used LM, TEM and NanoSAM to verify that the structures are indeed gas bubbles. These structures did not have an envelope, therefore they concluded that these structures are not cell organelles. Several different projects have been dedicated to search for gas bubbles in different organisms to prove the hypothesis that gas bubbles are present in all fermenting organisms. Up to date, gas bubbles have been found in 16 organisms (unpublished) during fermentation and the gas bubbles were absent or present in small numbers during respiration. Different parameters have been explored in the search for gas bubbles, but since the gas bubbles have only been found in experiments conducted at 30°C.

There is a need to explore gas bubble formation during fermentations at lower temperatures to determine the conserved status of gas bubble formation at lower temperatures. Several psychrophilic yeasts have been isolated from cold environments including Antarctica, the Arctic Circle, Asia and Argentina (Buzzini *et al*., 2012). The dominant yeasts isolated in these areas are *Cryptococcus*, *Mrakia*, *Candida* and *Rhodotorula* species (Buzzini *et al*., 2012; Hamid *et al*., 2014). Eight *Mrakia* species have been isolated from these cold environments and 7 of the 8 species have the ability to ferment glucose (Bab'eva *et al*., 2002; Thomas-Hall *et al*., 2010; Tsuji *et al*., 2013b; 2014). However, not much is known about the fermentation abilities of psychrophilic yeasts (Tsuji *et al*., 2013b; 2014). Most of the research done on psychrophilic yeasts are on the cold active enzymes they produce, the use of cold active enzymes in the industry and how psychrophilic yeasts are adapted to cold environments. The above information lead to several questions: What are the fermentation abilities of psychrophilic yeasts? Are gas bubbles produced in psychrophilic yeasts during low temperature fermentations?

1.8 Purpose of research

The information in the literature review on gas bubble formation and psychrophilic yeast lead to the purpose of this study which is as follows:

- To determine the fermentation abilities of psychrophilic yeasts and the different carbon sources these yeasts are able to ferment.
- To determine the conserved status of gas bubble formation in psychrophilic yeast at low temperature fermentations through light and electron microscopy.

1.9 References

Amaretti, A., Simone, M., Quartieri, A., Masino, F., Raimondi, S., Leonardi, A & Rossi, M. (2014). Isolation of Carotenoid-producing Yeasts from an Alpine Glacier. *Chem Eng Trans* **38**, 217-222.

Amato, P., Doyle, S. & Christner, B. C. (2009). Macromolecular synthesis by yeast under frozen conditions. *Environ Microbiol* **11**, 589-596.

Bab'eva, I. P., Lisichkina, G. A., Reshetova, I. S. & Danilevich, V. N. (2002). *Mrakia curviuscula* sp. nov.: A new psychrophilic yeast from forest substrates. *Microbiol* **71(4)**, 9-454.

Birgisson, H., Delgado, O., Arroyo, L. G., Hatti-Kaul, R. & Mattiasson, B. (2003). Cold-adapted yeasts as producers of cold active polygalacturonases. *Extremophiles* **7**, 185-193.

Buzzini, P., Branda, E., Goretti, M. & Turchetti, B. (2012). Psychrophilic yeasts from worldwide glacial habitats: diversity, adaption strategies and biotechnological potential. *FEMS Microbiol Ecol* **82**, 217-241.

Buzzini, P. & Margesin, R. (2013). Cold adapted yeasts: A Lesson from the Cold and a Challenge for the XXI Century. *Cold adapted Yeasts: Biodiversity, Adaption strategies and Biotechnological significance.* (Buzzini, P. & Margesin, R. eds). pp 3-22. Springer-Verlag, Berlin.

Caf, Y., Valipour, E. & Arikan, B. (2014). Study on Cold-Active and Acidophilic Cellulase (CMCase) from a novel psychrotrophic isolate *Bacillus sp*. K-11. *Int J Curr Microbiol Appl Sci* **3(5)**, 16-25.

Carrasco, M., Villarreal, P., Barahona, S., Alcaino, J., Cifuentes, V. & Baeza, M. (2016). Screening and characterization of amylase and cellulose activities in psychrotolerant yeasts. *BMC Microbiol* **16:21** DOI 10.1186/s12866-016-0640-8.

D'Amigo, S., Collins, T., Marx, J-C., Feller, G. & Gerday, C. (2006). Psychrophilic microorganisms: challenges for life. *EMBO Reports* **7(4)**, 385-389.

De Maayer, P., Anderson, D., Cary, C. & Cowan, D. A. (2014). Some like it cold: understanding the survival strategies of psychrophiles. *EMBO Reports* **15(5)**, 508-517.

De Maria, P. D., Carboni-Oerlemans, C., Tuin, B., Bargeman, G., van der Meer, A. B. & van Gemert, R. (2005). Biotechnological applications of *Candida Antarctica* lipase A: state-of-the-art. *J Mol Catal B Enzym* **37**, 36-46.

Deegenaars, M. L. & Watson, K (1997). Stress proteins and stress tolerance in an Antarctic, psychrophilic yeast, *Candida psychrophila*. *FEMS Microbiol Lett* **151**, 191-196.

Doerder, P. & Brunk, C. (2012). Natural Populations in Inbred Strains of Tetrahymena. Methods Cell Biology **109**, 277-300.

Di Menna, M. E. (1960). Yeasts from Antarctica. *J Gen Microbiol* **23**, 295-300.

Di Menna, M. E. (1966). Yeasts in Antarctic soil. *Antonie Leeuwenhoek* **32**, 29-38.

Feller, G. (2003). Molecular adaptions to cold in psychrophilic enzymes. *Cell Mol Life Sci* **60**, 648-662.

Feller, G. (2012). Psychrophilic enzymes: From Folding to Function and Biotechnology. *Scientifica* 2013, ID 512840.

Feller, G. & Gerday, C. (1997). Psychrophilic enzymes: molecular basis of cold adaption. *Cell Mol Life Sci* **53**, 830-841.

Gerday, C., Aittaleb, M., Arpigny, J. L., Baise, E., Chessa, J. P., Garsoux, G., Petrescu, I. & Feller, G. (1997). Psychrophilic enzymes: a thermodynamic challenge. *Biochim Biophys Acta* **1242**, 119-131.

Fugelsang, K. C. (2007). Wine Microbiology: Practical applications and procedures. Chapter 8: Fermentation and post-fermentation processing. Springer, New York, NY.

Hamid, B., Rana, R. S., Chauhan, D., Singh, P., Mohiddin, F. A., Sahay, S. & Abidi, I. (2014). Psychrophilic yeasts and their biotechnological applications – A review. *Afr J Biotechnol* **13(22)**, 2188-2197.

Hemmingsen, B. B., Ducoeur, L. C., Grapp, S. J., Skaug, V. & Hemmingsen, E. A. (1990). Gas supersaturation tolerance in amoeboid cells before and after ingestion of bubble-promoting particles. *Cell Biophys* **17**, 37-51.

Hemmingsen, B. B. & Hemmingsen, E. A. (1983). Intracellular Bubble Formation: Differences in Gas Supersaturation Tolerances Between Tetrahymena and Euglena1. *J Eukaryot Microbiol* **30**, 608-612.

Hemmingsen, B. B., Steinberg, N. A. & Hemmingsen, E. A. (1985). Intracellular gas supersaturation tolerance of erythrocytes and resealed ghosts. *Biophys J* **47**, 491-496.

Hemmingsen, E. A. & Hemmingsen, B. B. (1979). Lack of intracellular bubble formation in microorganisms at very high gas supersaturations. *J Appl Physiol Respir Environ Exerc Physiol* **47**, 1270-1277.

Hua, M-X., Chi, Z., Liu, G-L., Budzar, M. A. & Chi, Z-M. (2010). Production of a novel and cold-active killer toxin by *Mrakia frigida* 2E00797 isolated from sea sediment in Antarctica. *Extremophiles* **14**, 515-521.

Kawahara, H. (2008). Cryoprotectants and ice-binding proteins. *Psychrophiles: From Biodiversity to Biotechnology* pp. 229-246. Springer-Verlag, Berlin.

Klebahn, H. (1895). Gasvakuolen, ein Bestandteil der Zellen der Wasserblütebildenden Phycochromaceen. *Flora (Jena)* **80**, 241.

Kourkoutas, Y., Koutinas, A. A., Kanellaki, M., Banat, I. M., Marchant, R. (2002). Continuous wine fermentation using a psychrophilic yeast immobilized on apple cuts at different temperatures. *Food Microbiol* **19**, 127-134.

Lee. J. K., Park, K. S., Park, S., Park, H., Song, Y. H., Kang, S. H. & Kim, H. J. (2010). An extracellular ice-binding glycoprotein from an Arctic psychrophilic yeast. *Cryobiology* **60**, 222-228.

Liu, Y-S. & Wu, J-Y. (2007). Optimization of cell growth and carotenoid production of *Xanthophyllomyces dendrorhous* through statistical experiment design. *Biochem Eng J* **36**, 182-189.

Liu, Y-S., Wu, J-Y. & Ho, K-p. (2006). Characterization of oxygen transfer conditions and their effects on *Phaffia rhodozyma* growth and carotenoid production in shake flask cultures. *Biochem Eng J* **27**, 331-335.

Margesin, R., Fauster, V. & Fonteyne, P-A. (2005). Characterization of cold active pectate lyases from psychrophilic *Mrakia frigida*. *Lett Appl Microbiol* **40**, 453-459.

Margesin, R. & Miteva, V. (2011). Diversity and ecology of psychrophilic microorganisms. *Res Microbiol* **162**, 346-361

Margesin, R., Neuner, G. & Storey, K. B. (2007). Cold-loving microbes, plants, and animals-fundamental and applied aspects. *Naturwissenschaften* 94, 77-99.

Martinez, A., Cavello, I, Garmendia, G., Rufo, C., Cavalitto, S. & Vero, S. (2016). Yeasts from sub-Antarctic region: biodiversity, enzymatic activities and their potential as oleaginous microorganisms. *Extremophiles* **20**, 759-769.

Nakagawa, T., Nagaoka, T., Taniguchi, S., Miyaji, T. & Tomizuka, N. (2004). Isolation and characterization of psychrophilic yeasts producing cold adapted pectinolytic enzymes. *Lett Appl Microbiol* **38**, 383-387.

Padtare, S. & Inouye, M. (2008). Cold-shock proteins. *Psychrophiles: From Biodiversity to Biotechnology* pp 191-209. Springer-Verlag, Berlin.

Pfeiffer, T. & Morley, A. (2014). An evolutionary perspective on the crabtree effect. *Front Mol Biosci* **1**, 17 DOI:10.3389/fmolb.2014.00017.

Pronk, J. T., Steensma, H. Y. & Van Dijken, J. P. (1996). Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**, 1607-1633.

Raspor, P. & Zupan, J. (2006). Yeasts in extreme environments. In: Rosa, C. A. & Péter, G. (Editors). Biodiversity and Ecophysiology of Yeasts. Springer, Berlin, pp. 371-417.

Reynders, M. B., Rawlings, D. E. & Harrison, S. T. L. (1997). Demonstration of the crabtree effect in *Phaffia rhodozyma* during continuous and fed-batch cultivation. *Biotechnol Lett* **19(6)**, 549-552.

Rossi, M., Buzzini, P., Cordisco, L., Amaretti, A., Sala, M., Raimondi, S., Ponzoni, C., Pagnoni, U. M., & Matteuzzi, D. (2009). Growth, lipid accumulation, and fatty acids composition in obligate psychrophilic, facultative psychrophilic and mesophilic yeasts. *FEMS Microbiol Ecol* **69**, 363-372.

Ryan, W. L. & Hemmingsen, E. A. (1988). Gas supersaturation thresholds for bubble formation in and damage to sea urchin embryos. *Biol Bull* **174**, 83-89.

Shivaji, S. & Prasad, G. S. (2009). Antarctic yeasts: biodiversity and potential applications. *Yeast Biotechnology: Diversity and Applications* (Satyanarayana, T. & Kunze, G., eds) pp 3-16. Springer-Verlag, Berlin.

Struvay, C. & Feller, G. (2012). Optimization to Low Temperature Activity in Psychrophilic Enzymes. *Int J Mol Sci* **13**, 11643-11665.

Swart, C. W., Dithebe, K., Pohl, C. H., Swart, H. C., Coetsee, E., van Wyk, P. W. J., Swarts, J. C., Lodolo, E. J. & Kock, J. L. F. (2012). Gas bubble formation in the cytoplasm of a fermenting yeast. *FEMS Yeast Res* **12**, 867-869.

Swart, C. W., Dithebe, K., van Wyk, P. W. J., Pohl, C. H., Swart, H. C., Coetsee, E., Lodolo, E. & Kock, J. L. F. (2013). Intracellular gas bubbles deform organelles in fermenting brewing yeasts. *J Inst Brew* **119**, 15-16.

Thomas-Hall, S. R., Turchetti, B., Buzzini, P., Branda, E., Boekhout, T., Theelen, B. & Watson, K. (2010). Cold-adapted yeasts from Antarctica and the Italian Alps-description of three novel species: *Mrakia robertii* sp. nov., *Mrakia blollopis* sp. nov. and *Mrakiella niccombsii* sp. nov. *Extremophiles* **14**, 47-59.

Tsuji, M., Fujiu, S., Xiao, N., Hanada, Y., Kudoh, S., Kondo, H., Tsuda, S. & Hoshino, T. (2013a). Cold adaption of fungi obtained from soil and lake sediment in the Skarvsnes ice-free are, Antarctica. *FEMS Microbiol Lett* **346**, 121-130.

Tsuji, M., Goshima, T., Matsushika, A., Kudoh, S. & Hoshino, T. (2013b). Direct ethanol fermentation from lignocellulosic biomass by Antarctic basidiomycetous yeast *Mrakia blollopis* under low temperature condition. *Cryobiology* **67**, 241-243.

Tsuji, M., Yokota, Y., Kudoh, S. & Hoshino, T. (2014). Improvement of direct ethanol fermentation from woody biomasses by the Antarctic basidiomycetous yeast, *Mrakia blollopis*, under a low temperature condition. *Cryobiology* **68**, 303-305.

Turchetti, B., Buzzini, P., Goretti, M., Branda, E., Diolaiuti, G., Agata, C. D., Smiraglia, C. & Vaughan-Martini, A. (2008). Psychrophilic yeasts in glacial environments of Alpine glaciers. *FEMS Microbiol Ecol* **63**, 73-83.

Van Maris, A. J. A., Bakker, B. M., Brandt, M., Boorsma, A., Teixeira de Mattos, M. J., Grivell, L. A., Pronk, J. T., Blom, J. (2001). Modulating the distribution of fluxes among respiration and fermentation by overexpression of *HAP4* in *Saccharomyces cerevisiae*. *FEMS Yeast Res* **1**, 139-149.

Vishniac, H. S. (2006). Yeast biodiversity in the Antarctic. *The Yeast Handbook. Biodiversity and Ecophysiology of Yeasts* pp 419-440. Springer-Verlag, Berlin.

Visser, W., Scheffers, W. A., Batenburg-van der Vegte, W. H. & van Dijken, J. P. (1990). Oxygen requirements of yeast. *Appl Environ Microbiol* **56(12)**, 3785-3792.

Walsby, A. E. (1985). The permeability of heterocysts to the gasses nitrogen and oxygen. *Proc R Soc Lond* **226**, 345-366.

Walsby, A. E. (1994). Gas vesicles. *Micriobiol Rev* **58(1)**, 94-144.

Xin, M. & Zhou, P. (2007). *Mrakia psychrophila* sp. nov., a new species isolated from Antarctic soil. *J Zhejiang Univ Sci B* **8(4)**, 260-265.

Yang, J. P. P., Tan, H., Yang, R., Sun, X., Zhai, H. & Li, K. (2011). Astaxanthin production by *Phaffia rhodozyma* fermentation of cassava residues substrate. *Agric Eng Int* **13(2)**, 1-6.

Chapter 2

Fermentation by psychrophilic yeasts

2.1 Abstract

The ability to ferment carbon sources to carbon dioxide (CO_2) and ethanol is widespread amongst yeasts (Pronk *et al*., 1996). In literature, the Durham tube test is used to test for fermentation and relies on the production of gas which is trapped in the Durham tube, indicating a positive result (Barnett *et al*., 2000; Kurtzman & Fell, 1998). This test is not very sensitive and may have false negative results, however there are no easy alternative to test for fermentation (Pronk *et al*., 1996; van Dijken *et al*., 1986). The result of fermentation tests can be indicated as variable when some strains in a species test positive and other negative for gas production. Due to this the fermentation abilities of some yeast are vague when consulting literature (Barnett *et al*., 2000; Kurtzman & Fell, 1998). To expose gas bubbles in psychrophilic yeasts in Chapter 3, the fermentation abilities of individual yeast strains are important, therefore fermentation tests were done to determine the fermentation abilities of each individual strain.

2.2 Introduction

Yeasts can metabolise and/or ferment a variety of sugars, however the range of sugars varies between yeasts (Pronk *et al*., 1996). Wild type yeast strains can all be cultivated on glucose, but not all have the ability to convert glucose to ethanol via alcoholic fermentation (Pronk *et al*., 1996). The Kluyver rule states that an organism that is not able to ferment glucose will not be able to ferment other sugars (van Dijken *et al*., 1986).

