

Gas bubble formation in fermenting and non-fermenting yeasts

by

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Submitted in fulfilment of the requirements for the degree

Magister Scientiae

In the

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February 2016

*This dissertation is dedicated to my family: my mother,
P. S. Kgotle, my brother, M. E. Kgotle and my best friend,
M. D. Tsoene. My deceased grandmother, M. A. Tladi
and cousin, M. M. Mosia.*

Acknowledgements

It is imperative that I thank the following:

- ⊖ **God**, for providing me with the strength and ability to complete this M.Sc.

- ⊖ **Dr C. W. Swart**, for the advice and guidance, the patience and support she showed throughout the study and also being caring and understanding.

- ⊖ **My Family**, for the continued support and encouragement to keep pursuing my dreams and studies.

- ⊖ **Prof. J. L. F. Kock & Prof. C. H. Pohl**, for the opportunity, the guidance and believing in my M.Sc. project.

- ⊖ **Prof. P. W. J. van Wyk & Ms. H. Grobler**, for all the training and the valuable assistance with electron microscopy.

- ⊖ **Prof. H. C. Swart, Dr E. Coetsee & Dr M. M. Duvenhage**, for assistance with the NanoSAM and ToF-SIMS.

- ⊖ **Mrs. A. van Wyk**, for providing the yeasts that were used in the study.

- ⊖ **Mr. S. Collett**, for the graphics.

- ⊖ **National Research Fund (NRF)**, for the financial support.

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Chapter 1

Introduction

1.1. Motivation

Yeasts are the most commonly known eukaryotic organisms exploited for their metabolic capabilities and have become one of the main focal points in numerous food and biotechnological processes (Faria-Oliveira *et al.*, 2013). Certain yeasts play an important role in the production of bread, alcoholic beverages and dairy products, to name but a few, through the process of fermentation (Van Dijken *et al.*, 1986; Faria-Oliveira *et al.*, 2013).

Fermentation is a catabolic metabolism of sugar to provide energy, which is in turn used for the production of numerous compounds such as glycolytic compounds, enzymes and membrane-regulating proteins needed by the cell. Alcoholic fermentation includes the process of glycolysis and the pathway for ethanol production that specifically produces ethanol and carbon dioxide (CO₂) as by-products (Van Urk *et al.*, 1990; Faria-Oliveira *et al.*, 2013). Respiration, on the other hand, is also a catabolic process where sugars are utilized to provide energy for the yeast cells, yet it differs from fermentation in that it uses oxygen (O₂) as the primary electron acceptor. Unlike fermentation, respiration includes glycolysis, the citric acid cycle and the electron transport chain, where O₂ is the electron acceptor involved in the direct production of adenosine triphosphate (ATP) by oxidative phosphorylation. This process results in high energy production, which in turn results in high biomass production and accumulation of reserved carbohydrates (Van Urk *et al.*, 1990; Faria-Oliveira *et al.*, 2013).

Even though both fermentation and respiration are able to produce CO₂, there is a major difference in the quantity that each metabolism can produce. There are three different pathways of metabolism that can affect the mode of CO₂ production by yeasts i.e. (1) Crabtree-positive yeasts can undergo both fermentation and

respiration under oxic conditions with glucose excess; (2) Crabtree-negative yeasts can also undergo both fermentation and respiration, however fermentation only occurs under anoxic conditions, while respiration occurs under oxic conditions (Van Urk *et al.*, 1990; González Siso *et al.*, 1996) and (3) yeasts that can only respire under oxic conditions and are unable to grow under anoxic conditions (De Deken, 1966; Van Urk *et al.*, 1990). These modes of CO₂ production are distributed throughout the yeast domain, with Crabtree-positive yeasts, such as *Saccharomyces*, mostly used in industry where fermentation is the main goal. Since CO₂ is one of the main by-products of alcoholic fermentation, the expectation is that CO₂ would accumulate in the cytoplasm of vigorously fermenting yeasts in the form of gas bubbles.

Before 2012, there have however been no reports on the presence of gas bubbles inside cells. This could be ascribed to the work done by Hemmingsen and co-workers (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1990), which suggested that gas bubbles cannot be formed in the cytoplasm of microorganisms even under high gas supersaturation. Conversely in 2012, Swart and co-workers discovered areas inside the cytoplasm of the fermenting yeast *Saccharomyces* resembling vacuoles. However, these areas were found to lack surrounding membranes that are characteristics of true cell organelles and therefore it was concluded that these structures were gas bubbles (Swart *et al.*, 2012). Additional information about the newly discovered gas bubbles was obtained in 2013, when these inclusions were found to occupy a large portion of the cell, in turn compressing and deforming the membranes of other important cell organelles (Swart *et al.*, 2013). It was also uncovered that these gas bubbles occur in the cytoplasm of the distantly related fission yeast *Schizosaccharomyces pombe*.

Since it was observed that both the distantly related *Saccharomyces* and *Schizosaccharomyces* contain gas bubbles, it can be postulated that all yeasts in the yeast domain may contain gas bubbles. This leads to the question of whether the gas bubble formation phenomenon is conserved throughout the yeast domain. Another point of interest is the content of these bubbles i.e. if other compounds besides CO₂ are present.

Consequently, this study aims (1) to determine if gas bubble formation is conserved in the yeast domain by investigating both fermenting and non-fermenting yeasts, (2) to evaluate any differences in the number of gas bubbles produced in fermenting and non-fermenting yeasts and (3) to characterise the gas bubbles found in fermenting yeasts to identify any gaseous compounds other than CO₂. This work may provide additional information on the function of these gas bubbles, since it is well established that CO₂ formation and release plays an important role in yeast fermentation performance. This has great relevance to the brewing industry for example, where it can aid in optimisation of fermentation parameters to avoid the effects of CO₂ toxicity on fermentation performance and flavour formation. Furthermore, these gas bubbles were identified to exert pressure on the membranes of organelles in close proximity, which lead to the deforming of these organelles as well as the nucleus (Swart *et al.*, 2013). Therefore, it is proposed that these gas bubbles may not only impact cell metabolism but may also contribute to the increased pressure formed inside fermenting yeast cells (Vella *et al.*, 2012).

1.2. What are gas bubbles?

According to Blatteau and co-workers, gas bubbles are intracellular inclusions that can contain one type or a mixture of gases (Blatteau *et al.*, 2006). These structures are thought to be enclosed by an organic skin consisting of water molecules, which may be initially permeable, therefore allowing rapid diffusion of gas into the gas bubble (Fox & Herzfeld, 1954; Strauss & Kunkle, 1974; Yount, 1979). The formed gas bubbles can be symmetrical or asymmetrical spheres that range from a few nanometres to several millimetres in size (Blatteau *et al.*, 2006; Mahon, 2010). Gas bubbles were initially believed to have evolved from small particles filled with gas, called gas micronuclei, which can have a diameter less than 10 μm (Mahon, 2010). Therefore, gas micronuclei are the first step in gas bubble formation (Dean, 1944; Arieli *et al.*, 2002). If gas micronuclei are less than 2 μm in diameter they usually become dispersed in solution, yet if the diameter is greater than 2 μm it separates from the solution and forms the starting point of visible gas bubble formation. Early studies suggest that gas bubbles can be induced by gas supersaturation followed by decompression (Arieli *et al.*, 2002). The rate of bubble growth depends on gas diffusion, which can be influenced by numerous factors such as diffusion constant, pressure difference and gas solubility (Yount, 1979; Blatteau *et al.*, 2006). This would mean that during the expansion/growth of a gas bubble, the size and contents will eventually change according to diffusion of gas in and out of the bubble, where the rate of diffusion might be controlled by the concentration of a certain gas in the surrounding environment (Dean, 1944; Blatteau *et al.*, 2006). Another possibility is that the formation of bigger bubbles is induced by the coalescing of smaller gas bubbles (Mahon, 2010). This can be due to the fact that these gas bubbles are

separated from the environment by a thin skin of water which in turn stabilizes the formed gas bubble.