It is estimated that two thirds of all yeasts have the ability to perform alcoholic fermentation (Barnett *et al*., 2000; Van Dijken *et al*., 1986). Pronk and co-workers determined that at the time, 37% of the yeasts deposited in the Centraalbureau voor Schimmelcultures (CBS) did not have the ability of alcoholic fermentation (Pronk *et al*., 1996), however some yeasts listed as non-fermentative do contain the key enzyme of alcoholic fermentation, pyruvate decarboxylase (van Dijken *et al*., 1986).

The standard method of determining whether yeasts are able to undergo alcoholic fermentation is by measuring the production of gas in Durham tubes (Pronk *et al*., 1996; Van der Walt, 1970). The Durham test is not very sensitive and can have false negative results,

therefore the amount of yeasts able to ferment might be higher than stated in literature (Pronk *et al*., 1996; van Dijken *et al*., 1986). The absence of gas in the Durham tube might be due to the rate of CO_2 production being lower than the rate of CO_2 diffusion into the medium, since the Durham tube is an open vessel (van Dijken *et al*., 1986). However, there is no easy alternative that can be used to detect fermentation (van Dijken *et al*., 1986).

Temperature affects the length and the rate of alcoholic fermentation (Beltran *et al*., 2007; Lopez-Malo *et al*., 2013). Yeasts used in low temperature fermentations have a long fermentation time since low temperatures increases the lag phase and decreases the growth rate of the yeast (Beltran *et al*., 2007; Blateryon & Sablayrolles, 2000). Psychrophilic yeasts can be used in low temperature fermentations (Kourkoutas *et al*., 2002; Xin & Zhou, 2007), however most psychrophilic yeasts are basidiomycetous yeast (Shivaji & Prasad, 2009) and little is known about the fermentation ability of basidiomycetous yeasts (Tsuji *et al*., 2013; 2014). Psychrophilic yeast able to ferment includes *Mrakia* and *Mrakiella* species. Eight species of *Mrakia* have been reported: *Mrakia frigida*, *M. gelida*, *M. stokesii*, *M. nivalis*, *M. curviuscula*, *M. psychrophila*, *M. robertii* and *M. blollopis* (Tsuji *et al*., 2013; 2014). Seven of the 8 species are able to ferment glucose, with *Mrakia curviuscula* lacking this ability (Bab'eva *et al*., 2002; Thomas-Hall *et al*., 2010; Tsuji *et al*., 2013; 2014). *Saccharomyces bayanus* is a facultative psychrophile used industrially for the production of wine at low temperatures (Lopez-Malo *et al*., 2013) and *Xanthophyllomyces dendrorhous* is a Crabtree positive facultative psychrophilic yeast which can be used industrially for the production of a carotenoid pigment, astaxanthin, via fermentation (Liu & Wu, 2007; Liu *et al*., 2006; Reynders *et al*., 1997; Yang *et al*., 2011).

Traditionally, fermentation tests are done using 2% (w/v) sugar, the fermentation process is monitored frequently and the results recorded as positive (+), negative (-), variable (v), slow positive (s), delayed positive (D), weak positive (w), latent (l) or unknown result (?) depending on the time it takes to fill the Durham tube with gas (Barnett *et al*., 2000; Kurtzman & Fell, 1998). According to literature the yeasts listed in table 2.1 have the ability to undergo alcoholic fermentation, but the fermentation properties vary between the different authors. A variable or slow positive result may differ between strains therefore some species are listed as positive and negative for fermentation (Barnett *et al*., 2000).

Table 2.1: Combined fermentation properties of psychrophilic yeasts (Barnett *et al*. 2000; Kurtzman & Fell, 1998; Tsuji *et al*., 2013).

	Glucose	Galactose	Sucrose	Maltose
Mrakia frigida	+[1], s[2], D[3]	-[4], v[5], D	+, v, D	-, v, D
Mrakia gelida	+, D	-, D	+, D	-, D
Xanthophyllomyces dendrorhous	s, D	-	-, D, w[6]	-, D, w
Saccharomyces bayanus	+	+, -, v	+, -	+, -
Mrakiella aquatica	-, D	-, D	-	-, D
Mrakia curviuscula	-	-	-	-

1. Positive
2. Slow
3. Delayed
4. Negative
5. Variable
6. Weak

This project aims to expose gas bubbles in psychrophilic yeasts during fermentation, therefore the fermentation abilities of the yeast strains used is extremely important. Since the information about the fermentation properties of the yeast species used in this study varies among authors and literature does not include information about the precise time gas is produced in the Durham tube, the fermentation tests were performed to determine the fermentation abilities of each individual strain. The growth rate of psychrophilic yeasts are low, therefore different concentrations of sugar will be used to determine if an increase in sugar concentration reduces the fermentation time. In the search for gas bubbles in fermenting yeast, glycerol has been used as a non-fermentable carbon source (Swart *et al*., 2012). Since literature shows that some of these yeasts have a variable result for glycerol

assimilation (Barnett *et al*., 2000; Kurtzman & Fell, 1998), the yeasts used in this study will also be tested for glycerol assimilation. The information gathered in Chapter 2 will then be used to expose gas bubbles in psychrophilic yeasts in Chapter 3.

2.3 Materials and Methods

2.3.1 Strains used and cultivation

The yeasts used in this study (Table 2.2) were chosen according to their known fermentation abilities in literature as well as their fermentation temperature. All samples were cultivated at 15°C.

Table 2.2: A list of the yeasts and their strain numbers.

Genus name	Strain Number
Mrakia frigida	[1]NRRL Y-6989/[2]UOFS Y-2926
	NRRL Y-7211/UOFS Y-2927
	NRRL Y-7202/UOFS Y-2907
	NRRL Y-7203/UOFS Y-2929
	NRRL Y-7204/UOFS Y-2928
Mrakia gelida	NRRL Y-7102/UOFS Y-2904
	NRRL Y-7205/UOFS Y-2905
Mrakia curviuscula	NRRL Y-17367/UOFS Y-2909
Mrakiella aquatica	NRRL Y-6758/UOFS Y-2908
Xanthophyllomyces dendrorhous	[3]VKPM Y-1663/UOFS Y-0588
Saccharomyces bayanus var. *uvarum*	[4]CBS395/UOFS Y-1480
	[5]CSIR Y-0257/UOFS Y-0912

1. Agricultural Research Service (Northern Regional Research Laboratory) Culture Collection, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604
2. UNESCO MIRCEN Culture collection, Department of Microbial, Biochemical and Food Biotechnology, Faculty Natural and Agricultural Sciences, University of the Free State, South Africa
3. Russian National Collection of Industrial Microorganisms, 1-st Dorozhniy pr., 1, 117545, Moscow, Russia

4. Westerdijk Fungal Biodiversity Institute (Centraalbureau voor Schimmelcultures), Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
5. Council of Scientific and Industrial Research, Meiring Naudé Road, Brummeria, Pretoria, South Africa

The yeasts were maintained on glucose yeast malt (YM) (10 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract and 16 g l^{-1} agar) plates. An inoculum was prepared for each yeast strain in 50 ml glucose yeast malt (YM) broth in 250 ml Erlenmeyer flasks by inoculating a loopful cells in the media and incubating on a shaker at 160 rpm for 24 h at 15°C.

2.3.2 Fermentation tests

The fermentation tests were done using different carbon sources at different concentrations (Table 2.3). The media used for the fermentation tests were as follows: carbon source as indicated in Table 2.3, 20 g l^{-1} peptone and 10 g l^{-1} yeast extract. Test tubes containing Durham tubes and 5 ml media were inoculated with 200 µl prepared inoculum. The test tubes were incubated at 15°C for 30 days. Gas production in the Durham tubes was monitored daily.

Table 2.3: Carbon sources and concentrations used in this study

Carbon source	Concentration (g l^{-1})		
Glucose	20	40	60
Sucrose	20	40	60
Maltose	20	40	60
Galactose	20		
Glycerol	20		

2.3.3 Assimilation of glycerol

The yeasts were streaked out on Yeast Nitrogen Base (YNB) glycerol (6.7 g l^{-1} Yeast Nitrogen Base, 5 ml l^{-1} glycerol, 16 g l^{-1} agar) plates, incubated at 15°C for 48 h and the growth observed.

2.4 Results and discussions

The fermentation abilities of psychrophilic yeasts differ in literature (Barnett *et al*., 2000; Kurtzman *et al*., 1998). For the purpose of this study, fermentation tests were done to determine the fermentation abilities of the specific strains (Table 2.1), as well as the preferred carbon source concentration (Table 2.3). The fermentation tests were all carried out at 15°C. A positive (+) result was recorded when the Durham tube was filled with gas within 7 days of incubation, a latent (l) result was recorded when the Durham tube was filled with gas after 7 days of incubation, a weak (w) result was recorded if the Durham tube was filled ⅓ with gas after 7 days of incubation and a negative (-) result was recorded when no gas was observed in the Durham tube (Kurtzman & Fell, 1998).

As can be seen in Table 2.4, *Mrakia frigida*, *M. gelida*, *Xanthophyllomyces dendrorhous* and *Saccharomyces bayanus* strains tested positive for fermentation of glucose and sucrose at the different concentrations, filling the Durham tubes within 5 days of incubation. *Mrakiella aquatica* showed latent fermentation of glucose and sucrose, filling the Durham tube with gas after 9 days of incubation. *Mrakia curviuscula* did not ferment glucose or sucrose, confirming literature which states that this *Mrakia* species does not have fermentation abilities (Tsuji *et al*., 2013).

Saccharomyces bayanus strains showed a positive result for maltose and galactose fermentation at the different concentrations. *Mrakia frigida* UOFS Y-2927 and *M. gelida* UOFS Y-2904 showed a weak fermentation of 2% maltose and latent fermentation of 4% and 6% maltose. *Mrakia gelida* UOFS Y-2905 and *X. dendrorhous* showed latent maltose fermentation and the rest of the *M. frigida* strains and the *M. curviuscula* strain did not show any fermentation of maltose. Galactose fermentation was latent in *M. frigida* UOFS Y-2927, UOFS Y-2907, UOFS Y-2929, UOFS Y-2928, *M. gelida* UOFS Y-2904 and UOFS Y-2905, weak in *M. aquatica* and negative in *M. frigida* UOFS Y-2926, *M. curviuscula* and *X. dendrorhous*. As expected, no strains fermented glycerol.

The fermentation properties found in literature (Table 2.1) were compared to the results found in this study (Table 2.4). Some of the results were similar to the results found in literature e.g. *M. frigida* UOFS Y-2926 tested positive for fermentation of glucose in this study, which are similar to the result found by Barnett and co-workers, however Barnett, 2000, also

indicated a delayed result, and Kurtzmann and Fell, 1998, recorded a slow result, which was not observed in this study. It is clear that the fermentation abilities of psychrophilic yeasts vary among the different strains, which may influence the results obtained. When using yeasts for fermentation purposes, it is important to consider the fermentation properties of the individual strain to ensure accurate results.

The *Mrakia* and *Mrakiella* strains were cultivated on YNBGlycerol plates to determine whether they are able to assimilate glycerol, since literature states that these species have showed variable, delayed, slow or negative results for glycerol assimilation. As can be seen in Table 2.5, all of these strains were able to assimilate glycerol.

Table 2.4: Results of the fermentation tests for glucose, sucrose, maltose, galactose and glycerol at different concentrations

Yeast	Strain number	Glucose ($g\ l^{-1}$)			Sucrose ($g\ l^{-1}$)			Maltose ($g\ l^{-1}$)			Galactose ($g\ l^{-1}$)	Glycerol ($g\ l^{-1}$)
		20	40	60	20	40	60	20	40	60	20	20
M. frigida	UOFS Y-2926	+	+	+	+	+	+	-	-	-	-	-
	UOFS Y-2927	+	+	+	+	+	+	w	l	l	l	-
	UOFS Y-2907	+	+	+	+	+	+	-	-	-	l	-
	UOFS Y-2929	+	+	+	+	+	+	-	-	-	l	-
	UOFS Y-2928	+	+	+	+	+	+	-	-	-	l	-
M. gelida	UOFS Y-2904	+	+	+	+	+	+	w	l	l	l	-
	UOFS Y-2905	+	+	+	+	+	+	l	l	l	l	-
M. curviuscula	UOFS Y-2909	-	-	-	-	-	-	-	-	-	-	-
M. aquatica	UOFS Y-2908	l	l	l	l	l	l	w	w	l	w	-
X. dendrorhous	UOFS Y-0588	+	+	+	+	+	+	l	l	l	-	-
S. bayanus var. *uvarum*	UOFS Y-1480	+	+	+	+	+	+	+	+	+	+	-
	UOFS Y-0912	+	+	+	+	+	+	+	+	+	+	-

Table 2.5: Results of the tests for assimilation of glycerol

Yeast	Strain number	Growth observed
M. frigida	UOFS Y-2926	+
	UOFS Y-2927	+
	UOFS Y-2907	+
	UOFS Y-2929	+
	UOFS Y-2928	+
M. gelida	UOFS Y-2904	+
	UOFS Y-2905	+
M. curviuscula	UOFS Y-2909	+
M. aquatica	UOFS Y-2908	+
X. dendrorhous	UOFS Y-0588	+
S. bayanus var. *uvarum*	UOFS Y-1480	+
	UOFS Y-0912	+

2.5 Conclusions

Since the literature is unclear which strains of psychrophilic yeasts have the ability to ferment, stating that these yeasts have variable or delayed fermentation abilities, fermentation tests were carried out on all of the yeasts used in this study to determine the fermentation abilities of each individual strain. From the results obtained it is clear that the fermentation abilities of psychrophilic yeasts differ between different strains. *Mrakia frigida* strains showed a positive result for glucose and sucrose fermentation, variable result for maltose and galactose fermentation. *Mrakia gelida* strains showed a positive result for glucose and sucrose, latent

result for maltose fermentation and latent result for galactose fermentation. *Mrakiella aquatica* showed a latent result for glucose and sucrose fermentation and a weak result for maltose and galactose fermentation. *Xanthophyllomyces dendrorhous* showed a positive result for glucose and sucrose fermentation, latent result for maltose fermentation and negative result for galactose fermentation. *Saccharomyces bayanus* strains were positive for the fermentation of glucose, sucrose, maltose and galactose but were not able to ferment glycerol. *Mrakia curviuscula* did not ferment any carbon sources, similar to what was found in literature. All of the strains were able to assimilate glycerol, however, no strains were able to ferment glycerol. The results found in this chapter will be used to expose gas bubbles in psychrophilic yeasts in Chapter 3 of this study.

2.6 References

Barnett, J., Payne, R. & Yarrow, D. (2000). Yeasts: characteristics and identification, 3rd edn. Cambridge University Press, Cambridge, UK.

Beltran, G., Rozes, N., Mas, A. & Guillamon, J. M. (2007). Effect of low-temperature fermentation on yeast nitrogen metabolism. *World J Microbiol Biotechnol* **23**, 809-815.

Kourkoutas, Y., Koutinas, A. A., Kanellaki, M., Banat, I. M., Marchant, R. (2002). Continuous wine fermentation using a psychrophilic yeast immobilized on apple cuts at different temperatures. *Food Microbiol* **19**, 127-134.

Kurtzman, C. P. & Fell, J. W. (1998). The yeasts: a taxonomic study 4th edn. Elsevier, Amsterdam.

Liu, Y-S. & Wu, J-Y. (2007). Optimization of cell growth and carotenoid production of *Xanthophyllomyces dendrorhous* through statistical experiment design. *Biochem Eng J* **36**, 182-189.

Liu, Y-S., Wu, J-Y. & Ho, K-p. (2006). Characterization of oxygen transfer conditions and their effects on *Phaffia rhodozyma* growth and carotenoid production in shake flask cultures. *Biochem Eng J* **27**, 331-335.

Lopez-Malo, M., Chiva, R., Rozes, N. & Guillamon, J. M. (2013). Phenotypic analysis of mutant and overexpressing strains of lipid metabolism genes in *Saccharomyces cerevisiae*: Implication in growth at low temperatures. *Int J Food Microbiol* **162**, 26-36.

Molina, A. M., Swiegers, J. H., Varela, C., Pretorius, I. S. & Agosin, E. (2007). Influence of wine fermentation temperature on the synthesis of yeast-derived volatile aroma compounds. *Appl Microbiol Biotechnol* **77**, 675-687.

Pronk, J. T., Steensma, H. Y. & Van Dijken, J. P. (1996). Pyruvate metabolism in *Saccharomyces cerevisiae*. *Yeast* **12**, 1607-1633.

Reynders, M. B., Rawlings, D. E. & Harrison, S. T. L. (1997). Demonstration of the crabtree effect in *Phaffia rhodozyma* during continuous and fed-batch cultivation. *Biotechnol Lett* **19(6)**, 549-552.

Shivaji, S. & Prasad, G. S. (2009). Antarctic yeasts: biodiversity and potential applications. *Yeast Biotechnology: Diversity and Applications* (Satyanarayana, T. & Kunze, G., eds) pp 3-16. Springer-Verlag, Berlin.

Tsuji, M., Goshima, T., Matsushika, A., Kudoh, S. & Hoshino, T. (2013). Direct ethanol fermentation from lignocellulosic biomass by Antarctic basidiomycetous yeast *Mrakia blollopis* under low temperature condition. *Cryobiology* **67**, 241-243.

Tsuji, M., Yokota, Y., Kudoh, S. & Hoshino, T. (2014). Improvement of direct ethanol fermentation from woody biomasses by the Antarctic basidiomycetous yeast, *Mrakia blollopis*, under a low temperature condition. *Cryobiology* **68**, 303-305.