1.3. History of gas bubble formation in living cells

In 1979 Hemmingsen and Hemmingsen conducted a series of experiments to investigate the effects of high gas supersaturation on certain microorganisms in order to evaluate if gas supersaturation can induce intracellular gas bubble formation. From this study it was concluded that intracellular gas bubbles cannot be formed in the cytoplasm of microorganisms even at high gas supersaturation. Their study included eukaryotic, unicellular (yeasts) and simple multicellular organisms (protozoans) that were exposed to gas supersaturations of nitrogen (N), argon (Ar) and helium (He) followed by rapid decompression. Except for the yeast, the gas supersaturation caused cell death of a portion of the cells of the other organisms. Consequently Hemmingsen and Hemmingsen deduced that cell death may be caused by extracellular bubble formation or other factors involved (Hemmingsen & Hemmingsen, 1979). Extracellularly formed bubbles on the cell surface may initiate mechanical forces as a result of surface tension action and turbulences. Therefore this can lead to the bubbles rapidly expanding and rupturing the cells with no enveloping membrane or cell wall. This resistance to bubble formation may be due to the unique properties of the cytoplasm, which consists of 70 – 80 % water and the possibility that intracellular water may differ from external water (Hemmingsen *et al.*, 1985).

Later in 1990, Hemmingsen and co-workers still suggested that microorganisms cannot form intracellular gas bubbles even at high gas supersaturation. After

numerous experiments conducted on different microorganisms; eukaryotic, unicellular (yeasts) and simple multicellular organisms (protozoans), they proved that these organisms are not susceptible to intracellularly formed gas bubbles (Hemmingsen & Hemmingsen, 1979). Therefore their experiments focused on induction of gas bubbles through phagocytosis of bubble promoting particles (graphite, carmine, tobermorite, bone black and latex beads). These particles are believed to promote bubble formation by having gas adsorbed on their surfaces (Dean, 1944) or trapped within them (Hemmingsen *et al.*, 1990; Arieli *et al.*, 2002) due to their strong hydrophobicity (Dean, 1944). It was found that these particles, such as graphite, can lose the ability to promote bubbles by being outgassed through heating or being soaked in water (Henrici, 1873; Dean, 1944). In amoeboid cells, such as slime molds, phagocytosis of bubble promoting particles was expected to induce intracellular bubbles. Yet, it was reported that these particles were unable to induce bubble formation after being ingested by the cells (Hemmingsen *et al.*, 1990). Since the difference in the external and internal environment of the cell has an effect on gas bubbles formation (Hemmingsen *et al.*, 1985), the phagocytosis of these particles by amoeboid cells did not produce gas bubbles (Hemmingsen *et al.*, 1990).

Interestingly, information on gas vesicles have been available for over a decade (Walsby, 1994) and provided the first evidence that gas can be accumulated and stored inside cells. In 1994, Walsby wrote an extensive review about gas vesicles and even though these intracellular inclusions are different from gas bubbles, they may provide additional information about what can be expected from gas bubbles / gas stored inside cells. Gas vesicles have a very distinct structure, i.e. cylindrical with conical caps at the ends. These cylinders are hollow, gas-filled and solely made of proteins. Similar to gas bubbles, gas vesicles are highly permeable to gases which

allow them to be constantly filled through gaseous diffusion (Cohen-Bazire *et al.*, 1969; Walsby, 1994). These organelles are unique in that they can prevent gas diffusion and gas dissipation on their own accord (Bowen & Jensen, 1964). Gas vesicles were initially identified in prokaryotes (mostly non-motile) from aquatic habitats, such as Cyanobacteria. They are important organelles that assist the organism in maintaining buoyancy in water by lowering the density of the cell. This in turn affects the rate at which a cell can float or sediment, respectively in water (Bowen & Jensen, 1964; Cohen-Bazire *et al.*, 1969; Walsby, 1974; 1994). This manipulation of buoyancy provides these aquatic microorganisms with the ability to survive in these habitats, such as enabling the Cyanobacteria to float up towards the light for photosynthesis and allowing aerophilic bacteria to float into oxygenated surface water for respiration (Walsby, 1994). A group of gas vesicles become components of a compound cell organelle called a gas vacuole (Cohen-Bazire *et al.*, 1969). When viewed with the light microscope (LM) these gas vesicles appear as granules inside the cell due to the fact that they are highly refractile organelles resembling air bubbles (Bowen & Jensen, 1964; Walsby, 1974; 1994).

In 2012, gas bubbles were discovered as intracellular inclusions in the budding yeast *Saccharomyces* (Swart *et al.*, 2012). This was an unexpected discovery since it has previously been stated by Hemmingsen and Hemmingsen (1979) that intracellular gas bubbles could not be formed in the cytoplasm of microorganisms. Swart and co-workers (2012) conducted a series of experiments to investigate the formation of gas bubbles in yeasts, which provided information about these intracellular inclusions. Experiments included microscopic techniques such as LM, which was used to identify light scattering granules similar to the gas vesicles observed in the Cyanobacteria. These observations led to the use of transmission electron

microscopy (TEM) to verify the existence of gas bubbles inside yeast cells. With TEM the light scattering granules observed with LM could be seen as electron transparent structures (Fig. 1a) (Swart *et al.*, 2012) that lack a surrounding membrane (Fig. 1b) (Swart *et al.*, 2013), which is a key characteristic of true cell organelles. The final verification step involves the use of nano scanning Auger microscopy (NanoSAM), which allows for the 3-dimensional (3-D) evaluation of cells. This technique uses scanning electron microscopy (SEM) and scanning auger microscopy (SAM) to provide both an image and element composition of the sample being viewed. The sample is bombarded with an argon (Ar^+) gun that removes 27 nm thick slices of the sample per minute, after which the SEM mode is used to capture an image of the etched sample. A nanoprobe bombards the sample to excite auger electrons that are used to determine the element composition of the sample, where the SAM mode is used to detect the Auger profile of the different elements. Bubble-like structures were observed inside the etched yeasts cells (Fig. 1c) and this was the final indication of gas bubble formation in the yeast *Saccharomyces* (Swart *et al.*, 2012). The next question that came to mind was the nature of the content of these bubbles.

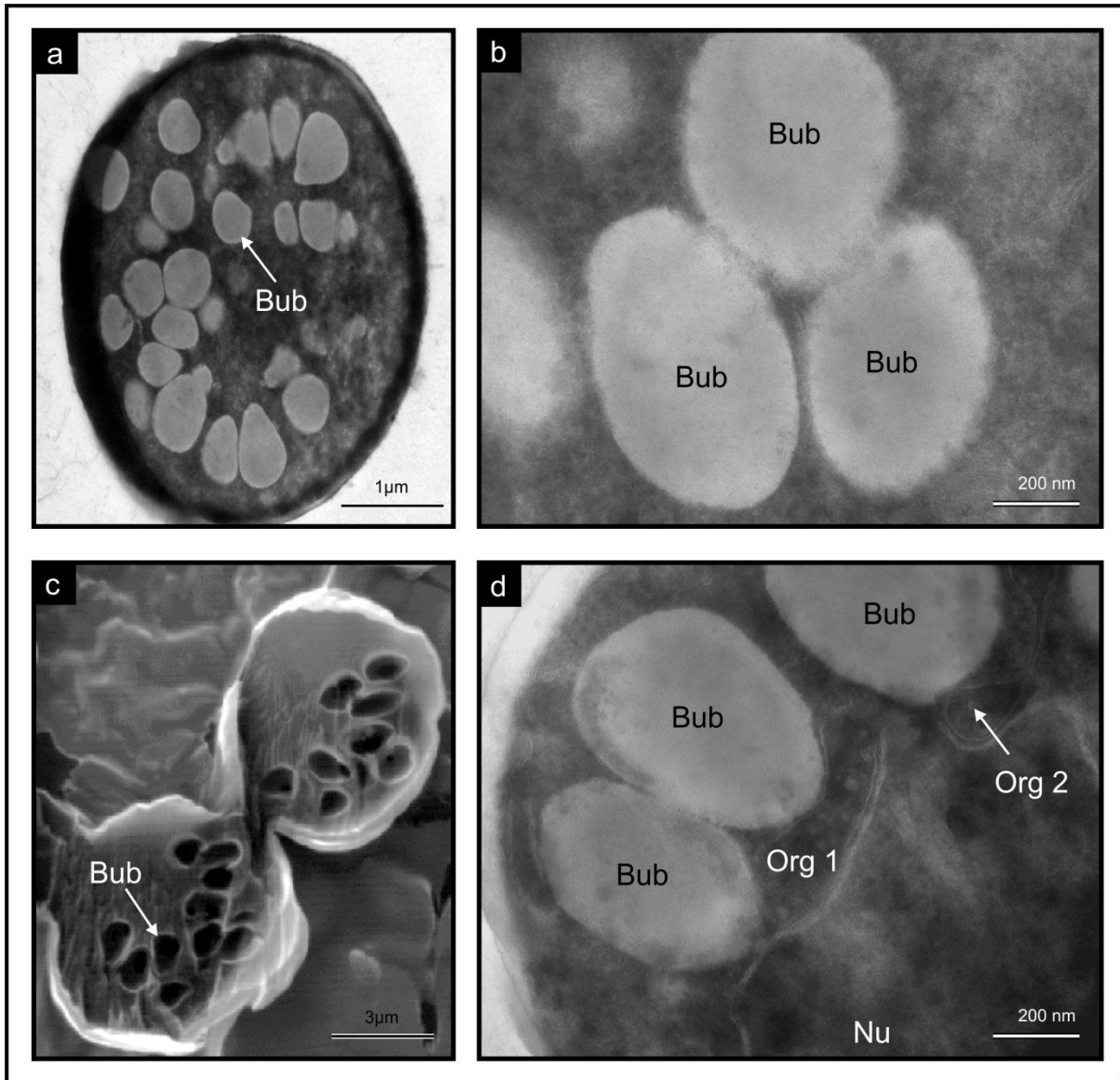


Figure 1: The yeast *Saccharomyces pastorianus* analysed for gas bubble formation. (a) Electron transparent structures observed in the cytoplasm of the budding yeast, *S. pastorianus* after transmission electron microscopy (TEM) analysis, (b) The less electron dense structures at high magnification indicating the lack of surrounding plasma membranes, (c) Nano scanning Auger microscopy (NanoSAM) analysis showing bubble-like structures observed by Swart and co-workers (2012), which they concluded as gas bubbles. [Taken with permission from Swart *et al.*, 2012]. (d) Analysis with TEM indicating gas bubbles deforming cell organelles and therefore in turn deforming the nucleus, which can affect proper cell functions. [Taken with permission from Swart *et al.*, 2013]. Bub = Gas bubbles. Org = Organelle. Nu = Nucleus.

It is well established that the process of fermentation produces large amounts of CO₂ and since *Saccharomyces* is highly fermentative, it would be expected that the primary gas to accumulate as gas bubbles would be CO₂. It has also been stated in literature that CO₂ can form gas bubbles more readily than other gases commonly found in the cytoplasm such as nitrogen (N₂), oxygen (O₂) and hydrogen (H₂) (Metschul, 1924; Dean, 1944). This could be ascribed to the fact that CO₂ is highly soluble in water and can diffuse more rapidly in and out of gas bubbles than the other gases mentioned (Dean, 1944; Blatteau *et al.*, 2006). In order to identify the gas contained in these gas bubbles, the yeast cells were grown in yeast malt broth (YM) containing a metal salt; zinc sulphate heptahydrate (ZnSO₄ · 7H₂O). The cells were then analysed with NanoSAM using the element mapping component of the instrument. From the initial description of a gas bubble, it was stated that these structures are thought to be enclosed with a thin layer of water molecules for stability (Yount, 1979). Therefore, due to the reaction: $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ (Shen *et al.*, 2004), it was expected that carbonic acid should be formed at high concentrations around the boundary of the bubbles if these bubbles indeed contained CO₂. The results indicated that the boundary of the bubbles inside these cells contained concentrated amounts of an insoluble metal bicarbonate. This compound was formed by the Zinc reacting with the carbonic acid at the boundary of the bubbles at neutral pH, which is typical of the yeast cell cytoplasm. From these observations it was concluded that this yeast produces gas bubbles that contain CO₂ (Swart *et al.*, 2012).

In 2013 Swart and co-workers provided additional information regarding these gas bubbles, reporting that the bubbles tend to occupy a large part of the cell, leaving limited space for other important cell organelles. Swart and co-workers observed that these gas bubbles compress and deform membranes of nearby cell organelles (Fig. 1d), in turn deforming the membrane of the nucleus (Swart *et al.*, 2013). This leads one to wonder what the effects of these indentations are on the cell health and lifespan.

Gas bubble formation was initially studied in the budding yeast *Saccharomyces*, thus to broaden the understanding of this phenomenon, the fission yeast *Schizosaccharomyces pombe* (*Schizo. pombe*) was also investigated for the presence of gas bubbles. This yeast was selected due to its ability to reproduce via fission, which is different from *Saccharomyces* and that it is also only distantly related to *Saccharomyces*. Both these yeasts are highly fermentative, Crabtree-positive and very often used as workhorses in industry. The results of the study performed on *Schizo. pombe* indicated that gas bubbles are also present in this distantly related yeast, yet tend to be smaller compared to bubbles formed in *Saccharomyces* (Fig. 2). This could be ascribed to the fact that *Schizo. pombe* is not such a strong fermenter as compared to *Saccharomyces* (Piškur *et al.*, 2006).

Interestingly, the gas bubbles in this fission yeast can be found at the terminal ends of fully elongated cells about to divide (Fig. 2a). This could be due to the fact that, during fission a septum forms in the middle of the cell, thus driving the gas bubbles to the terminal ends of the cell. However in cells that are not dividing, gas bubbles are observed in the middle of (Fig. 2b) or dispersed throughout the cell.

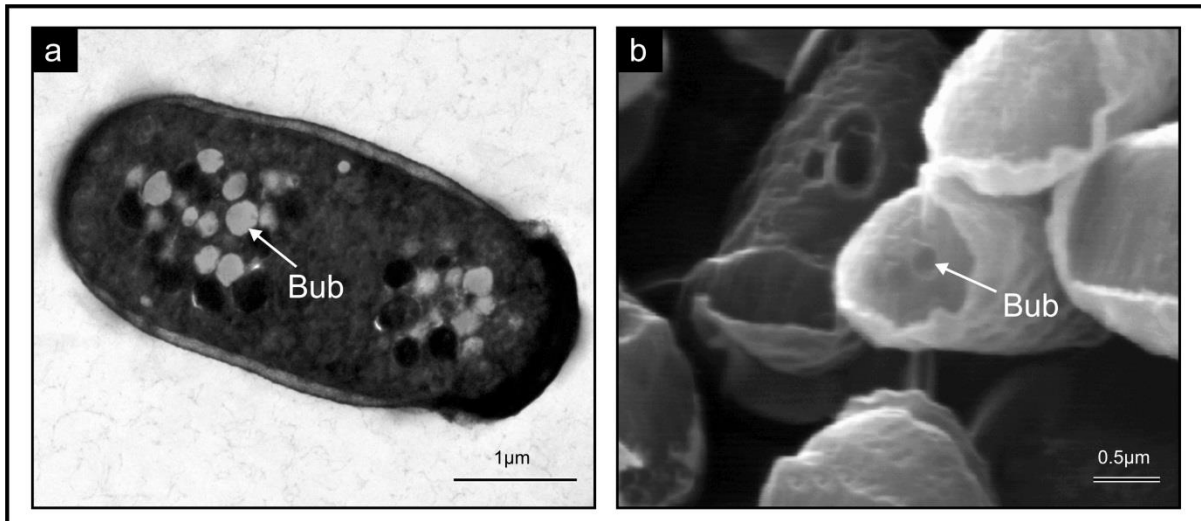


Figure 2: *Schizosaccharomyces pombe* analysis for gas bubbles formation. (a) Transmission electron microscopy (TEM) showing smaller electron transparent structures as compared to *Saccharomyces* and (b) Nano scanning Auger microscopy (NanoSAM) indicating less bubble-like structures formed than in *Saccharomyces*. Interestingly, the bubbles in *Schizo. pombe* cells that were preparing for fission, were located at the terminal end of the cells. Bub = Gas bubbles.