Van der Walt, J. P. (1970). Criteria and methods used in classification. The Yeasts: A taxonomic study. North-Holland Publishing Company, Amsterdam.

Van Dijken, J. P., Van den Bosch, E., Hermans, J. J., De Miranda, L. R. & Scheffers, W. A. (1986). Alcoholic fermentation by 'non-fermentative' yeasts. *Yeasts* **2**, 123-127.

Yang, J. P. P., Tan, H., Yang, R., Sun, X., Zhai, H. & Li, K. (2011). Astaxanthin production by *Phaffia rhodozyma* fermentation of cassava residues substrate. *Agric Eng Int* **13(2)**, 1-6.

Chapter 3

Gas bubble formation in psychrophilic yeasts

3.1 Abstract

Gas bubbles have been found in several organisms during fermentations at 30°C. In this chapter, gas bubbles have been found during fermentations at 15°C in psychrophilic yeasts. The yeasts were cultivated in fermentable and non-fermentable media. Gas bubbles were present in the yeasts cultivated in fermentable media, and absent or present in low numbers when cultivated in non-fermentable media when observed with Light Microscopy, Transmission Electron Microscopy and Nano Scanning Auger Microscopy. An increase in bubble-like structures was observed when the glucose concentration of the media was increased. To confirm whether the structures are gas bubbles or lipid droplets, lipid extraction was done. No significant increase in the total lipid content was observed with an increase in glucose concentration, suggesting these structures are not lipid droplets. *Mrakiella aquatica* showed the highest total lipid content, therefore this yeast was stained with BODIPY stain and subjected to Confocal Laser Scanning Microscopy. The results showed the presence of lipids and bubble-like structures in the cell. The weight of one fermenting yeast cell was determined in order to verify if fermenting yeast cells containing gas bubbles weigh less than non-fermenting yeast cells without gas bubbles. The results of this chapter indicated that gas bubbles are present in fermenting psychrophilic yeasts and the structures observed are not lipid droplets and an increase in glucose concentration results in an increase in bubble production.

3.2 Introduction

Carbon dioxide (CO_2) is one of the main products of alcoholic fermentation (van Dijken *et al*., 1986). During alcoholic fermentation in yeasts, CO_2 is produced via the alcoholic fermentation pathway in the cytoplasm of the cell and is released vigorously into the environment. Due to the rapid production of CO_2, the release of the gas is expected to form a bottleneck, resulting in the formation of gas bubbles in the cytoplasm of the cell (Swart *et al*., 2012).

Gas bubbles are defined as symmetrical to asymmetrical spheres in an aqueous medium. They may coalesce and can consist of one or more gasses (Blatteau *et al*., 2006). The size of gas bubbles range from a few nanometers to several millimeters. When they are smaller than

10µm they are suspended and called gas nuclei but when they are larger than 10µm they are not suspended and called gas bubbles (Mahon, 2010).

Gas bubbles were found for the first time in living cells in 2012 in the fermenting yeast *S. cerevisiae* and *S. pastorianus* (Swart *et al*., 2012) using Light Microscopy (LM), Transmission Electron Microscopy (TEM) and Nano Scanning Auger Microscopy (NanoSAM). Since 2012, gas bubbles have been found in several organisms: *Saccharomyces cerevisiae*; *Saccharomyces pastorianus*; *Saccharomyces bayanus* var. *uvarum*; *Saccharomyces kudriavzevii*; *Saccharomyces mikatae*; *Saccharomyces paradoxus*; *Schizosaccharomyces pombe*; *Rhizopus oryzae*; *Torulaspora globosa*; *Zygosaccharomyces bailli*; *Kluyveromyces marxianus*; *Debaryomyces hansenii*; *Lipomyces starkeyi*; *Pichia membranifaciens*; *Yarrowia lipolytica* and *Rhodotorula glutinis* (Unpublished). These gas bubbles were present during fermentation and absent or present in low numbers during respiration and were all found during growth at 30°C.

Different parameters have been explored in the search for gas bubbles, but since all previous experiments were conducted at 30°C, there is a need to explore gas bubble formation during low temperature fermentation to determine the conserved status of gas bubble formation at lower temperatures. The focus of this chapter will be to expose gas bubbles formed during fermentation in psychrophilic yeasts.

3.3 Materials and methods

3.3.1 Strains used and cultivation

The yeasts used in this study (Table 3.1) were chosen according to their fermentation abilities as well as their fermentation temperature (Barnett *et al*., 2000; Kurtzman & Fell, 1998; Tsuji *et al*., 2013).

Table 3.1: A list of the yeasts and their strain numbers as well as the cultivation temperature.

Yeast	Strain Number
Mrakia frigida	[1]NRRL Y-6989/[2]UOFS Y-2926
	NRRL Y-7211/UOFS Y-2927
	NRRL Y-7202/UOFS Y-2907
	NRRL Y-7203/UOFS Y-2929
	NRRL Y-7204/UOFS Y-2928
Mrakia gelida	NRRL Y-7102/UOFS Y-2904
	NRRL Y-7205/UOFS Y-2905
Mrakia curviuscula	NRRL Y-17367/UOFS Y-2909
Mrakiella aquatica	NRRL Y-6758/UOFS Y-2908
Xanthophyllomyces dendrorhous	[3]VKPM Y-1663/UOFS Y-0588
Saccharomyces bayanus var. *uvarum*	[4]CBS395/UOFS Y-1480
	[5]CSIR Y-0257/UOFS Y-0912

1. Agricultural Research Service (Northern Regional Research Laboratory) Culture Collection, National Center for Agricultural Utilization Research, 1815 N. University Street, Peoria, IL 61604
2. UNESCO MIRCEN Culture collection, Department of Microbial, Biochemical and Food Biotechnology, Faculty Natural and Agricultural Sciences, University of the Free State, South Africa
3. Russian National Collection of Industrial Microorganisms, 1-st Dorozhniy pr., 1, 117545, Moscow, Russia
4. Westerdijk Fungal Biodiversity Institute (Centraalbureau voor Schimmelcultures), Uppsalalaan 8, 3584 CT, Utrecht, The Netherlands
5. Council of Scientific and Industrial Research, Meiring Naudé Road, Brummeria, Pretoria, South Africa

The yeasts were maintained on glucose yeast malt (YM) (10 g l^{-1} glucose, 5 g l^{-1} peptone, 3 g l^{-1} yeast extract, 3 g l^{-1} malt extract and 16 g l^{-1} agar) plates. An inoculum was prepared for each yeast strain in 50 ml glucose yeast malt (YM) broth in 250 ml Erlenmeyer flasks by inoculating a loopful cells in the media and incubating on a shaker at 160 rpm for 24 h at 15°C (Table 3.1). The inoculum was used to inoculate fermentable and non/weak-fermentable media in 100 ml Erlenmeyer flasks with 20 ml media. The fermentable media consisted of a 20 g l^{-1} glucose YM broth and a 60 g l^{-1} glucose YM broth. The non/weak-fermentable media consisted of 20 g l^{-1} yeast extract peptone (YP) containing galactose or glycerol (10 g l^{-1} yeast extract, 20 g l^{-1}

peptone, 1 g l^{-1} galactose/10 ml l^{-1} glycerol) media depending on the yeast (Table 3.2). The flasks were then incubated at 15°C for the specific time (Table 3.2) as determined by the fermentation tests in Chapter 2. After the fermentation time was reached, the samples were retrieved and analyzed with Light Microscopy (LM), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM), Nano Scanning Auger Microscopy (NanoSAM) (Swart *et al.,* 2010; Kock *et al.,* 2011) and prepared for lipid extraction.

Table 3.2: Fermentable and non/weak fermentable carbon sources as well as the incubation time for each yeast used in the study

Yeast	Strain Number	Fermentable	Non/Weak fermentable	Incubation Time
M. frigida	UOFS Y-2926	Glucose	Galactose	5 days
	UOFS Y-2927	Glucose	Galactose	5 days
	UOFS Y-2907	Glucose	Galactose	5 days
	UOFS Y-2929	Glucose	Galactose	5 days
	UOFS Y-2928	Glucose	Galactose	5 days
M. gelida	UOFS Y-2904	Glucose	Galactose	5 days
	UOFS Y-2905	Glucose	Galactose	5 days
M. curviuscula	UOFS Y-2909	Glucose	Galactose	5 days
M. aquatica	UOFS Y-2908	Glucose	Galactose	9 days
X. dendrorhous	UOFS Y-0588	Glucose	Glycerol	5 days
S. bayanus var. *uvarum*	UOFS Y-1480	Glucose	Glycerol	18 h
	UOFS Y-0912	Glucose	Glycerol	18 h

3.3.2 Light Microscopy (LM)

The 12 yeast strains cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media were subjected to light microscopy (Axioplan, Zeiss, Germany) coupled to a Colourview Soft Digital imaging system (Münster, Germany). Light Microscopy was used in this study to determine the presence of light scattering granules, similar to what was observed by Swart and co-workers in *S. cerevisiae* and *S. pastorianus* (Swart *et al*., 2012). The detection of light scattering granules serves as the first step in determining the presence of gas bubbles.

3.3.3 Transmission Electron Microscopy (TEM)

The 12 yeast strains cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media were subjected to TEM evaluation as the second step in the determination of presence of gas bubble formation (Swart *et al.,* 2012). The yeast suspensions were centrifuged at 3000 rpm for 5 min. The pellet was retrieved and fixated for 3 h with 3% (v/v) buffered glutardialdehyde (Merck, Darmstadt, Germany). The buffer used in the TEM preparation was 0.1 M (pH 7) sodium phosphate buffer. The samples were then fixated with buffered 1% (v/v) osmium tetroxide (Merck, Darmstadt, Germany) for 1 h. Both fixation steps were followed by rinsing the sample for 5 min in the buffer, followed by centrifuging the sample for 1 min at 1500 g. After fixation the cells were immobilized in 1% agar. After the agar solidified, the agar was cut into 2mm x 2mm x 2mm cubes. The next step in TEM preparation consists of dehydrating the samples with an acetone gradient: 20 minutes in 50%, 70% and 95% acetone and twice for 1 h in 100% acetone. After dehydration, the acetone was removed and replaced with epoxy resin by adding acetone and epoxy in a 1:1 ratio for 8 h. After 8 h the samples were placed in 100% epoxy for 8 h followed by another 8 h in 100% epoxy in a vacuum. The samples were then embedded in specialized molds and polymerized for 8 h at 70°C. A Leica ultracut UM7 microtome was used to cut the embedded samples with a glass knife in 60nm sections. The sections were mounted onto copper grids and stained with uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany) for 10 min and 5 min respectively. The cells were viewed using a transmission electron microscope [FEI (Phillips) CM 100, Netherlands].

3.3.4 Scanning Electron Microscopy (SEM)

The 12 yeast strains cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media were subjected to SEM analyses due to the lack of SEM images available for psychrophilic yeasts. The fixation process in SEM preparation is similar to what was described in section 3.3.3. The cells were dehydrated using an ethanol gradient: 20 minutes in 50%, 70% and 95% ethanol and twice for 1 h in 100% ethanol. After dehydration, the cells were dried using a Tousimis critical point dryer (Rockville, Maryland, USA) and mounted onto stubs. The cells were sputter coated with gold to increase the electron conductivity of the cells after which the cell were subjected to SEM (Shimadzu SSX-550, Kayoto, Japan) analyses.

3.3.5 Nano Scanning Auger Microscopy (NanoSAM)

The samples prepared for SEM analyses were subjected to NanoSAM (PHI 700 Nanoprobe, Japan) analyses with SEM and SAM facilities. The field emission electron gun used for the SEM analyses was set at: 2.258 A filament current; 4.94 kV extractor voltage and 176 µA extractor current. A 20 kV, 10 nA electron beam with a diameter of 12 nm was obtained for SEM imaging. The upper pressure of the electron gun unit was 9.9E-10 Torr. The pressure in the main chamber was 3.47E-10 Torr. The Nanoprobe was also equipped with an Argon (Ar^{+}) ion sputtering gun set at: 2kV beam voltage, 1.2 µA ion beam current and a 1 mm x 1 mm raster area, giving a sputter rate of 12 nm min^{-1}.

3.3.6 Lipid extraction

Lipid extraction was done on the 12 yeast strains cultivated in 2% glucose YM, 6% glucose YM and 2% YPGalactose/YPGlycerol. Freeze dried biomass was used to extract the lipids using chloroform and methanol to a ratio of 2:1 (Folch *et al*., 1957). The extracts were washed with dH_20 and organic solvents evaporated under vacuum. The lipids were dissolved with 10 ml diethyl ether and dried using a nitrogen stream. The samples were dried to constant weight overnight at 100°C. The total lipid content of the yeast cells was calculated by weight measurement and an Analysis of Variance (ANOVA) was performed on the data to determine whether there is a significant difference between the total lipid content of the different strains cultivated on fermentable and non-fermentable media. The data of the strains cultivated on galactose as non-fermentable media and glycerol as non-fermentable media was separated and analyzed separately. There is no significant difference when cultivating the yeast on different carbon sources when the p value is 0.05 or more.

3.3.7 Confocal Laser Scanning Microscopy (CLSM)

Mrakiella aquatica cells cultivated as described in section 3.3.1 were stained with 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY) stain (Harris *et al*., 2013; Rumin *et al*., 2015). A stock solution BODIPY 493/503 stain was prepared with a concentration of 1mg ml^{-1} dissolved in dimethyl sulfoxide (DMSO) and stored at -20°C. The stock solution was added to the yeast suspension to a final concentration of 1 µl ml^{-1}. The samples were incubated at room temperature for 30min and subjected to Confocal Laser Scanning Microscopy (CLSM).

3.3.8 Weight determination

The yeast *S. bayanus* var. *uvarum* UOFS Y-1480 was used to determine the average weight of a single yeast cell during fermentation and respiration. An inoculum was prepared as described in section 3.3.1. The inoculum was used to inoculate 100 ml Erlenmeyer flasks with 20 ml 2% YMGlucose, 6% YMGlucose and 2% YPGlycerol media. The flasks were incubated at 15°C for up to 24 h. At 2 h intervals, one flask 2% YMGlucose, one flask 6% YMGlucose and one flask 2% YPGlycerol were retrieved. For each flask the total cell count was determined using a hemocytometer. The samples were then centrifuged at 4000 x g, the supernatant discarded and the pellets freeze dried and weighed.

3.4 Results and discussions

The yeasts used in this study were cultivated in two different glucose concentrations to determine the effect of an increased glucose concentration on gas bubble formation. The yeasts were also cultivated in a carbon source that is weakly or non-fermentable by these yeasts to serve as a control for fermentation. *Mrakia curviuscula* was also used in this study as a control since this yeast does not have the ability to ferment. Figure 3.1, Fig. 3.2, Fig. 3.3 and Fig. 3.4 are representative.

3.4.1 Cultivation

The yeasts were cultivated in 2% YMGlucose, 6% YMGlucose and 2% YMGalactose/YMGlycerol media. The rate of fermentation decreases with temperature. As was observed in Chapter 2, some of the psychrophilic yeasts fill the Durham tubes with CO_2 gas produced during fermentation after 5 days of incubation, therefore the incubation time used in this study was determined by the time required for the yeasts to fill the Durham tubes with gas (Table 3.2). The non/weak fermentable carbon sources were not expected to lead to gas bubble formation, since the yeasts are not able to ferment glycerol and the fermentation of galactose is latent or weak as observed in chapter 2, therefore only producing gas in the Durham tubes after 7 days of incubation and the tests for gas bubbles were carried out at the times indicated in table 3.2.

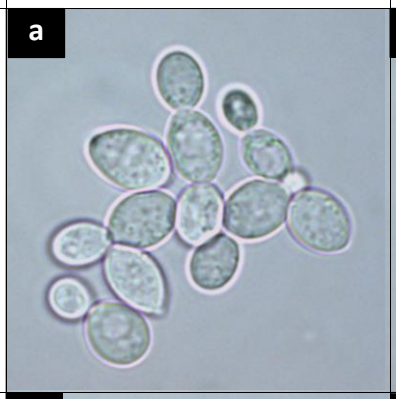
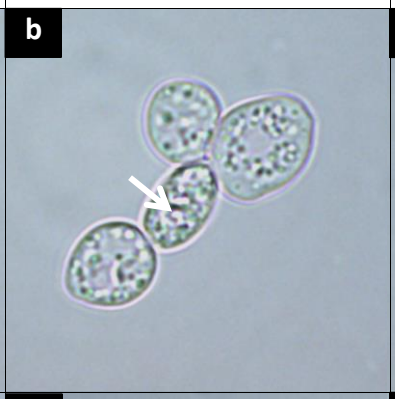
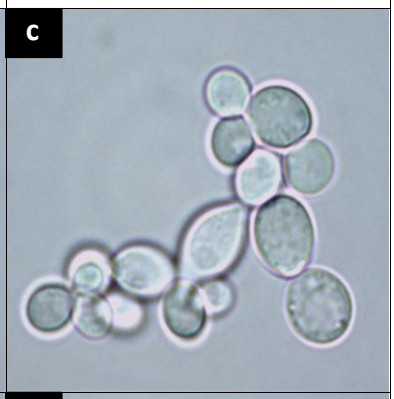
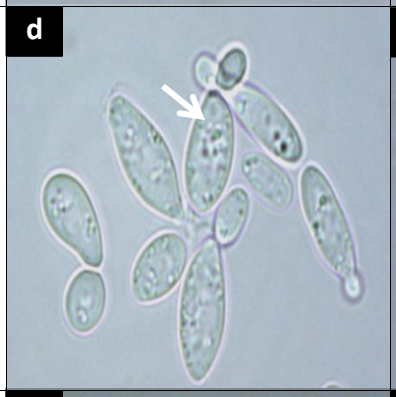
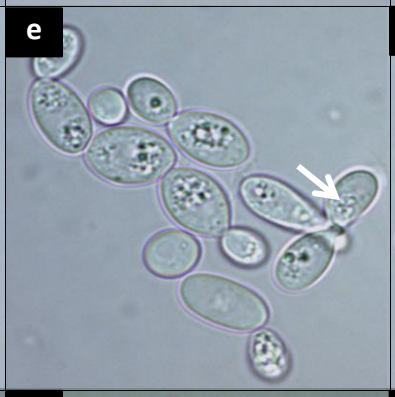
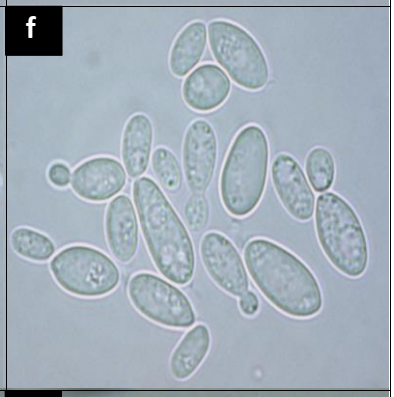
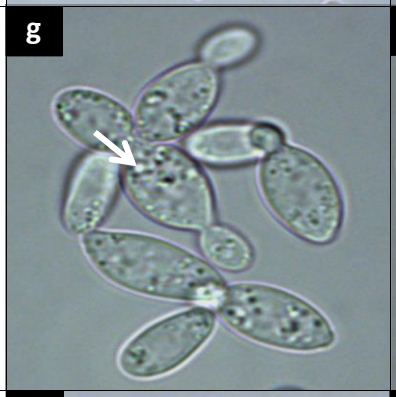
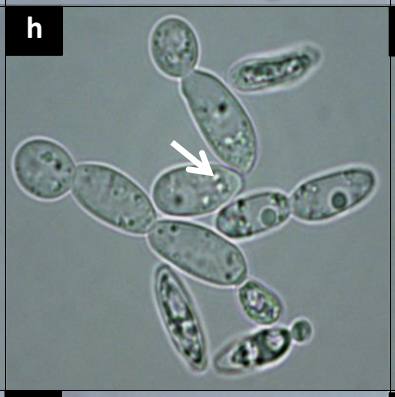
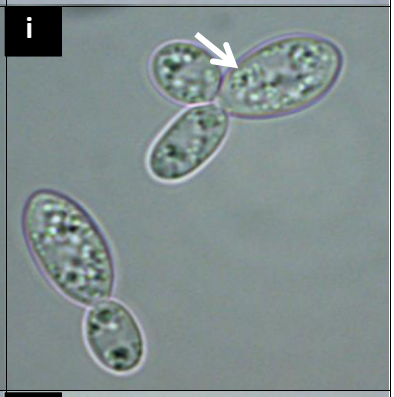
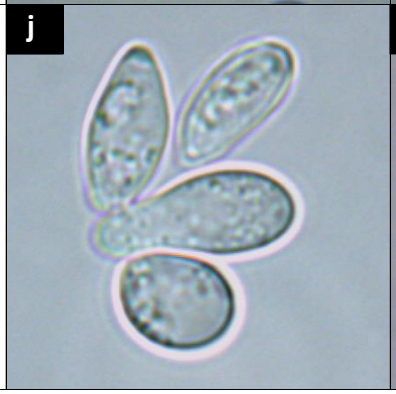
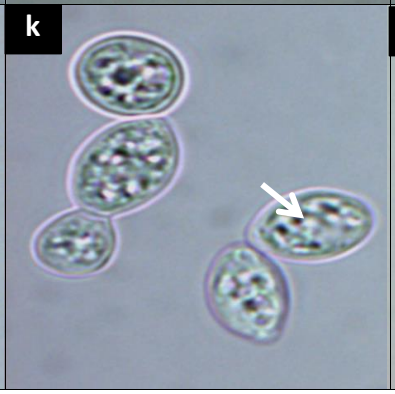
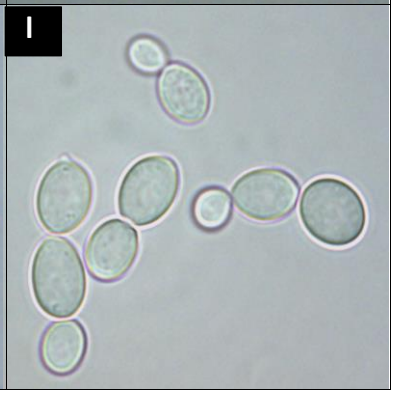
3.4.2 Light Microscopy

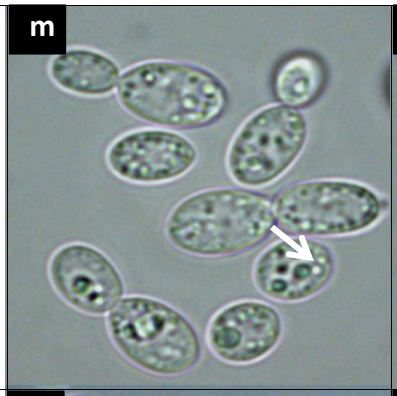
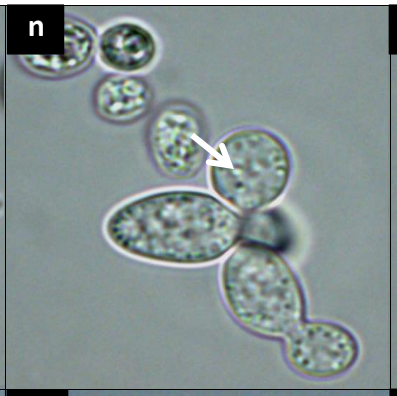
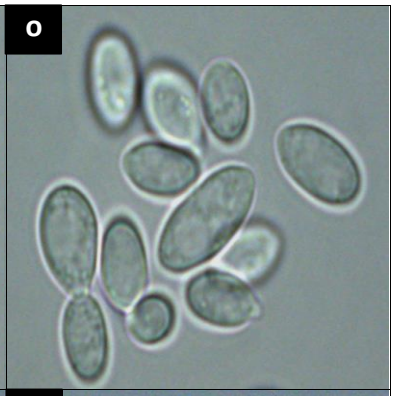
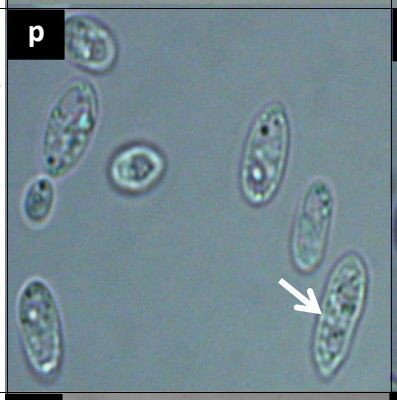
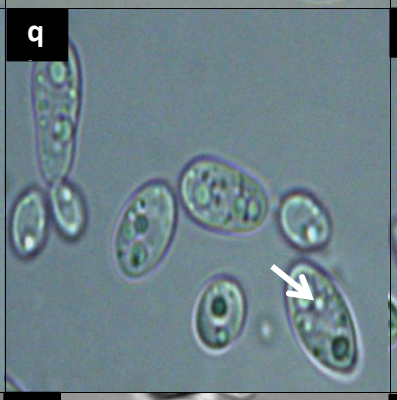
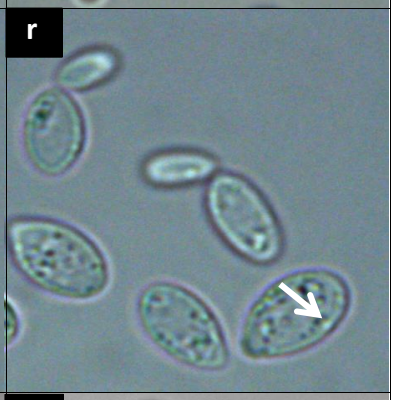
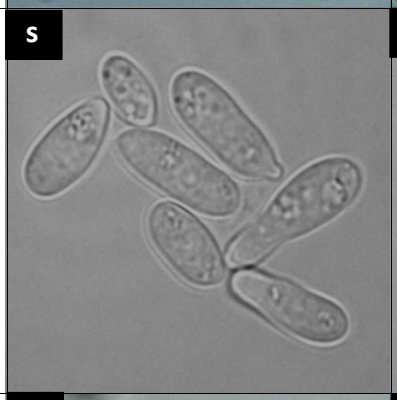
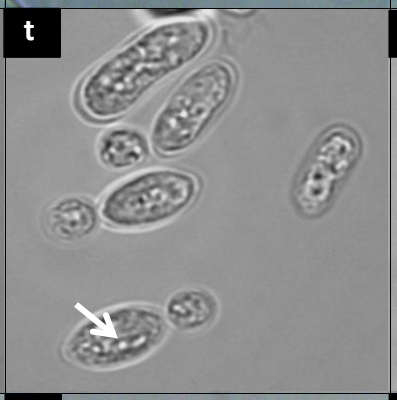
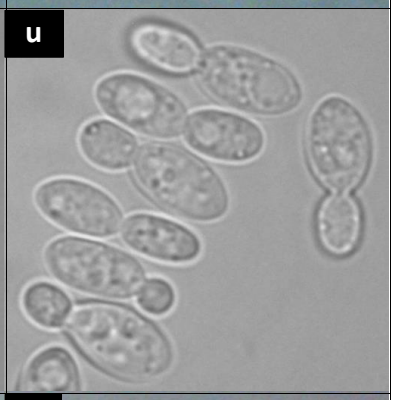
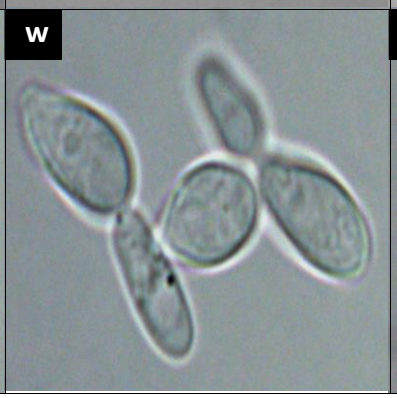
The density of biological material is higher than the density of gas, therefore it is expected that the light from the microscope will be scattered by the gas inside the gas bubbles, giving the gas bubbles a granular appearance (Swart *et al*., 2012). This was observed in cyanobacteria as glistening refractile areas and in *Saccharomyces* as light scattering granules (Swart *et al*., 2012; Walsby, 1994), therefore the presence of light scattering granules in yeast cells serves as the first step in the search for gas bubbles in living cells.

Figure 3.1 consists of light micrographs of psychrophilic yeasts cultivated in 2% YMGlucose, 6% YMGlucose and 2% YMGalactose/YMGlycerol. *Mrakia frigida* UOFS Y-2927 (Fig. 3.1d), UOFS Y-2907 (Fig. 3.1g), UOFS Y-2928 (Fig. 3.1m), *M. gelida* UOFS Y-2904 (Fig. 3.1p), *M. aquatica* UOFS Y-2908 (Fig. 3.1y), *X. dendrorhous* UOFS Y-0588 (Fig. 3.1bb), *S. bayanus* var. *uvarum* UOFS Y-1480 (Fig. 3.1ee) and UOFS Y-0912 (Fig. 3.1hh) contained light scattering granules when cultivated in 2% YMGlucose media, this was expected, since these yeasts have the ability to ferment glucose. Four yeast strains did not contain light scattering granules when cultivated in 2% YMGlucose media including *M. frigida* UOFS Y-2926 (Fig. 3.1a), UOFS Y-2929 (Fig. 3.1j), *M. gelida* UOFS Y-2905 (Fig. 3.1s) and the control *M. curviuscula* UOFS Y-2909 (Fig. 3.1v). The structures observed in Fig. 3.1a, Fig. 3.1j and Fig. 3.1s appear granular, but there is no light scattering observed at 2% glucose concentration, therefore these structures are not gas bubbles. Since these yeasts, except *M. curviuscula*, are expected to ferment in 2% YMGlucose, the absence of light scattering granules might be due to the lack of a bottleneck of CO_2 production, therefore the buildup of gas in the cell is delayed or too small to cause the scattering of light at this stage.

As expected, light scattering granules were present in all the yeasts cultivated in 6% YMGlucose media, except in *M. curviuscula*, the control. This suggests the strains that did not contain light scattering granules when cultivated in 2% YMGlucose media has a higher fermentation rate when the glucose concentration is increased. *Mrakia frigida* UOFS Y-2907, *M. gelida* UOFS Y-2904, *M. aquatica* UOFS Y-2908 and *X. dendrorhous* UOFS Y-0588 contained light scattering granules when cultivated in a weak or non-fermentable carbon source (Fig. 3.1i, Fig. 3.1r, Fig. 3.1aa, Fig. 3.1dd). This might be due to CO_2 buildup during respiration, even though the rate of respiration is much slower than fermentation, six molecules of CO_2 are produced during respiration where two molecules of CO_2 are produced during fermentation,

which might lead to a buildup of CO_2 in the cell. Since light scattering granules were present in the light micrographs, transmission electron microscopy was done to view the gas bubbles at a higher magnification and to confirm the LM results.

Yeast	2%Glucose	6%Glucose	2%Galactose/2%Glycerol
M. frigida UOFS Y-2926	 a	 b	 c
M. frigida UOFS Y-2927	 d	 e	 f
M. frigida UOFS Y-2907	 g	 h	 i
M. frigida UOFS Y-2929	 j	 k	 l

M. frigida UOFS Y-2928	 m	 n	 o
M. gelida UOFS Y-2904	 p	 q	 r
M. gelida UOFS Y-2905	 s	 t	 u
M. curviuscula UOFS Y-2909	 v	 w	 x