1.4. Carbon dioxide production in yeast cells

Carbon dioxide production in yeasts can be achieved through two major glucose dissimilation metabolisms i.e. respiration and fermentation (Pronk *et al.*, 1996; Kregiel, 2008). The initial pathway for the degradation of glucose in yeast is glycolysis, which occurs in both respiration and fermentation and through this pathway glucose is converted into pyruvate.

During respiration, pyruvate is fed into the citric acid cycle by oxidative decarboxylation to acetyl-CoA by the enzyme pyruvate dehydrogenase (Pronk *et al.*, 1996; Van Dijken *et al.*, 1993; Kregiel, 2008). The acetyl-CoA is oxidised to produce one molecule of CO₂. In addition, during the citric acid cycle, two decarboxylation reactions occur; then two additional CO₂ molecules are produced. Respiration

produces in total six molecules of CO₂ since one glucose molecule produces two molecules of pyruvate. This respiratory dissimilative route produces CO₂ and water after the full oxidation of pyruvate as by-products and due to the growing activity of the cell, some of the generated intermediates will be channelled into biosynthetic pathways for accumulation of reserve carbohydrates (Pronk *et al.*, 1996). During alcoholic fermentation of glucose, the pyruvate is initially decarboxylated into acetaldehyde (Van Dijken *et al.*, 1993) with the coupled production of one molecule of CO₂ by the enzyme pyruvate decarboxylase (Pronk *et al.*, 1996). Thereafter the acetaldehyde is finally reduced to ethanol by the enzyme alcohol dehydrogenase (Kregiel, 2008). Overall the process of alcoholic fermentation produces two molecules of CO₂; ethanol and CO₂ are the by-products of alcoholic fermentation after the full dissimilation of pyruvate (Pronk *et al.*, 1996). However CO₂ fixing reactions can also occur during cellular metabolism which may affect the concentrations of CO₂ and in turn gas bubble formation (Van Dijken *et al.*, 1993; Pronk *et al.*, 1996). The fact that fermentation has a significantly shorter route to get to CO₂ production than respiration and that fermentation is much more rapid than respiration may be some of the reasons why fermentation produces larger quantities of CO₂ than respiration. Yeasts can be categorized into several different groups depending on their modes of CO₂ production; fermentation or respiration (Pronk *et al.*, 1996; Kregiel, 2008).

According to the modes of CO₂ production, the yeast domain can also be classified into three different groups; Crabtree-positive yeasts, Crabtree-negative yeasts and yeasts that are non-fermentative (only respire) (Veiga *et al.*, 2000). Crabtree-positive yeasts exhibit the Crabtree effect, where alcoholic fermentation can occur under aerobic conditions grown in glucose excess. These yeasts go through respiration

under aerobic conditions however they can also go through fermentation under aerobic conditions when there is an access of glucose in the environment. In contrast, Crabtree-negative yeasts do not exhibit the Crabtree response (Van Urk *et al.*, 1989) and here fermentation occurs under anoxic conditions while respiration occurs under oxic conditions (Van Dijken *et al.*, 1986; Pronk *et al.*, 1996; Wardrop *et al.*, 2004). Non-fermentative yeasts are unable to ferment (only respire). This could be due to some inability of these yeasts to produce important enzymes that are needed for the fermentation pathway (Van Dijken *et al.*, 1993; González Siso *et al.*, 1996; Kręgiel, 2008).

The most basic definition of the Crabtree effect in yeast is that it is the repression of respiration by fermentation, where alcoholic fermentation occurs under fully aerobic conditions (Van Dijken *et al.*, 1993; González Siso *et al.*, 1996; Pronk *et al.*, 1996; Veiga *et al.*, 2000; Wardrop *et al.*, 2004; Kręgiel, 2008). Thus, yeasts that can exhibit this effect mainly go through the fermentation pathway rather than respiration pathway (De Deken *et al.*, 1966). The Crabtree effect can be encountered in two different forms: 1) Short-term Crabtree effect is the immediate occurrence of aerobic alcoholic fermentation induced by a change from a sugar-limited growing culture to a sugar excess culture (Kręgiel, 2008). That could be caused by the over saturation of the respiratory route, inducing a bottle neck at the pyruvate point, 2) long-term Crabtree effect is the occurrence of aerobic alcoholic fermentation regardless of cultivation in sugar excess or sugar limitation. This could be due to the respiratory route being insufficient in pyruvate dissimilation (Van Urk *et al.*, 1989; 1990; Pronk *et al.*, 1996; Wardrop *et al.*, 2004).

Different studies on the Crabtree effect have indicated that numerous parameters are responsible for the differences in yeasts that are Crabtree-positive and Crabtree-

negative and these factors include (1) the mechanism of glucose uptake, (2) the route of pyruvate metabolism (enzyme concentration and activity) (Van Dijken *et al.*, 1993) and (3) the accumulation of reserve carbohydrates (Van Urk *et al.*, 1990). For Crabtree-positive yeasts it was found that glucose uptake is facilitated by diffusion which entails unrestricted glucose entry (higher glucose consumption rate) into the cell, therefore leading to aerobic fermentation. Although for Crabtree-negative yeasts the entry is regulated by a H⁺-symport system which will lead to a favoured respiration pathway (Van Urk *et al.*, 1989; Kęgiel, 2008). It was also observed for Crabtree-positive yeasts that the concentration of the key enzyme in alcoholic fermentation, pyruvate decarboxylase, increased excessively while its activity also became six-fold higher than normal. In contrast, the concentration and activity of this enzyme remained low in Crabtree-negative yeasts (Van Dijken *et al.*, 1993). Lastly Crabtree-positive yeasts were observed not to have an increased growth rate meaning their biomass remained constant. Alternatively, while for Crabtree-negative yeasts their biomass production rapidly increased under oxic conditions (Van Urk *et al.*, 1990; Pronk *et al.*, 1996; Wardrop *et al.*, 2004) and this could be caused by synthesis of intracellular reserve carbohydrates (Van Urk *et al.*, 1990; Kęgiel, 2008).

1.5. Conclusions

Gas bubbles were initially defined as symmetrical to asymmetrical spheres that are enclosed by a thin enveloping skin of water. These structures may contain one type or a mixture of multiple gases (Dean, 1944) and can range in size from a few nanometres to several millimetres (Blatteau *et al.*, 2006; Mohan, 2010). The work done by Hemmingsen and co-workers provided numerous studies that led them to conclude that gas bubbles cannot be formed in the cytoplasm of microorganisms, even at high gas supersaturation (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1985; Hemmingsen *et al.*, 1990). They later reported on gas bubble induction by phagocytosis of bubble-promoting particles in organisms with amoeboid cells such as slime mold and macrophages. Nonetheless the process proved difficult to carry out due to the loss of the bubble-promoting properties of the particles after phagocytosis by the cells (Hemmingsen *et al.*, 1990). The discovery of gas bubbles in the budding yeast *Saccharomyces* (Swart *et al.*, 2012) led to the disproval of the previously reported conclusion by Hemmingsen and co-workers (1979). This discovery incited further examination of gas bubbles in yeasts, where it was found that these gas bubbles lack surrounding membranes and can occupy a large part of the cell, leading to the deforming of membranes of other important cell organelles (Swart *et al.*, 2012; 2013). The fission yeast *Schizosaccharomyces pombe* was also investigated for the occurrence of gas bubbles and exhibited structures similar to that found in *Saccharomyces*. All this information on gas bubbles raised many questions about this phenomenon. Since these gas bubbles were observed in two distantly related yeasts *Saccharomyces* and *Schizosaccharomyces*, is this a conserved characteristic in the yeast domain? Are there any differences that can be encountered if these gas bubbles are characterised in different yeasts? Additional

research needs to be carried out to address these lingering questions, some of which will be the focus of this thesis (see 1.6).

1.6. Purpose of research

The purpose of this study, according to the aforementioned relevant literature study, is outlined by the following aims:

The determination of the conserved status of gas bubbles in yeasts, differences in the amount of gas bubbles produced by each type of yeast and the characterization of these observed gas bubbles.