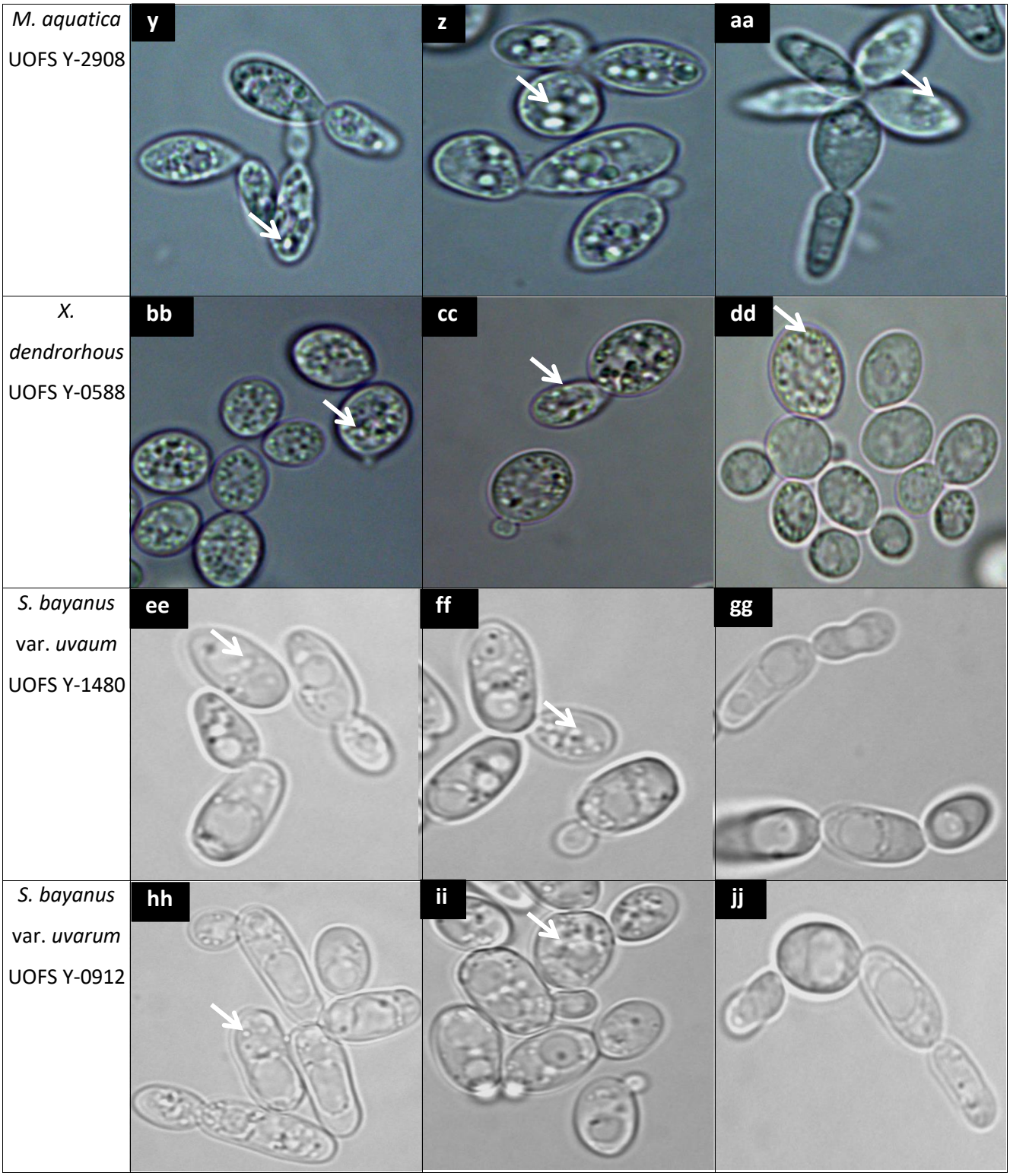


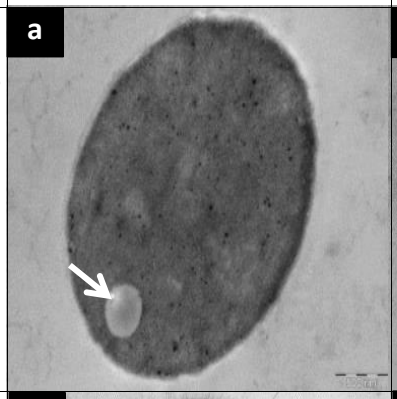
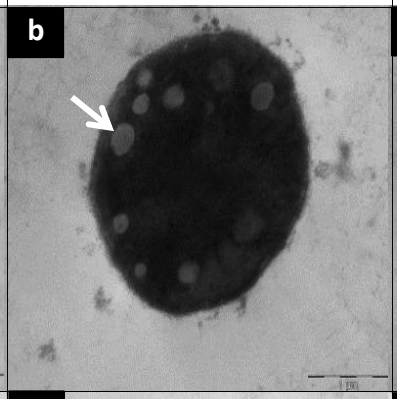
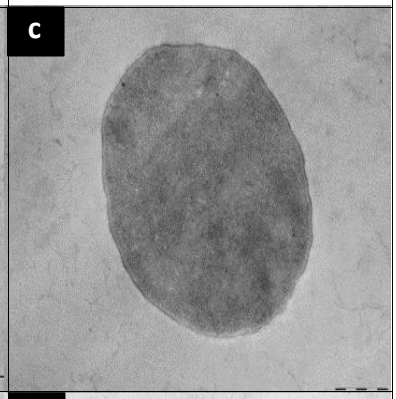
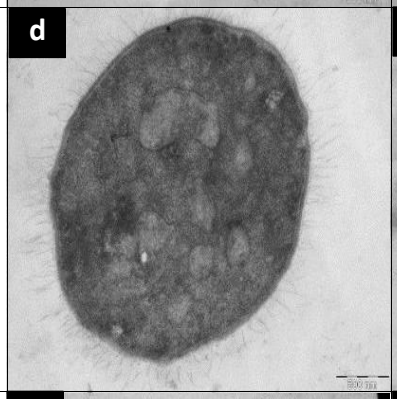
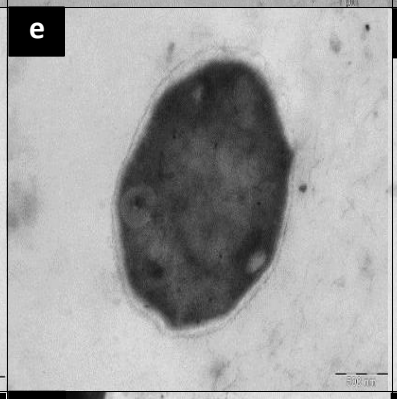
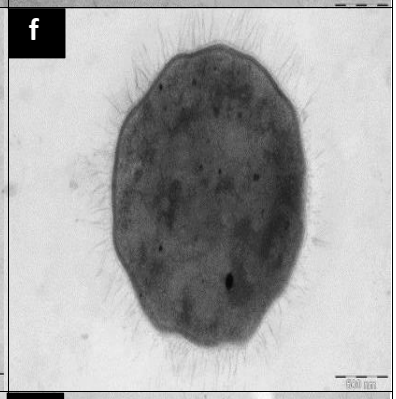
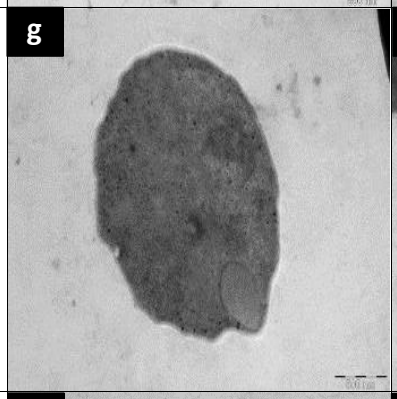
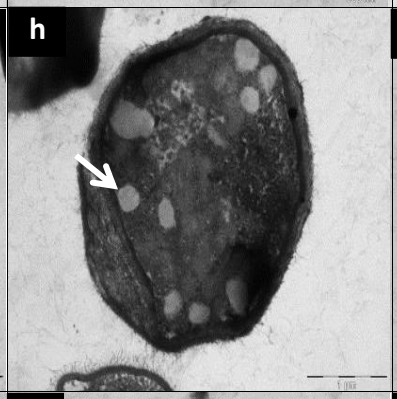
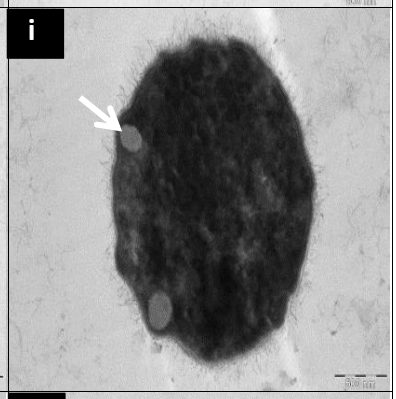
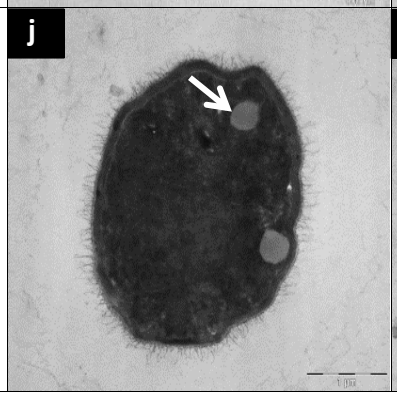
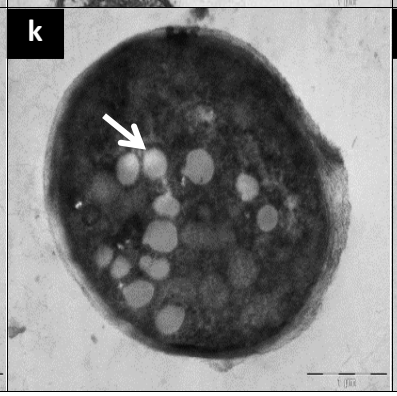
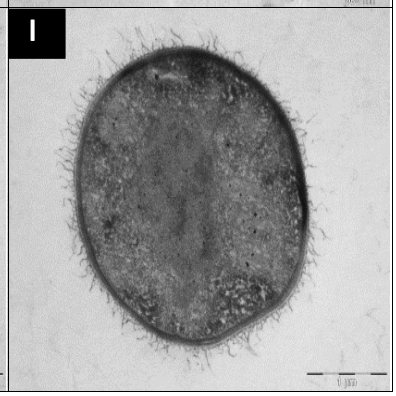
Figure 3.1: Light micrographs of psychrophilic yeast cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose. Arrows indicate light scattering granules in the cell.

3.4.3 Transmission Electron Microscopy (TEM)

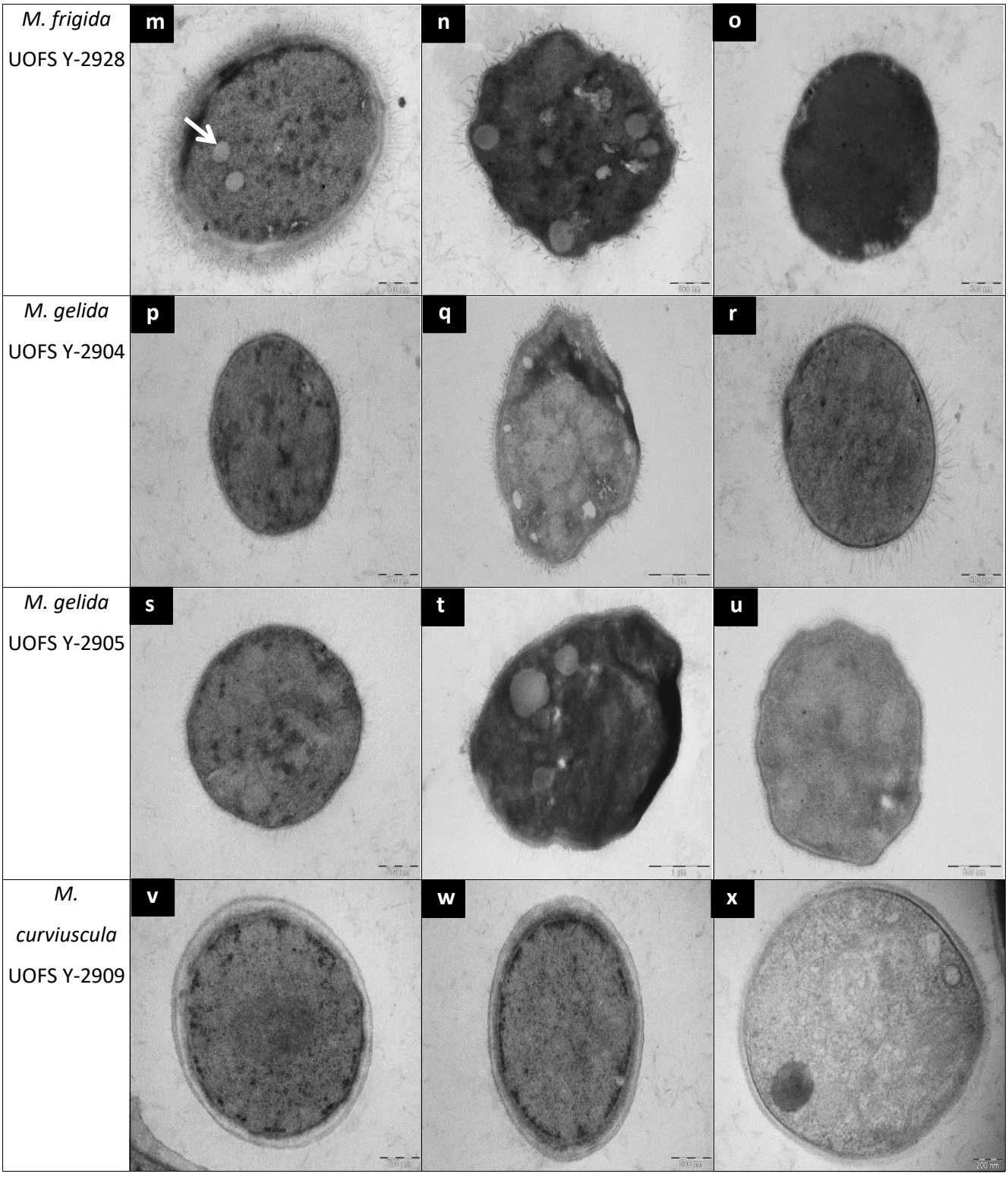
Transmission Electron Microscopy was used to verify the results obtained with LM and view the two dimensional (2D) ultrastructure of the yeast cells. During TEM analysis electrons are beamed through the samples which are electron dense due to metal staining in the preparation process. Electron dense cell material is darker in color and electron transparent gas bubbles are light in color as can be seen in figure 3.2. Non-enveloped, electron transparent structures were observed in the following yeasts when cultivated in 2% YMGlucose media: *M. frigida* UOFS Y-2926 (Fig. 3.2a), UOFS Y-2929 (Fig. 3.2j), UOFS Y-2928 (Fig. 3.2m), *M. aquatica* UOFS Y-2908, *X. dendrorhous* UOFS Y-0588 (Fig. 3.2bb), *S. bayanus* var. *uvarum* UOFS Y-1480 (Fig. 3.2ee) and UOFS Y-0912 (Fig. 3.2hh). No electron transparent structures were observed in *M. frigida* UOFS Y-2927 (Fig. 3.2d, Fig. 3.3), UOFS Y-2907 (Fig. 3.2g), *M. gelida* UOFS Y-2904 (Fig. 3.2p), UOFS Y-2905 (Fig. 3.2s) and *M. curviuscula* UOFS Y-2909 (Fig. 3.2v), the control.

Several non-enveloped electron transparent structures were observed in the yeasts when cultivated in media with an increased glucose concentration as can be seen in *M. frigida* UOFS Y-2926 (Fig. 3.2b), UOFS Y-2907 (Fig. 3.2h), UOFS Y-2929 (Fig. 3.2k), UOFS Y-2928 (Fig. 3.2n), *M. gelida* UOFS Y-2904 (Fig. 3.2q), UOFS Y-2905 (Fig. 3.2t), *M. aquatica* UOFS Y-2908 (Fig. 3.2z), *X. dendrorhous* UOFS Y-0588 (Fig. 3.2cc), *S. bayanus* var. *uvarum* UOFS Y-1480 (Fig. 3.2ff) and UOFS Y-0912 (Fig. 3.2ii). No electron transparent structures were observed in *M. frigida* UOFS Y-2927 (Fig. 3.2e) and the control *M. curviuscula* UOFS Y-2909 (Fig. 3.2w) when cultivated in 6% YMGlucose media.

A few electron transparent structures were observed in *M. frigida* UOFS Y-2907 (Fig. 3.2i), *M. aquatica* UOFS Y-2908 (Fig. 3.2aa) and *X. dendrorhous* UOFS Y-0588 (Fig. 3.2dd) when cultivated in a weak or non-fermentable carbon source. No electron transparent structures were observed in the other yeast strains when cultivated in 2% YPGalactose/YPGlycerol media (Fig. 3.2c, Fig. 3.2f, Fig. 3.2l, Fig. 3.2o, Fig. 3.2r, Fig. 3.2u, Fig. 3.2x, Fig. 3.2gg, Fig. 3.2jj). Some of the yeasts observed with TEM are surrounded by finger-like structures, however the purpose of these structures are still unknown (Fig. 3.2d, Fig. 3.2f, Fig. 3.2i, Fig. 3.2j, Fig. 3.2l, Fig. 3.2m, Fig. 3.2n, Fig. 3.2p, Fig. 3.2q, Fig. 3.2r, Fig. 3.2s, Fig. 3.3).

Yeast	2% Glucose	6% Glucose	2% Galactose/ 2% Glycerol
M. frigida UOFS Y-2926			
M. frigida UOFS Y-2927			
M. frigida UOFS Y-2907			
M. frigida UOFS Y-2929			

M. frigida UOFS Y-2928	m	n	o
M. gelida UOFS Y-2904	p	q	r
M. gelida UOFS Y-2905	s	t	u
M. curviuscula UOFS Y-2909	v	w	x



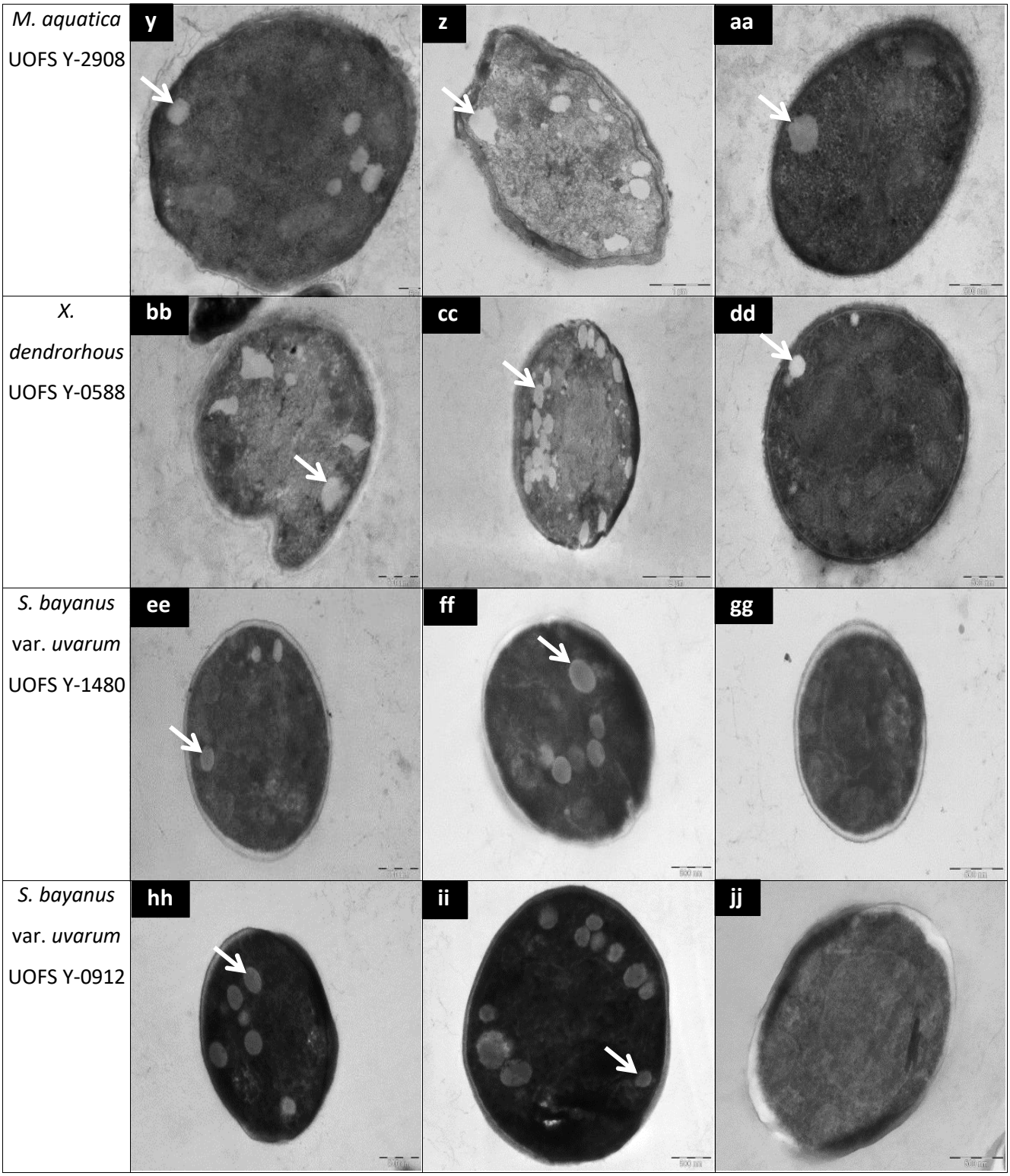


Figure 3.2: TEM micrographs of psychrophilic yeasts cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media. Arrows indicate electron transparent structures.

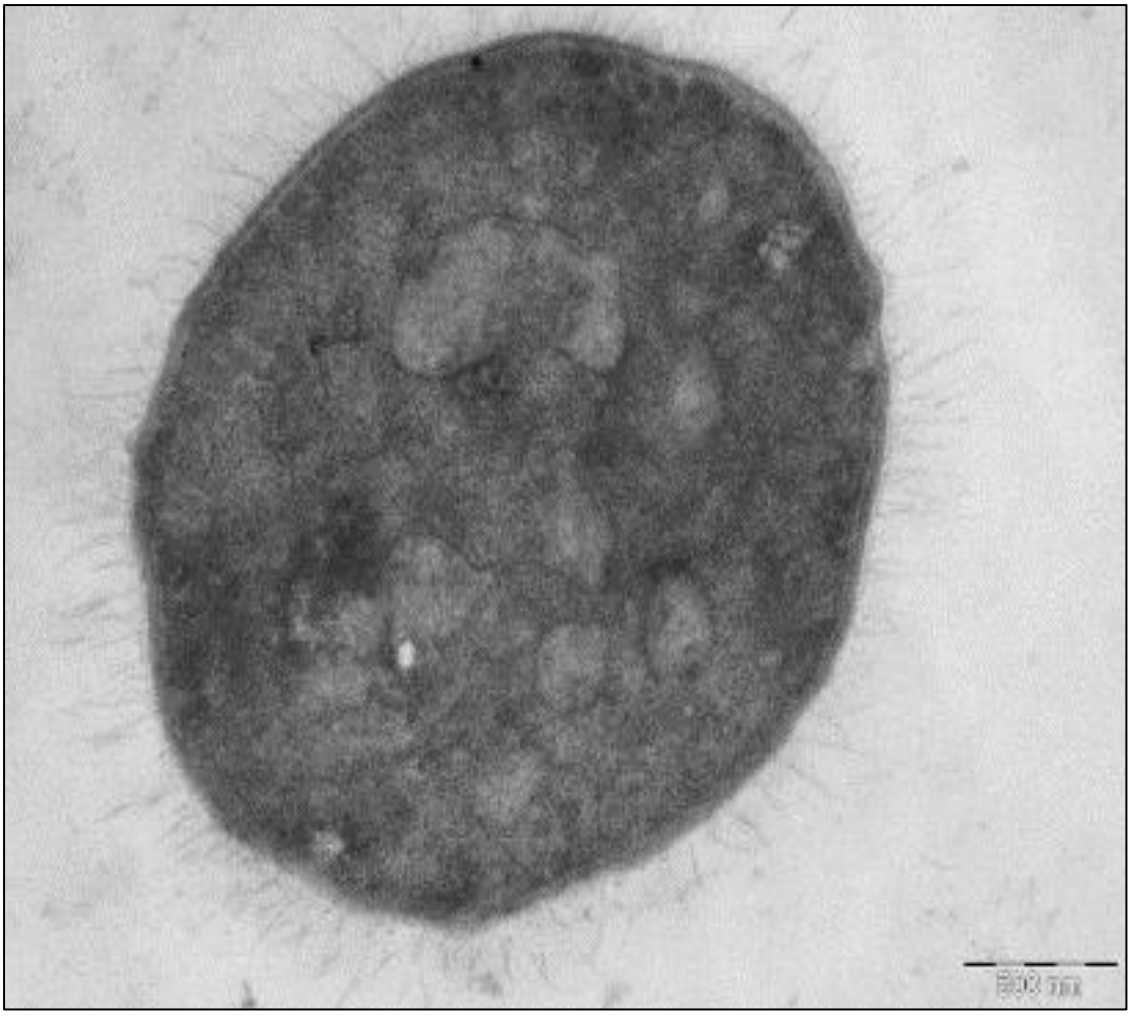
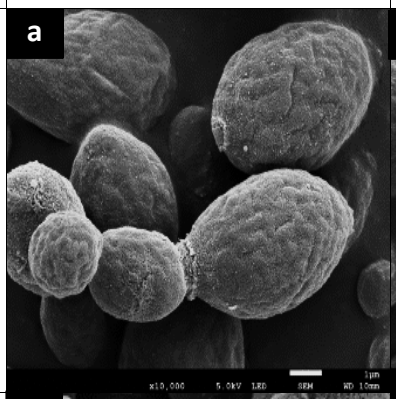
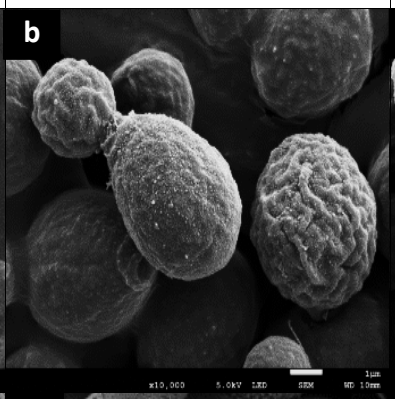
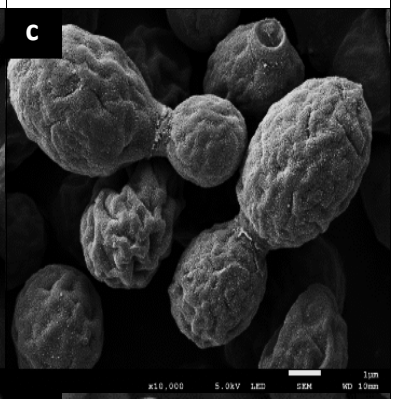
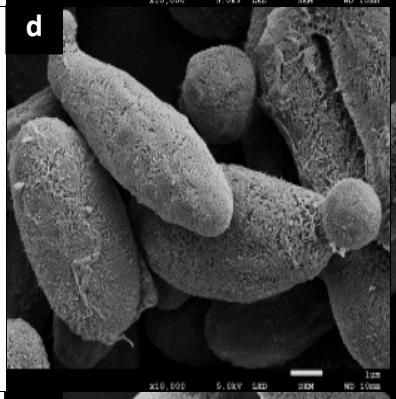
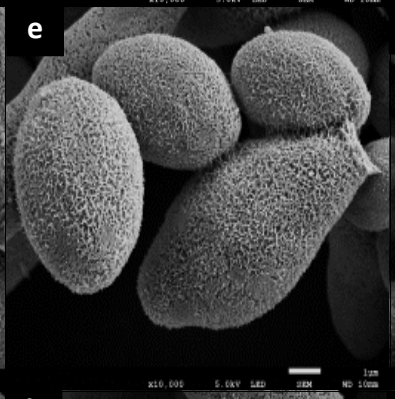
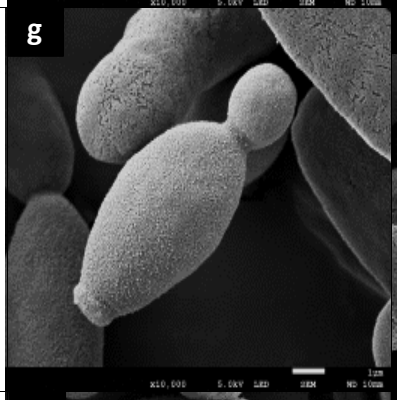
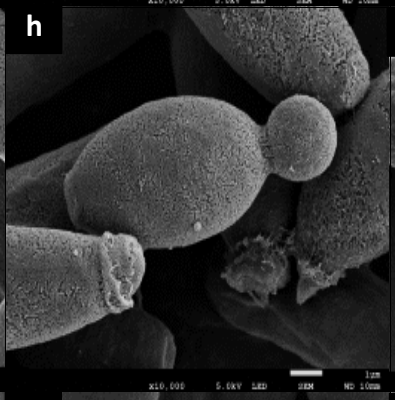
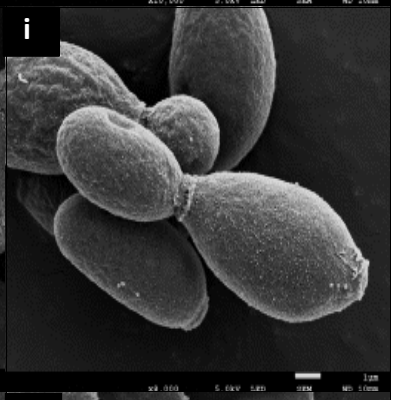
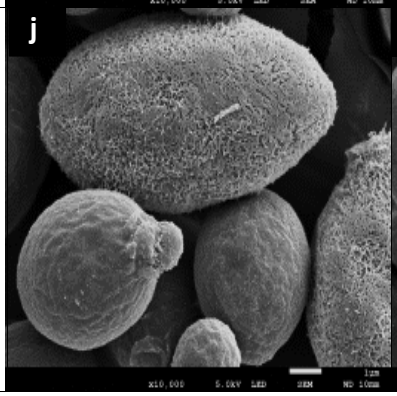
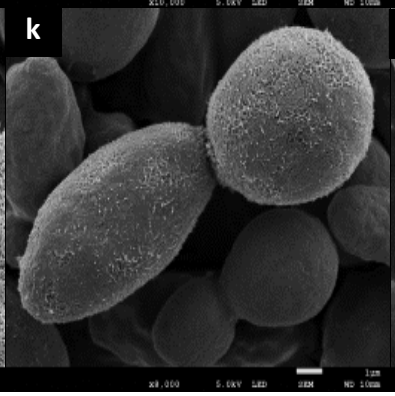
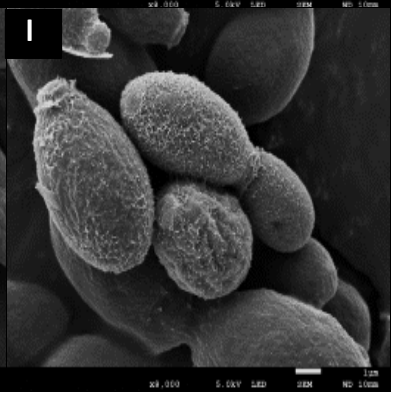


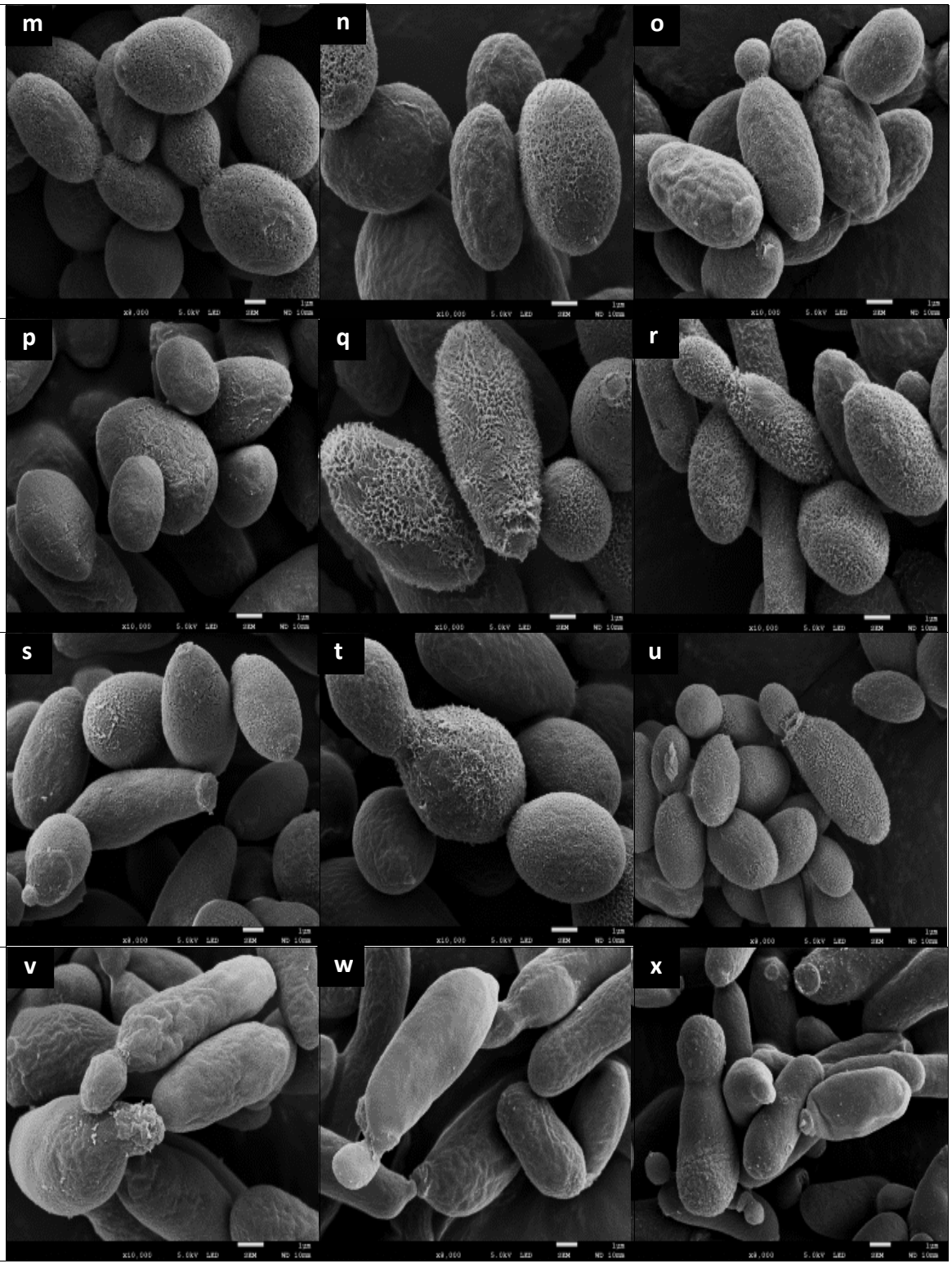
Figure 3.3: TEM micrograph of *M. frigida* UOFS Y-2927 cultivated in 2% YMGlucose media.

3.4.4 Scanning Electron Microscopy (SEM)

Scanning Electron Microscopy was done on all the yeasts in this study due to the lack of SEM images of psychrophilic yeasts in literature. The surface of these cells shows finger-like structures (Fig. 3.4e, Fig. 3.4f, Fig. 3.4j, Fig. 3.4k, Fig. 3.4l, Fig. 3.4n, Fig. 3.4q, Fig. 3.4r, Fig. 3.4t, Fig. 3.4cc), similar to the structures observed during TEM analysis (Fig. 3.2d, Fig. 3.2f, Fig. 3.2i, Fig. 3.2j, Fig. 3.2l, Fig. 3.2m, Fig. 3.2n, Fig. 3.2p, Fig. 3.2q, Fig. 3.2r, Fig. 3.2s, Fig. 3.3). Even though SEM does not contribute to the detection of intracellular gas bubbles, SEM was performed on the psychrophilic yeasts as little to no SEM imaging are available in literature of these yeasts.