1. Specific yeasts will be selected according to their mode of CO₂ production, focusing mainly on the difference between fermenting and non-fermenting yeasts and further dividing the fermenting yeasts into Crabtree-positive and Crabtree-negative yeasts.
2. Yeast selected for this study will be initially grown in yeast malt broth (YM) under oxic conditions.
3. Microscopic techniques which will be used to investigate the prepared yeast cells include:
 - 3.1. Light microscopy (LM) to determine the presence of light scattering granules inside the cells.
 - 3.2. Transmission electron microscopy (TEM) to provide the two dimensional (2D)-ultrastructure of the yeast cells in order to observe the presence of electron transparent structures and whether they contain membranes.

3.3. Nano scanning Auger microscopy (NanoSAM) to provide the three dimensional (3D)-ultrastructure of the yeast cells enabling the visualization of CO₂-gas bubbles and to carry out elemental analysis on the sample of yeast cells.

3.4. Time-of-flight secondary ion mass spectrometry (ToF-SIMS) to provide visualization of the elements and organic molecules in the sample, by production of a secondary ion image and a mass spectrum of the compounds.

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Chapter 2

Determination of the conserved status of gas bubble formation in yeasts

2.1. Abstract

The recent discovery of gas bubble formation in the Crabtree-positive baker's yeast, *Saccharomyces cerevisiae* and brewer's yeast, *Saccharomyces pastorianus* resolved the missing link between intracellular carbon dioxide (CO₂) production through alcoholic fermentation and the eventual release of CO₂ into the surrounding environment. Since the study mentioned focused on Crabtree-positive yeasts, the question arose whether the gas bubble phenomena is conserved amongst yeasts, irrespective of their mode of CO₂ production. This study aims to determine the conserved status of gas bubbles amongst yeast species that are Crabtree-positive, Crabtree-negative and also species that can only respire. Similar to what was observed with the Crabtree-positive *Saccharomyces*, when CO₂ bubbles were discovered, it is expected that other Crabtree-positive yeasts should also contain a large amount of gas bubbles depending on their fermentation capabilities. In contrast, yeasts that are Crabtree-negative (grown under oxic conditions) and yeasts that strictly respire, should contain significantly less bubbles since respiration produces much less CO₂ as compared to fermentation. Results obtained indicated that Crabtree-positive fermenting yeasts contained large amounts of gas bubbles when grown on fermentable media, yet little to none when grown on non-fermentable media. Furthermore, the Crabtree-negative fermenting and strictly respiring yeasts contained less gas bubbles on both fermentable and non-fermentable media. From the results obtained it was concluded that gas bubble formation is conserved in yeasts, however the amount and size of the formed gas bubbles are affected by whether the mode of CO₂ production is fermentation, respiration or both. The implication of these findings may impact fermentation biotechnology, since these gas bubbles may affect cell physiology and organelle function.

2.2. Introduction

During alcoholic fermentation, alcohol and carbon dioxide (CO₂) are produced as by-products by yeasts cells, after which these compounds are excreted into the surrounding environment (Van Dijken *et al.*, 1993; Pronk *et al.*, 1996; Faria-Oliveira *et al.*, 2013). However, even with the extensive studies on the process of fermentation, there has not been any report of CO₂ accumulation inside the cell prior to excretion. The lack of these reports could be ascribed to studies performed by Hemmingsen and co-workers (1979), suggesting that intracellular gas bubbles cannot be formed in the cytoplasm of any type of microorganism, even under high gas supersaturation (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1990). In contrast, Swart and co-workers (2012) recently proved the occurrence of gas bubbles inside the highly fermentative Crabtree-positive yeast *Saccharomyces* (Swart *et al.*, 2012). The study focused on using novel imaging techniques, including the recently established field of Auger-architectomics (https://en.wikipedia.org/wiki/auger_architectomics) to expose gas bubbles inside the budding yeasts *Saccharomyces cerevisiae* and *Saccharomyces pastorianus*. These researchers found that gas bubbles fill a significant part of the cell, leaving limited space for other cell organelles. This in turn led to the distortion or contortion of other cytoplasmic organelles, possibly influencing organelle function and cell physiology (Swart *et al.*, 2013). Another important characteristic of gas bubbles is that they lack surrounding membranes (Swart *et al.*, 2012), which is an indication that they are not true cell organelles.

These findings in the yeast *Saccharomyces* by Swart and co-workers (2012) led us to wonder about the status of gas bubble formation in Crabtree-negative and non-fermentative (strictly respiring) yeasts. When evaluating the difference between fermenting and non-fermenting yeasts, the main distinguishing factor is oxygen gas (O₂) availability since it is well established that fermentation does not require O₂ to occur (Pronk *et al.*, 1996). Furthermore, fermentation produces more CO₂ than respiration since fermentation is more rapid than respiration (Van Dijken *et al.*, 1986; 1993; Van Urk *et al.*, 1990; Pronk *et al.*, 1996). As a consequence, this study focuses on investigating the difference in bubble formation of differently metabolizing yeasts, i.e. Crabtree-positive, Crabtree-negative and strictly respiring yeasts. The Crabtree-positive yeasts can go through both fermentation and respiration for CO₂ production (even when O₂ is present), while Crabtree-negative yeasts only uses fermentation under anoxic conditions and respiration under oxic conditions for CO₂ production. Strictly respiring yeasts rely only on respiration for CO₂ production (De Deken *et al.*, 1966; Van Dijken *et al.*, 1986; 1993; Van Urk *et al.*, 1989; 1990). These yeasts will be used for the determination of the conserved status of gas bubbles formation.

2.3. Materials and Methods

2.3.1. Strains used and Cultivation

The yeasts used in this study (Table 1) were preserved in the UNESCO MIRCEN Culture Collection of the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, South Africa, 9300. These yeasts were chosen according to their CO₂ production activities.

Table 1. A list of the yeast strains used in the experiments together with their preferred mode of carbon dioxide (CO₂) production.

Yeast Names	Strain Number	Mode of carbon dioxide production
<i>Torulaspora globosa</i>	CY 130 8/95, UOFS Y-0847	Crabtree-positive
<i>Zygosaccharomyces bailli</i>	IGC 4245, UOFS Y-1535	Crabtree-positive
<i>Debaryomyces hansenii</i>	BCY 06, UOFS Y-219	Crabtree-negative
<i>Kluyveromyces marxianus</i>	IGC 2671, UOFS Y-1191	Crabtree-negative
<i>Pichia membranifaciens</i>	CBS 76, UOFS Y-823	Strictly respire
<i>Lipomyces starkeyi</i>	CBS 1807, UOFS Y-1999	Strictly respire
<i>Yarrowia lipolytica</i>	CBS 599, UOFS Y-1138	Strictly respire
<i>Rhodotorula glutinis</i>	CBS 20, UOFS Y-0519	Strictly respire

Yeasts were streaked on glucose yeast malt (YM) agar plates (Wickerham, 1951) and incubated for 48 h at 30 °C. The cells were then used to prepare an inoculum for both fermentable media; glucose yeast malt broth (10 g l⁻¹ glucose, 3 g l⁻¹ yeast extract, 3 g l⁻¹ malt extract, 5 g l⁻¹ peptone) and non-fermentable media; yeast peptone glycerol broth (YPG) (30 ml l⁻¹ glycerol, 10 g l⁻¹ yeast extract, 20 g l⁻¹ peptone) media (Sherman, 2002). The inoculum was prepared by inoculating 100 ml of the two different media in 500 ml conical flasks with a loopful of the cells. The inoculum was incubated at 30 °C for 24 h, while shaking at 160 rpm. Experimental flasks were then prepared by inoculating 500 ml conical flasks, containing 100 ml of the two different media respectively, with 1 ml of the inoculum. These were incubated for 48 h at 30 °C, while shaking at 160 rpm and after 48 h, samples were drawn and analysed. The samples grown on YM were analysed with light microscopy (LM), transmission electron microscopy (TEM) and nano scanning Auger

microscopy (NanoSAM) as described by Swart *et al.* (2010) and Kock *et al.* (2011), while the samples grown on YPG were analysed with only LM and TEM.

2.3.2. Light microscopy (LM)

Samples from all eight yeasts grown on both fermenting and non-fermenting media for 48 h were subjected to LM (Axioplan, Zeiss, Germany) coupled to a Colourview Soft Digital imaging system (Münster, Germany). This was performed to evaluate the purity of the samples and to determine whether light scattering granules are present inside the cells. These light scattering granules have been previously noted as gas bubbles by Swart and co-workers (2012) during their study on the yeast *Saccharomyces*. Observation of light scattering granules serves as the first determination step for gas bubble formation.

2.3.3. Transmission electron microscopy (TEM)

Suspensions of the yeasts after 48 h were centrifuged at 1450 g for 5 min, as to harvest and prepare the cells for TEM evaluation according to Swart *et al.* (2010). The first step is fixation with 3 % glutaraldehyde (Merck, Darmstadt, Germany) buffered with 0.1 M (pH 7) sodium phosphate buffer for 3 h. This then is followed by a second fixation with a buffered solution of 1 % osmium tetroxide (Merck, Darmstadt, Germany) for 1 h. Each of the fixation steps were followed with rinsing of the cells with the same sodium phosphate buffer for 5 min, twice after both the glutaraldehyde fixation and after the osmium tetroxide fixation steps. The cells were there after dehydrated with acetone in the following series of concentrations; 50 %, 70 %, 95 %, with each step for 20 min and twice in 100 % acetone for 1 h each step.

After the dehydration process the cells were embedded in epoxy resin and polymerized at 70 °C for 8 h in special moulds. The embedded samples were then sliced into 60 nm thick sections by a Leica ultracut UM7 microtome using a glass knife. The sections are then mounted on copper grids and subsequently double stained using uranyl acetate (Merck, Darmstadt, Germany) and lead citrate (Merck, Darmstadt, Germany). Each step was performed for 10 min and 5 min respectively followed by rinsing after each staining. The sections were viewed using TEM [FEI (Phillips) CM 100, Netherlands].

2.3.4. Nano scanning Auger microscopy (NanoSAM)

Centrifugation at 1450 g for 5 min was used to harvest cells from the eight yeasts cultivation after 48 h. The cells were prepared for NanoSAM in scanning electron microscopy (SEM) mode as described by Swart *et al.* (2010) and Kock *et al.* (2011). The cells were subjected to fixation with 3 % glutaraldehyde (Merck, Darmstadt, Germany) buffered with 0.1 M (pH 7) sodium phosphate buffer followed by a second fixation with a buffered solution of 1 % osmium tetroxide for 1 h. After each fixation step the cells were rinsed twice with sodium phosphate buffer for 5 min. The next step is dehydration of the fixed cells with a series of ethanol concentrations; 50 %, 70 %, 95 %, for 20 min per step. Then followed with two 100 % ethanol steps, each performed for 1 h. Thereafter the cells were critically point dried, mounted on metal stubs and coated with gold to make them electron conductive. Samples were then examined with a PHI 700 Nanoprobe (Japan) equipped with SEM and scanning auger microscopy (SAM) facilities. The field emission gun was set at: 2.34 A filament current for both the SEM and SAM analyses, with 4 kV extractor voltage and 238.1 µA extractor current. A 20 kV, 10 nA electron beam with a diameter of 12 nm, was

obtained with these settings for the Auger analyses and SEM imaging. The pressure of the main chamber was at 2.29E-10 Torr, while the electron gun unit had an upper pressure of 8.8E-10 Torr, the measurements were taken by Aperture A. The Field of View (FOV) was 8 μm for the SEM mode, with the number of frames being 4. To obtain the Auger point analyses, ten cycles were used per survey, 1 eV per step and 20 ms per step. The nanoprobe was also equipped with an argon (Ar^+) ion sputtering gun, which was set at: 2 kV beam voltage, 2 μA ion beam current and a 1 x 1 mm raster area, giving a sputter rate of about 27 $\text{nm}\cdot\text{min}^{-1}$. The ion emission current was set at 15 mA. An alternating sputter mode with sputter intervals and sputter time of 1 min and 2 min respectively was used without any rotation.

2.4. Results and Discussion

2.4.1. Light scattering granule detection by LM

Light micrographs indicated that both the Crabtree-positive yeasts, *Torulasporea globosa* and *Zygosaccharomyces bailli*, when grown on YM fermentable media, appeared to be filled with light scattering granules (Fig. 1a, c). However the cells grown on YPG non-fermentable media appeared to contain substantially less light scattering granules when compared to the YM grown cells (Fig. 1b, d). In contrast to Crabtree-positive yeasts, it was observed that both the Crabtree-negative yeasts, *Kluyveromyces marxianus* and *Debaryomyces hansenii*, grown under oxic conditions, contained less light scattering granules on both YM and YPG media (Fig. 2). This could be ascribed to the fact that these yeasts only respire under oxic conditions, therefore producing less CO_2 . The four respiring yeasts, *Lipomyces starkeyi*, *Pichia membranifaciens*, *Yarrowia lipolytica* and *Rhodotorula glutinis* were

observed to contain little to no light scattering granules, which is the opposite of what was observed for the Crabtree-positive yeasts but similar to Crabtree-negative yeasts (Fig. 3). This observation was expected since both Crabtree-negative and strictly respiring yeasts rely on respiration for CO₂ production in oxic conditions.