Yeast	2% Glucose	6% Glucose	2% Galactose/2% Glycerol
M. frigida UOFS Y-2926			
M. frigida UOFS Y-2927			
M. frigida UOFS Y-2907			
M. frigida UOFS Y-2929			

M. frigida UOFS Y-2928	m	n	o
M. gelida UOFS Y-2904	p	q	r
M. gelida UOFS Y-2905	s	t	u
M. curviuscula UOFS Y-2909	v	w	x



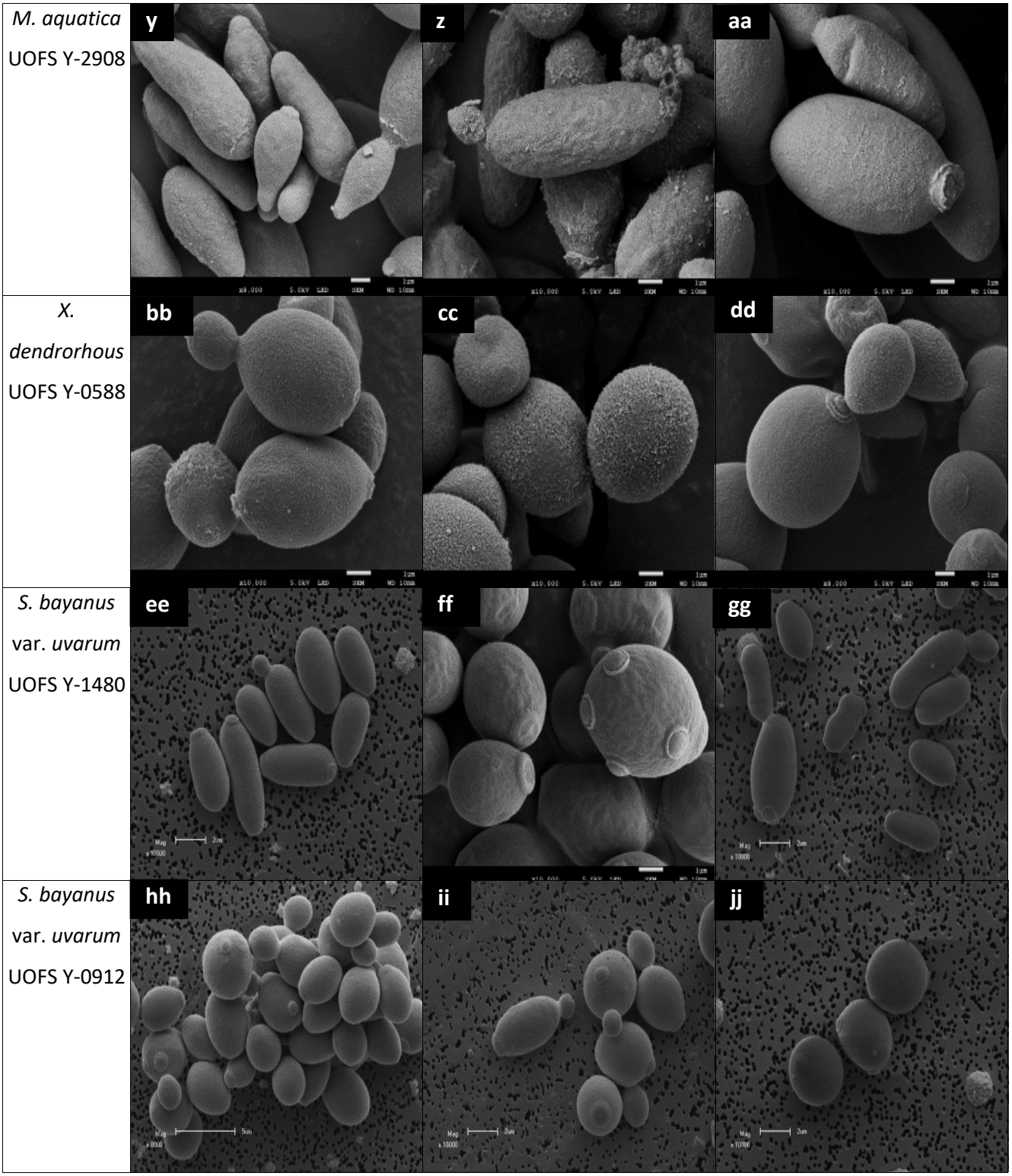


Figure 3.4: Scanning Electron Microscopy images of psychrophilic yeasts cultivated in 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media.

3.4.5 Nano Scanning Auger Microscopy (NanoSAM)

Nano Scanning Auger Microscopy (NanoSAM) was used to analyze the yeast cells in order to verify the results obtained with LM and TEM. During NanoSAM analyses the cell is etched with an argon gun followed by SEM imaging to obtain a 3-Dimentional image of the cell. *Mrakia frigida* UOFS Y-2926 cultivated in 6% YMGlucose and 2% YPGalactose were selected for NanoSAM analyses. Gas bubbles were observed in the fermenting cells (Fig. 3.5a) and were absent in non-fermenting cells (Fig. 3.5b).

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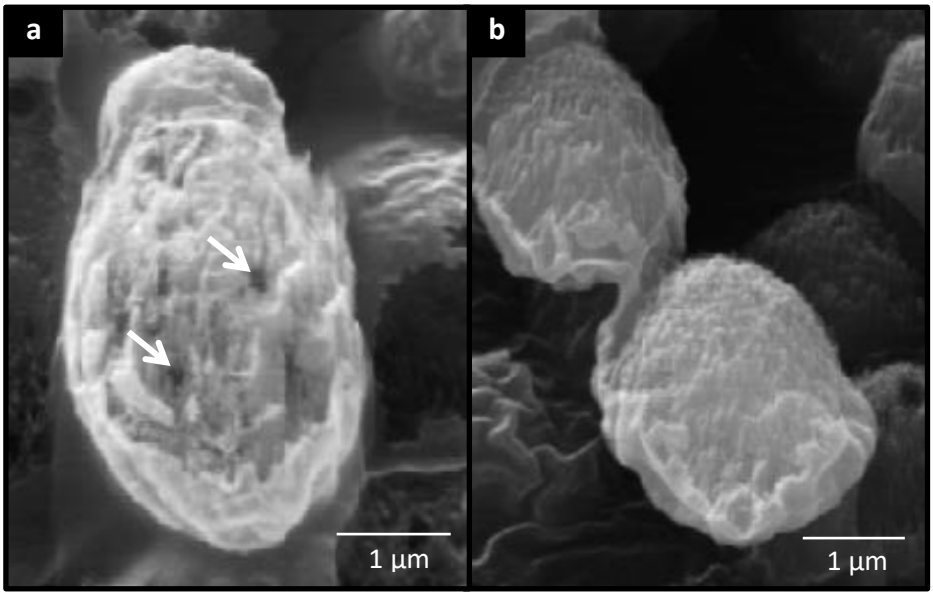

Figure 3.5: Nano Scanning Auger Microscopy images. a) *Mrakia frigida* UOFS Y-2926 cultivated in 6% YMGlucose media, b) *Mrakia frigida* UOFS Y-2926 cultivated in 2% YMGalactose media. Arrows indicate the presence of gas bubbles in the cells.

3.4.6 Lipid Extraction

In literature, light scattering granules observed with LM and electron transparent structures observed with TEM have been described and labelled as lipid droplets. The main difference between gas bubbles and lipid droplets are that gas bubbles are not surrounded by a membrane and consist of gas (Swart *et al*., 2012), where lipid droplets consist of neutral lipids and are surrounded by a phospholipid layer and proteins (Jacquier *et al*., 2011). Since gas bubbles and lipid droplets are similar in appearance, it is necessary to distinguish between gas bubbles and lipid droplets and determine whether the structures observed in section 3.3.3 and 3.3.4 are gas bubbles or lipid droplets. If the structures observed are lipid droplets, it is

expected that the total lipid content of the cells containing these structures would be more per gram biomass than the cells without these structures. In addition, if the structures observed are lipid droplets, it is expected that the total lipid content per gram biomass would increase when there is an increase in glucose concentration. To determine the total lipid content of the yeast cells, lipid extraction was done on all the yeasts strains (Fig. 3.6). The p-value was used to determine whether the data obtained is statistically significant. A p-value smaller or equal to 0.05 indicates the hypothesis was rejected and a p-value greater than 0.05 indicates the hypothesis was accepted. Even though the standard deviations are large, the statistical analyses confirm that the structures observed within the yeast cells are not lipid droplets (Table 3.3).

Table 3.3: Analysis of variance (ANOVA) on the effect of carbon source on total fat content when cultivated on glucose compared to galactose as non-fermentable carbon source. The yeast strains used were *M. frigida* UOFS Y-2926, UOFS Y-2927, UOFS Y-2907, UOFS Y-2929, UOFS Y-2928, *M. gelida* UOFS Y-2904, UOFS Y-2905, *M. curviuscula* UOFS Y-2909 and *M. aquatica* UOFS Y-2908 and the values in the table are collective values of the total fat content of the yeasts mentioned.

Carbon Source	**Total Fat (%)**
2%Gal (n = 36)	10.67 ± 6.65
2%Gluc (n = 36)	11.16 ± 6.39
6%Gluc (n = 36)	10.00 ± 9.13
Significance level	p = 0.8045

Means with different superscripts in the same column differ significantly.

Table 3.4: Analysis of variance (ANOVA) on the effect of carbon source on total fat content when cultivated on glucose compared to glycerol as non-fermentable carbon source. The yeast strains used were *S. bayanus* var. *uvarum* UOFS Y-1480, UOFS Y-0912 and *X. dendrorhous* UOFS Y-0588 and the values in the table are collective values of the total fat content of the yeasts mentioned.

Carbon Source	**Total Fat (%)**
2%Glyc (n = 12)	11.27 ± 6.55
2%Gluc (n = 12)	10.91 ± 6.73
6%Gluc (n = 12)	7.90 ± 2.87
Significance level	p = 0.2937

Means with different superscripts in the same column differ significantly.

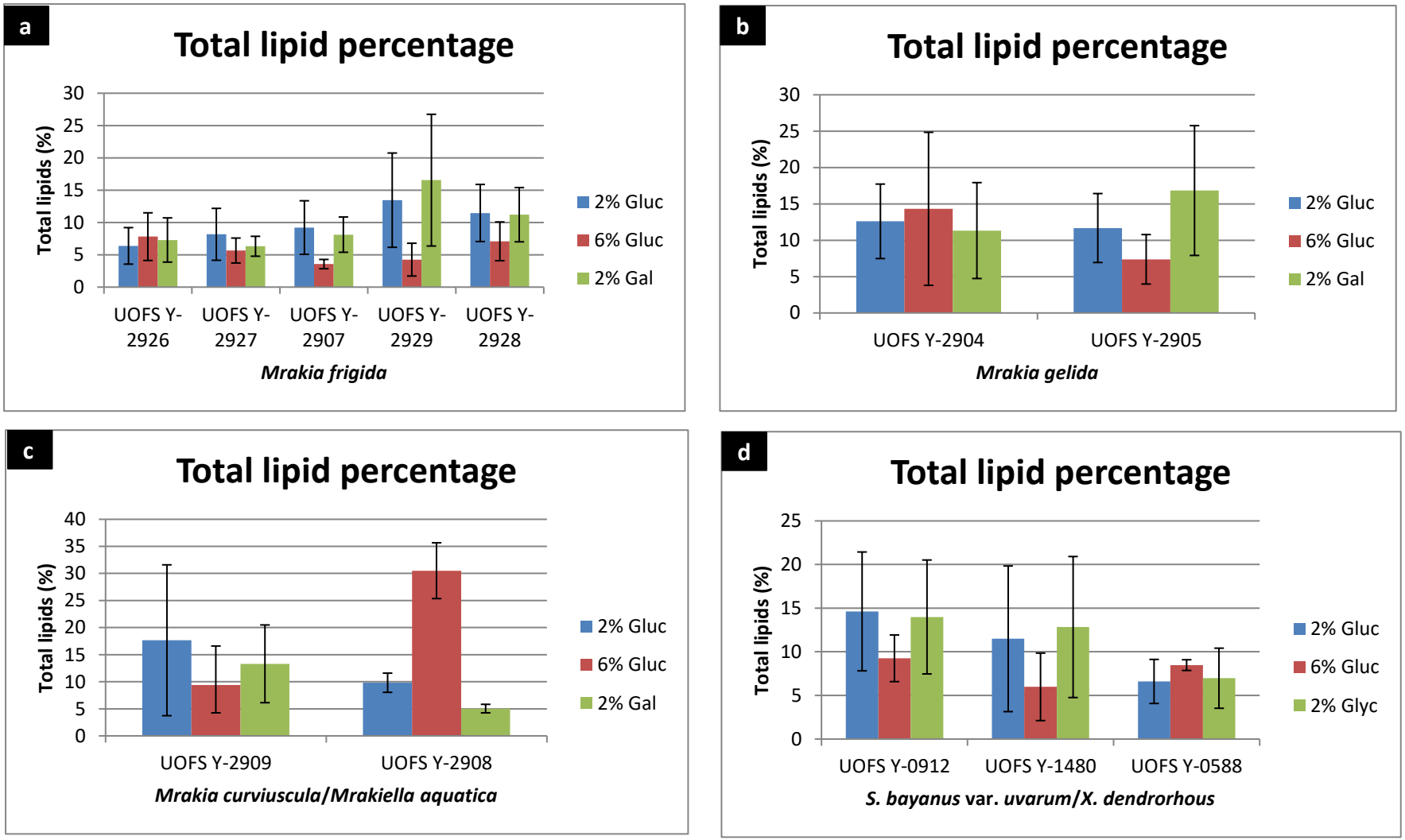

Figure 3.6: Lipid extraction results of psychrophilic yeasts cultivated on 2% YMGlucose, 6% YMGlucose and 2% YPGalactose/YPGlycerol media.

4.3.7 Confocal Laser Scanning Microscopy

The lipid extraction results showed an increase in the total lipid content with an increase in glucose concentration in *Mrakiella aquatica* (Fig. 3.6c), therefore the structures observed in Fig. 3.1y, Fig. 3.1z, Fig. 3.1aa, Fig. 3.2z and Fig. 3.2aa might be lipid droplets rather than gas bubbles. To confirm this, *M. aquatica* cells were stained with 4,4-Difluoro-1,3,5,7,8-Pentamethyl-4-Bora-3a,4a-Diaza-s-Indacene (BODIPY) to confirm the lipid extraction results in *M. aquatica*. The BODIPY stain stains the lipids within the cells. Green fluorescence is observed when subjected to Confocal Laser Scanning Microscopy (CLSM). *Mrakiella aquatica* cultivated in fermentable 2% YMGlucose media contained unstained light scattering granules and green lipid droplets within the cell (Fig. 3.7a, Fig. 3.7b, Fig. 3.7c). Similar results were obtained when this yeast was cultivated in fermentable 6% YMGlucose media (Fig. 3.8a, Fig. 3.8b, Fig. 3.8c). When cultivated in non-fermentable 2% YPGalactose media, only green lipid droplets were observed and no unstained light scattering granules (Fig. 3.9a, Fig. 3.9b, Fig. 3.9c). These results show that even though there is an increase in lipid production with the increase in glucose concentration, the gas bubbles are also present in the fermenting cells. Since no membrane is observed in the structures observed in Fig. 3.2y, Fig. 3.2z and Fig. 3.2aa, and the CLSM results show the presence of light scattering granules and lipids in the cell, the structures observed are not only gas bubbles, but lipid droplets as well.

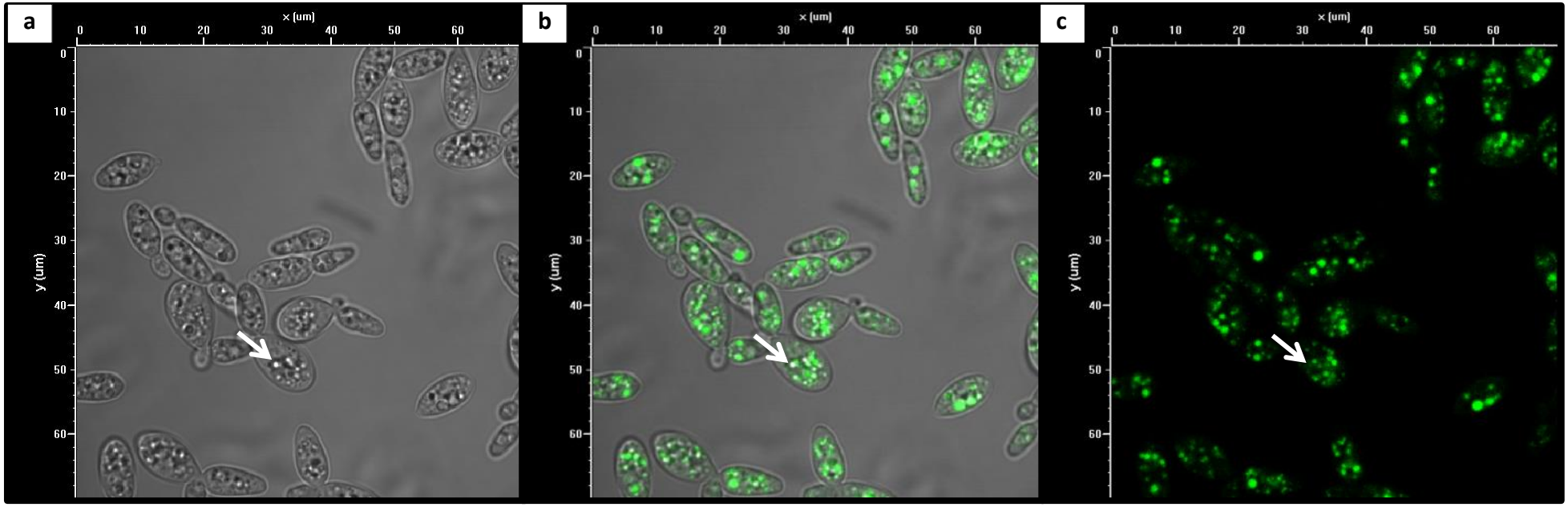

Figure 3.7: Confocal Laser Scanning Microscopy images of *M. aquatica* cultivated in 2% YMGlucose media and stained with BODIPY stain. Green fluorescence indicates lipids within the cells and arrows indicate light scattering granules. a) Light micrograph, b) light micrograph with green fluorescence, c) green fluorescence micrograph.

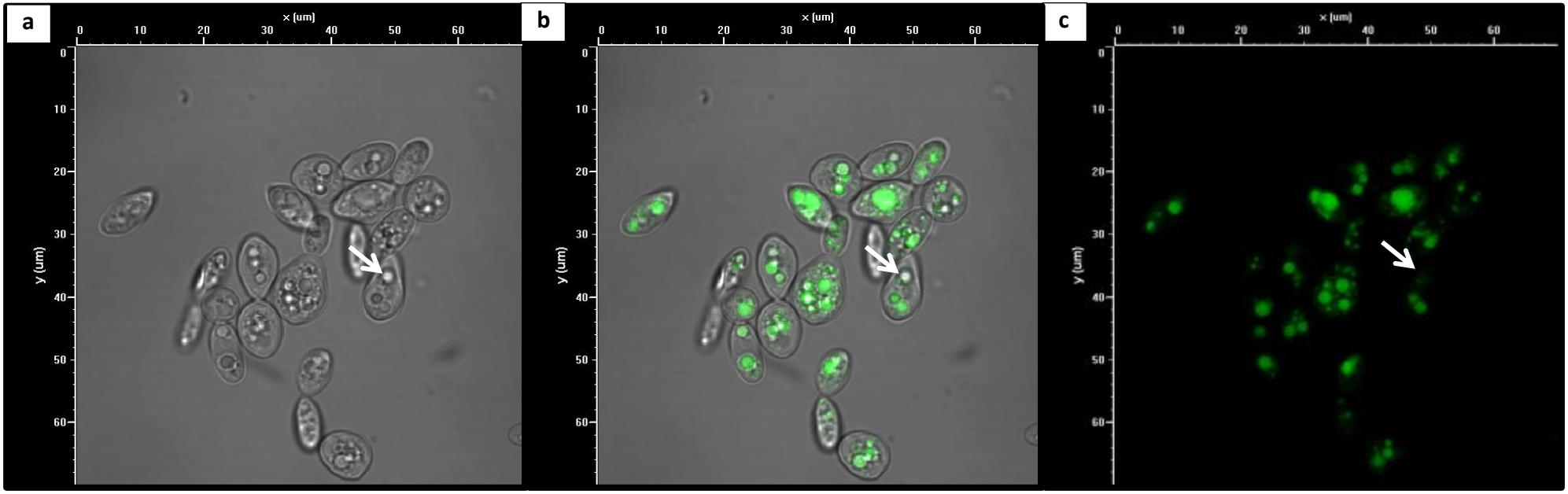

Figure 3.8: Confocal Laser Scanning Microscopy images of *M. aquatica* cultivated in 6% YMGlucose media and stained with BODIPY stain. Green fluorescence indicates lipids within the cells and arrows indicate light scattering granules. a) Light micrograph, b) light micrograph with green fluorescence, c) green fluorescence micrograph.

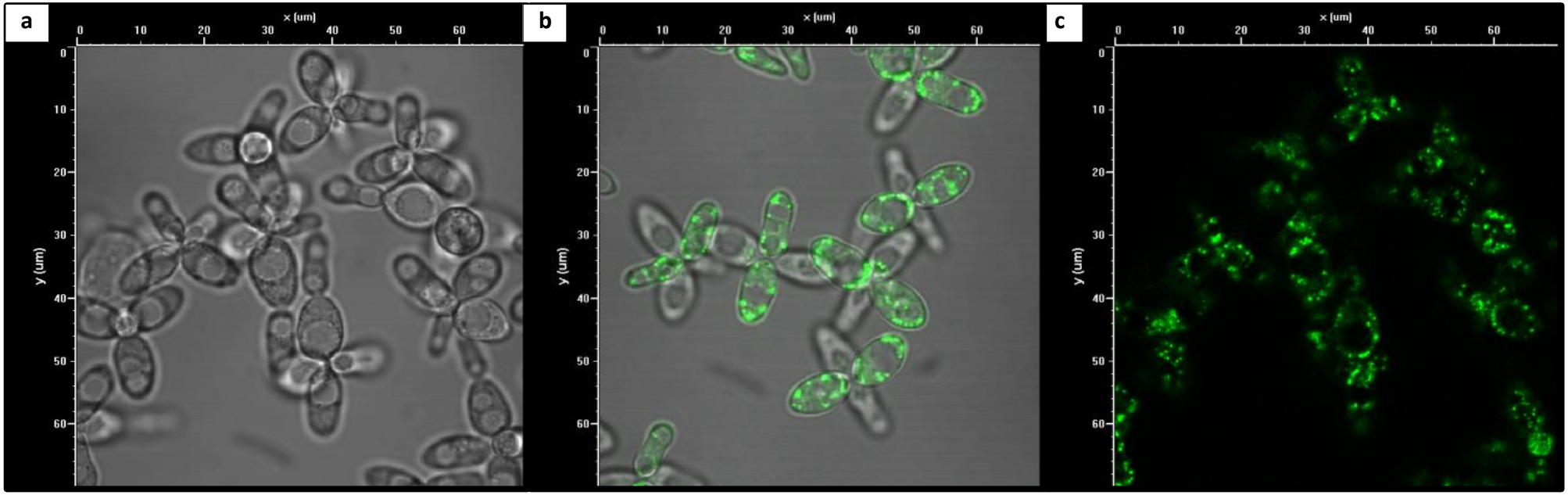

Figure 3.9: Confocal Laser Scanning Microscopy images of *M. aquatica* cultivated in 2% YPGalactose media and stained with BODIPY stain. Green fluorescence indicates lipids within the cells. a) Light micrograph, b) light micrograph with green fluorescence, c) green fluorescence micrograph.

4.3.8 Weight determination

The weight of a single cell of *Saccharomyces bayanus* var. *uvarum* UOFS Y-1480 was calculated to determine whether the presence of gas bubbles influences the weight of a yeast cell. The cell count was determined using a hemocytometer and the cells were freeze dried and weighed. It is expected that a fermenting yeast cell containing gas bubbles would weigh less than a respiring yeast cell with no gas bubbles since the gas bubbles occupy large areas of the cell. From the dataset the average weight of one fermenting yeast cell and one respiring yeast cell was calculated (Table 3.5) and the data was plotted on graphs (Fig. 3.10 & Fig. 3.11). No significant difference could be observed between fermenting and respiring cells.

Table 3.5: Weight of one cell of *Saccharomyces bayanus* var. *uvarum* UOFS Y-1480.

6% YMGlucose		2% YPGlycerol	
Average weight (mg)	Standard deviation	Average weight (mg)	Standard deviation
2.33×10^{-7}	3.01×10^{-7}	2.61×10^{-7}	2.69×10^{-7}

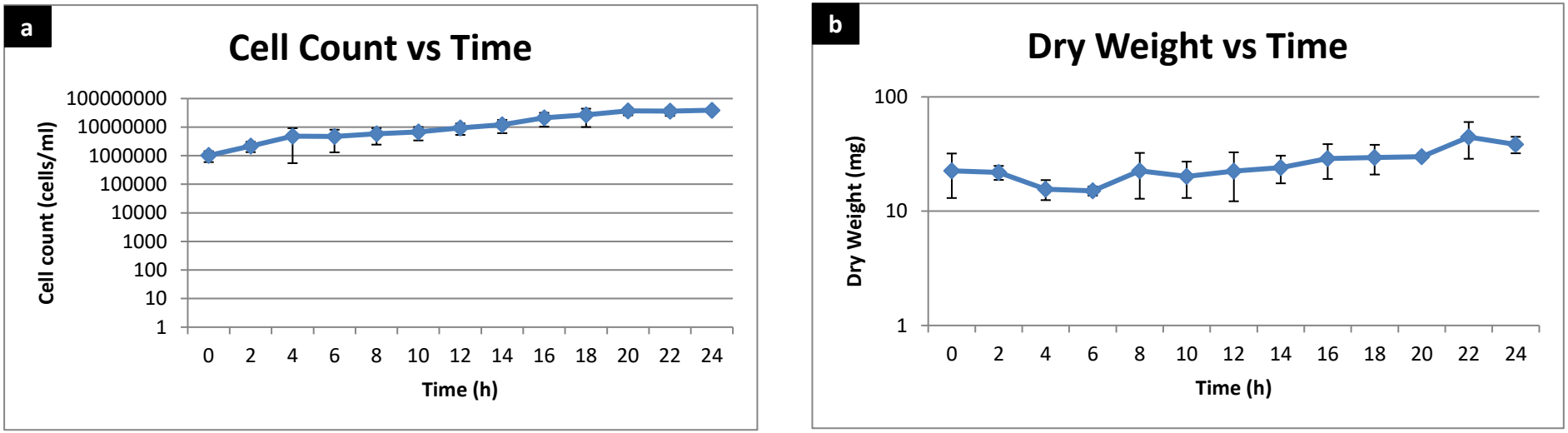

Figure 3.10: Weight determination graphs of *Saccharomyces bayanus* var. *uvarum* UOFS Y-1480 cultivated in 6% YMGlucose media.

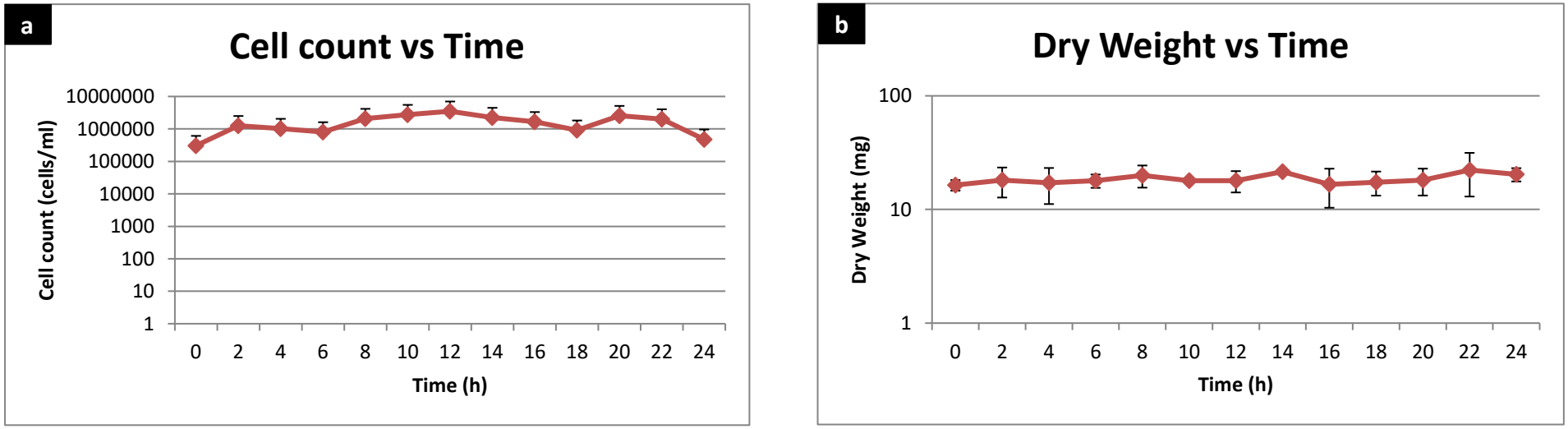

Figure 3.11: Weight determination graphs of *Saccharomyces bayanus* var. *uvarum* UOFS Y-1480 cultivated in 2% YPGlycerol media.

3.6 Conclusions

Gas bubbles have been found in several organisms during fermentations at 30°C. This lead to the question whether gas bubbles is also present during low temperature fermentations. Psychrophilic yeasts were cultivated in fermentable 2% YMGlucose and 6% YMGlucose media and non-fermentable 2% YPGalactose/YPGlycerol media and subjected to Light Microscopy (LM), Transmission Electron Microscopy (TEM), Scanning Electron Microscopy (SEM) and Nano Scanning Auger Microscopy (NanoSAM). Gas bubbles were observed as light scattering granules when viewed with LM, non-enveloped electron transparent structures when viewed with TEM and holes in the cells when viewed with NanoSAM. These results are similar to the results found by Swart and Co-workers in *Saccharomyces* (Swart *et al*., 2012). The SEM and TEM images show the presence of finger-like structures on the surface of these cells. An increase in bubble-like structures was observed with the increase in glucose concentration in the TEM results. The question arose whether these structures are gas bubbles or lipid droplets. Lipid extraction was done, and the total lipid content of the cells was calculated to verify whether there is an increase in lipid production with the increase in glucose concentration. No significant difference was observed between the lipid content when cultivated in different media, suggesting these structures are gas bubbles rather than lipid droplets. *Mrakiella aquatica* was stained with BODIPY stain to which confirmed the presence of lipids and gas bubbles during fermentation and the presence of only lipids during respiration. The weight of one yeast cell during fermentation and respiration was calculated, however the results showed no significant difference. The presence of gas bubbles in psychrophilic yeasts confirms that these structures are conserved in yeast. Future research might include the biotechnological effect of gas in yeast cell and investigating the mechanism of release of the gas bubbles to the cell environment.

3.7 References

Barnett, J., Payne, R. & Yarrow, D. (2000). Yeasts: characteristics and identification, 3rd edn. Cambridge University Press, Cambridge, UK.

Blatteau, J-E., Souraud, J-B., Gempp, E. & Boussuges, A. (2006). Gas nuclei, their origin, and their role in bubble formation. *Aviat Space Environ Med* **77(10)**, 1068-2076.

Butte, W. (1983). Rapid method for the determination of fatty acid profiles from fats and oils using trimethyl sulphonium hydroxide for transesterification. *J Chromatogr* **261**, 142-145.

Folch, J., Lees, M. & Sloane-Stanley, G. H. (1957). A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem* **226**, 497-509.

Harris, L-A. L. S., Skinner, J. R. & Wolins, N. E. (2013). Imaging of neutral lipids and neutral lipid associated proteins. *Methods Cell Biol* **116**, DOI: 10.1016/B978-0-12-408051-5.00011-5.

Jacquier, N., Choudhary, V., Mari, M., Toulmay, A., Reggiori, F. & Schneiter, R. (2011). Lipid droplets are functionally connected to the endoplasmic reticulum in *Saccharomyces cerevisiae*. *J Cell Sci* **124**, 2424-2437.

Kock, J. L. F., Swart, C. W. & Pohl, C. H. (2011). The anti-mitichondrial antifungal assay for the discovery and development of new drugs. *Expert Opin Drug Discov* **6**, 671-681.

Kurtzman, C. P. & Fell, J. W. (1998). The yeasts: a taxonomic study 4th edn. Elsevier, Amsterdam.

Mahon, R. T. (2010). Tiny bubbles. *J Appl Physiol* **108**, 238-239.

Mishra, P. & Prasad, R. (1989). Relationship between ethanol tolerance and fatty acyl composition of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* **30**, 294-298.

Rumin, J., Bonnefond, H., Saint-Jean, B., Rouxel, C., Sciandra, A., Bernard, O., Cadoret, J-P. & Bougaran, G. (2015). The use of fluorescent Nile red and BODIPY for lipid measurement in microalgae. *Biotechnol Biofeul* **8:42**, DOI: 10.1186/s13068-015-0220-4.

Swart, C. W., Dithebe, K., Pohl, C. H., Swart, H. C., Coetsee, E., Van Wyk, P. W. J., Swarts, J., Lodolo, E. J. & Kock, J. L. F. (2012). Gas bubble formation in the cytoplasm of a fermenting yeast. *FEMS Yeast Res* **12,** 867-869.

Swart, C. W., Swart, H. C., Coetsee, E., Pohl, C. H., Van Wyk, P. W. J. & Kock, J. L. F. (2010). 3-D architechture and elemental composition of fluconazole treated yeast asci. *Sci Res Essays* **5(22)**, 3422-3417.

Van Dijken, J. P., Van den Bosch, E., Hermans, J. J., De Miranda, L. R. & Scheffers, W. A. (1986). Alcoholic fermentation by 'non-fermentative' yeasts. *Yeasts* **2**, 123-127.

Chapter 4

Summary

Summary

In recent research on the production of intracellular gas bubbles in yeast during fermentation, bubble-like structures were observed in 16 organisms during fermentation. These structures were absent or present in small numbers during respiration. Different parameters have been explored in the search for gas bubbles, but bubble-like structures have only been observed during 30°C fermentations. In this study, the production of gas bubbles during low temperature fermentations in psychrophilic yeasts was explored. The yeasts were cultivated in fermentable and non-fermentable media. The fermentable media was used at two different concentrations. Bubble-like structures were observed in the yeasts cultivated in fermentable media using Light Microscopy, Transmission Electron Microscopy (TEM) and Nano Scanning Auger Microscopy. Additionally, finger-like structures were observed on the cell surface of some of the psychrophilic yeast cells with TEM and Scanning Electron Microscopy. When cultivating the yeasts in non-fermentable media, the gas bubbles were absent or present in low numbers. *Mrakia curviuscula* was used as a control since this yeast does not have the ability to ferment. An increase in bubble-like structures were observed when the glucose concentration of the fermentable media was increased, therefore to confirm that the bubble-like structures are gas bubbles rather than lipid droplets, the total lipid content of the yeast cells was determined and Confocal Laser Scanning Microscopy (CLSM) using BODIPY stain was done. No significant increase in lipid content was observed, suggesting that the structures are gas bubbles and a clear difference between lipid droplets and gas bubbles were observed with CLSM. The difference between the weight of a fermenting and non-fermenting yeast cell was determined to determine fermenting cells containing gas weigh less than a respiring yeast cell, however the results were not significant. The bubble-like structures observed in this study were similar to the structures observed by Swart and Co-workers in *Saccharomyces*. The presence of gas bubbles in psychrophilic yeasts confirms that these structures are conserved in yeast. Future research might include the biotechnological effect of gas in yeast cells and investigating the mechanism of release of the gas bubbles to the cell environment.

Keywords: Carbon dioxide, Fermentation, Intracellular gas bubbles, NanoSAM, Psychrophilic, Yeast