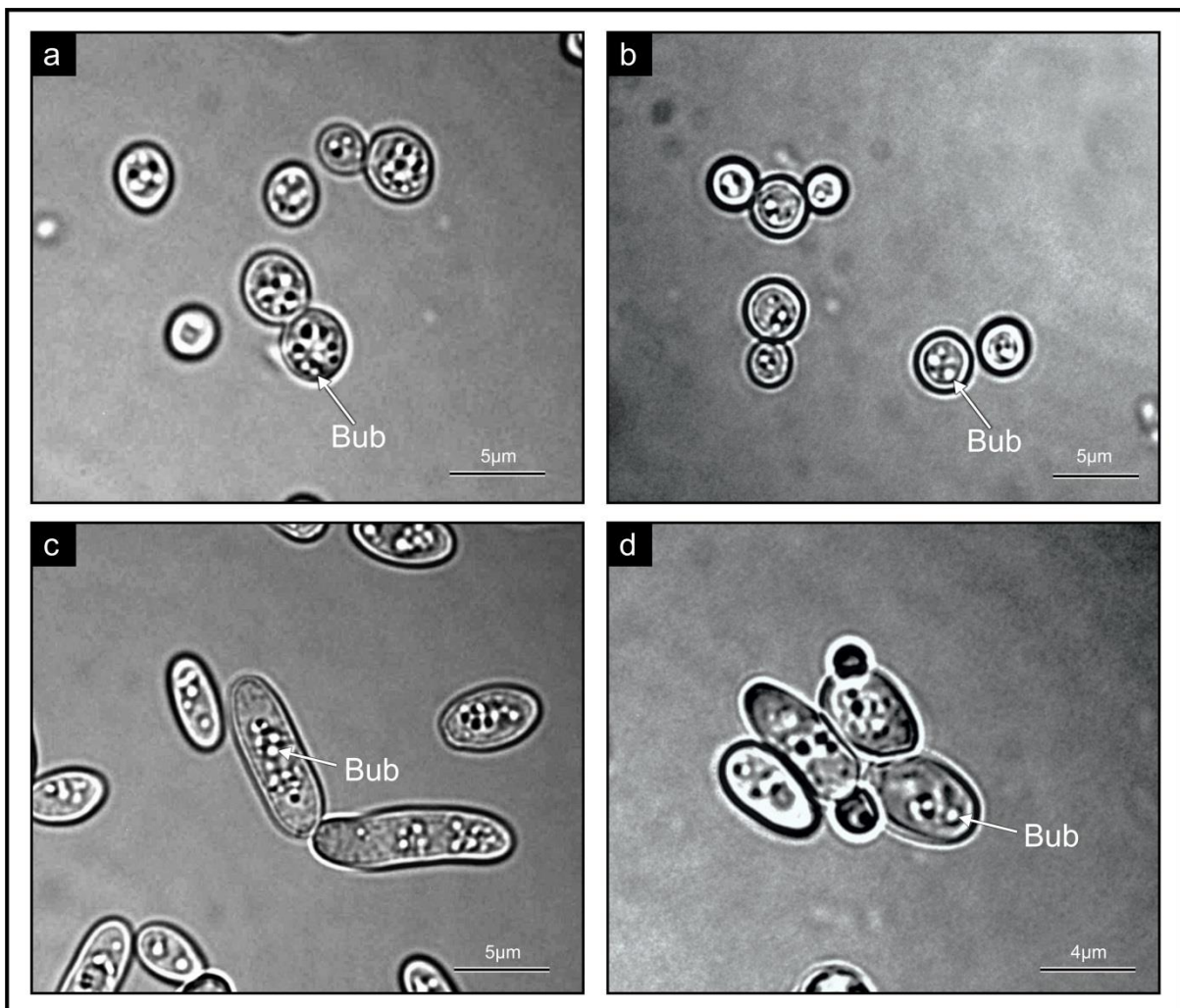


Figure 1. Light micrographs of the Crabtree-positive yeasts indicating light scattering granules in the cytoplasm of the cells grown on different media. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of *Torulaspora globosa* and c) YM grown cells and d) YPG grown cells of the yeast *Zygosaccharomyces bailli*. Bub = Gas bubbles.

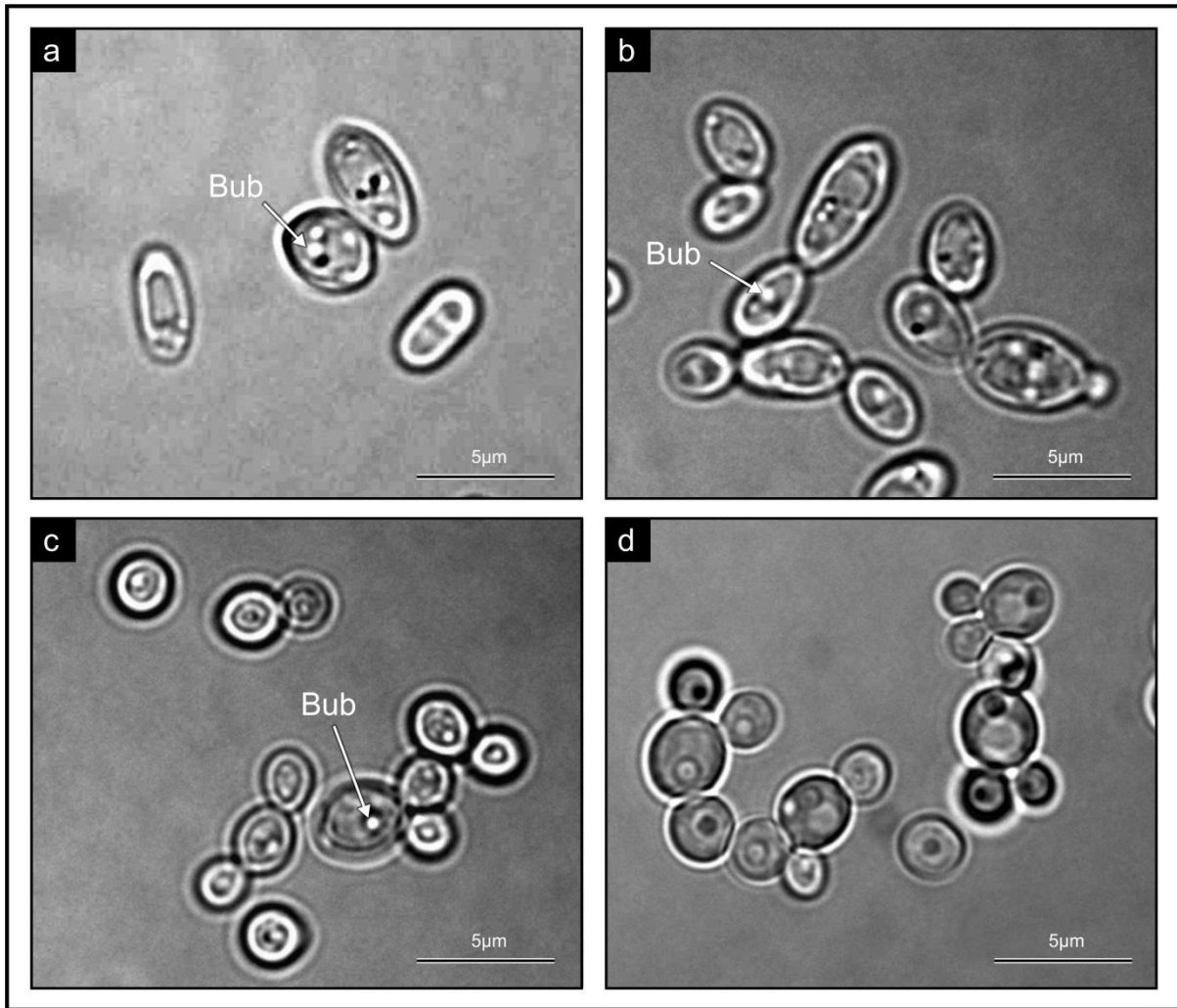


Figure 2. Light micrographs of the Crabtree-negative yeasts indicating a significantly smaller number of light scattering granules compared to Crabtree-positive yeasts. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of *Kluyveromyces marxianus*. c) YM grown cells and d) YPG grown cells of *Debaryomyces hansenii* respectively. Cells grown on both media appeared to exhibit similar results regardless whether the media is fermentable or non-fermentable, which is expected since these yeasts respire on both media under oxic conditions. Bub = Gas bubbles.

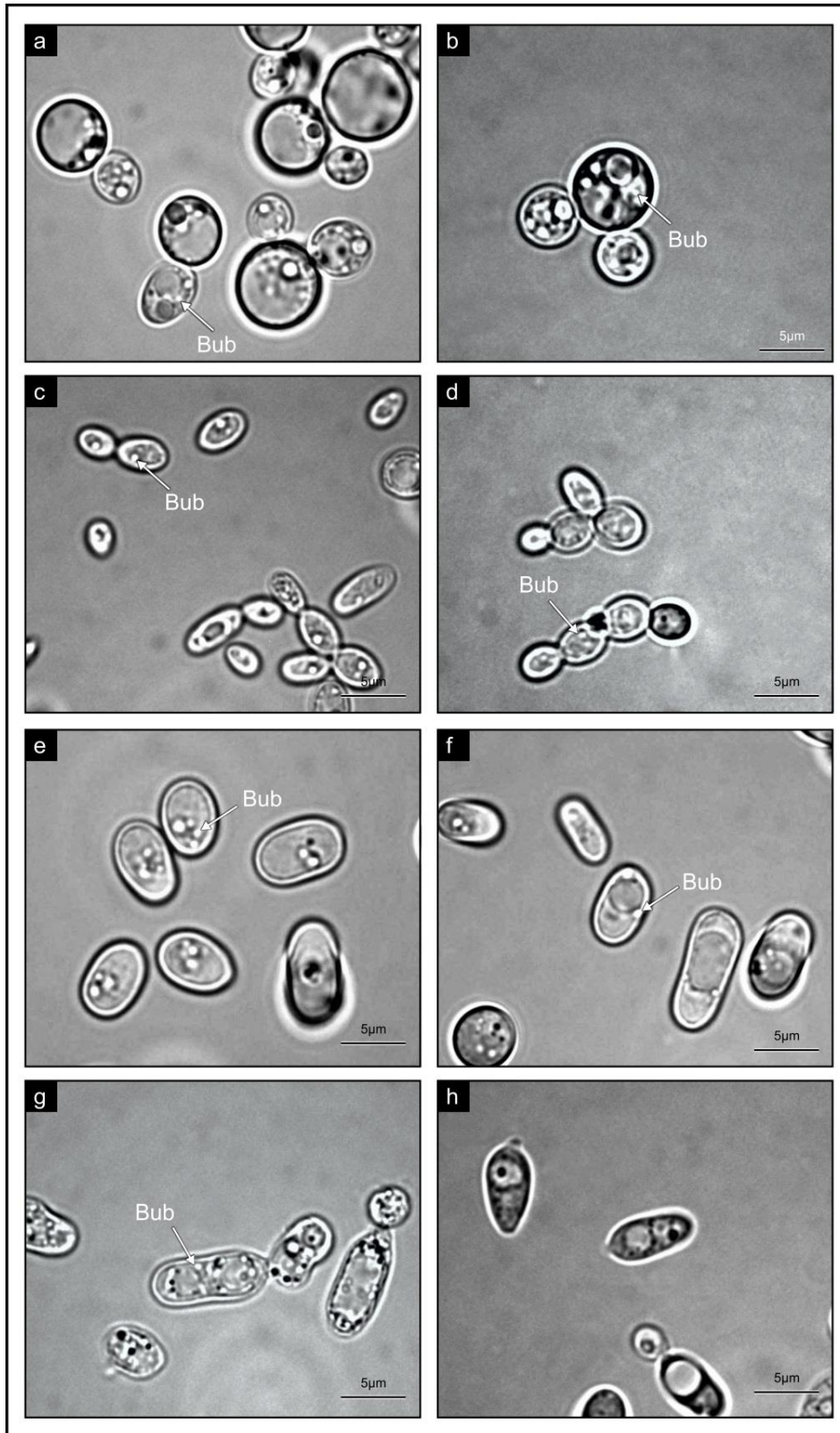


Figure 3. Light scattering granules observed in strictly respiring yeasts when viewed with the light microscope. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of the yeast *Lipomyces starkeyi*. c) YM grown cells and d) YPG grown cells of the yeast *Pichia membranifaciens*. e) YM grown cells and f) YPG grown cells of the yeast *Yarrowia lipolytica*. g) YM grown cells and h) YPG grown cells of the yeast *Rhodotorula glutinis*. Bub = Gas bubbles.

2.4.2. Gas bubble verification by TEM

Transmission electron microscopy analysis on the Crabtree-positive yeasts grown on YM media indicated that cells contained large amounts of electron transparent structures, believed to be gas bubbles due to the lack of surrounding membranes (Fig. 4a, c). This was expected since the initial discovery of gas bubbles was observed in *S. pastorianus* and *S. cerevisiae*, which are both Crabtree-positive yeasts. These Crabtree-positive yeasts can simultaneously ferment and respire under oxic conditions, therefore resulting in the production of a large amount of CO₂. The results for Crabtree-positive yeasts grown on YPG media indicated the formation of significantly less electron transparent structures (Fig. 4b, d). This was expected since the non-fermentable media contains glycerol that forces the cells to only respire, thus less CO₂ is produced and less gas bubbles are formed. The analysis on Crabtree-negative yeasts grown on YM media indicated the occurrence of electron transparent structures, which were significantly less than what was observed with Crabtree-positive yeasts (Fig. 5a, c). This observation is to be expected since Crabtree-negative yeasts grown under oxic conditions undergo respiratory metabolism, resulting in the production of less CO₂, therefore less gas bubbles are formed. The same results were also observed with growth on YPG media (Fig. 5b, d), which was expected since this media intentionally forces the yeasts to respire instead of ferment. All the respiring yeasts exhibited similar results with what was observed with Crabtree-negative yeasts. The results for YM grown strictly respiring yeasts indicated less gas bubbles formed than the Crabtree-positive yeasts (Fig. 6a, c, e, g), which is similar to Crabtree-negative yeasts. The results for the YPG cultivation were similar to what was observed with the YM growth (less gas bubbles were formed) (Fig. 6b, d, f, h). This is understandable since these yeasts

never ferment and respiration is the only metabolism responsible for CO₂ production. It is interesting to note that the yeast *Lipomyces starkeyi* contains other electron transparent structures however these are not gas bubbles since they are membrane bound (Fig. 6a, b). These structures can possibly be lipid droplets accumulated inside the cells since *Lipomyces* is an oleaginous yeast.

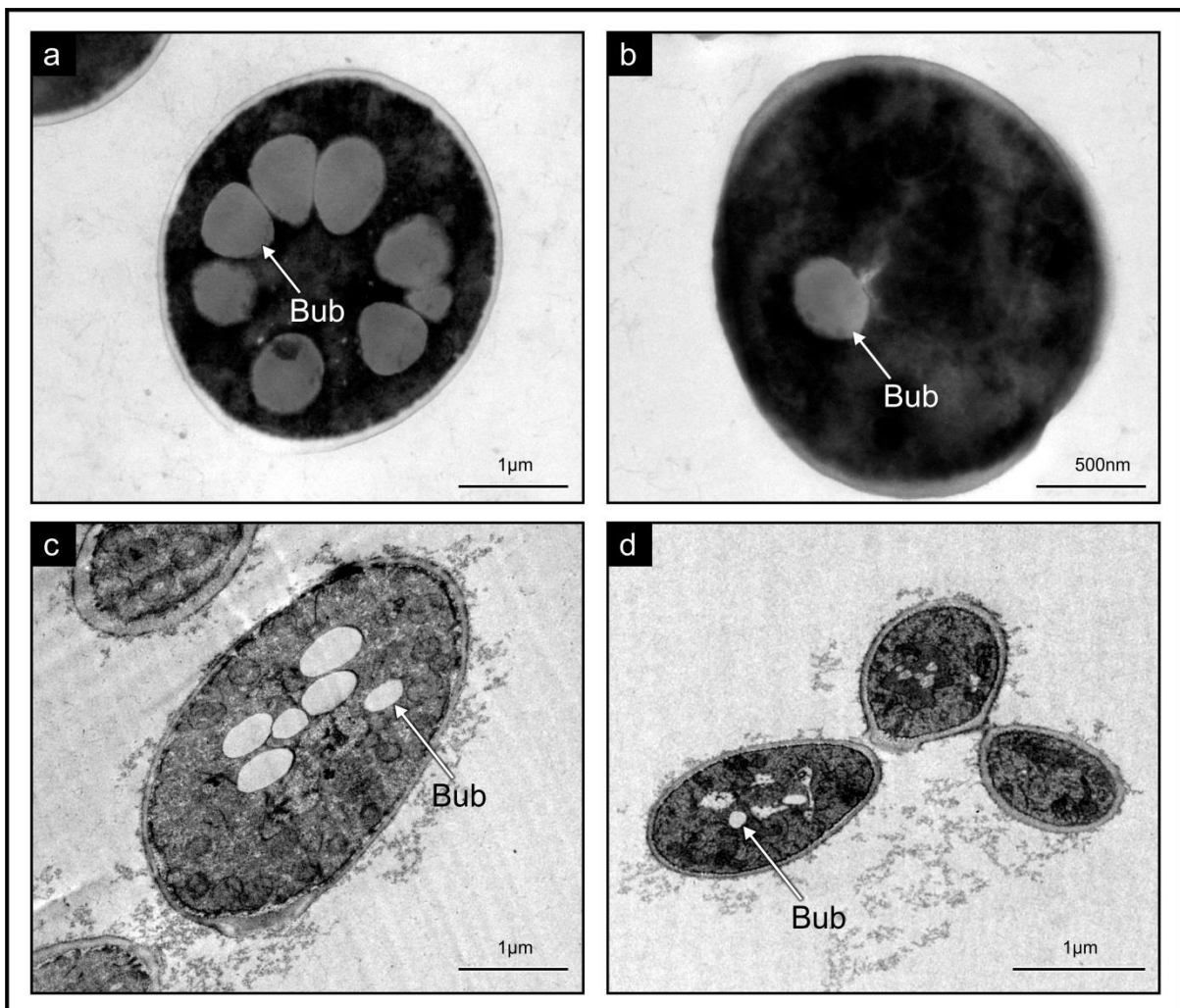


Figure 4. Transmission electron micrographs for the Crabtree-positive yeasts indicating electron transparent structures believed to be gas bubbles. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of *Torulasporea globosa* and c) YM grown cells and d) YPG grown cells of the yeast *Zygosaccharomyces bailli*. Bub = Gas bubbles.

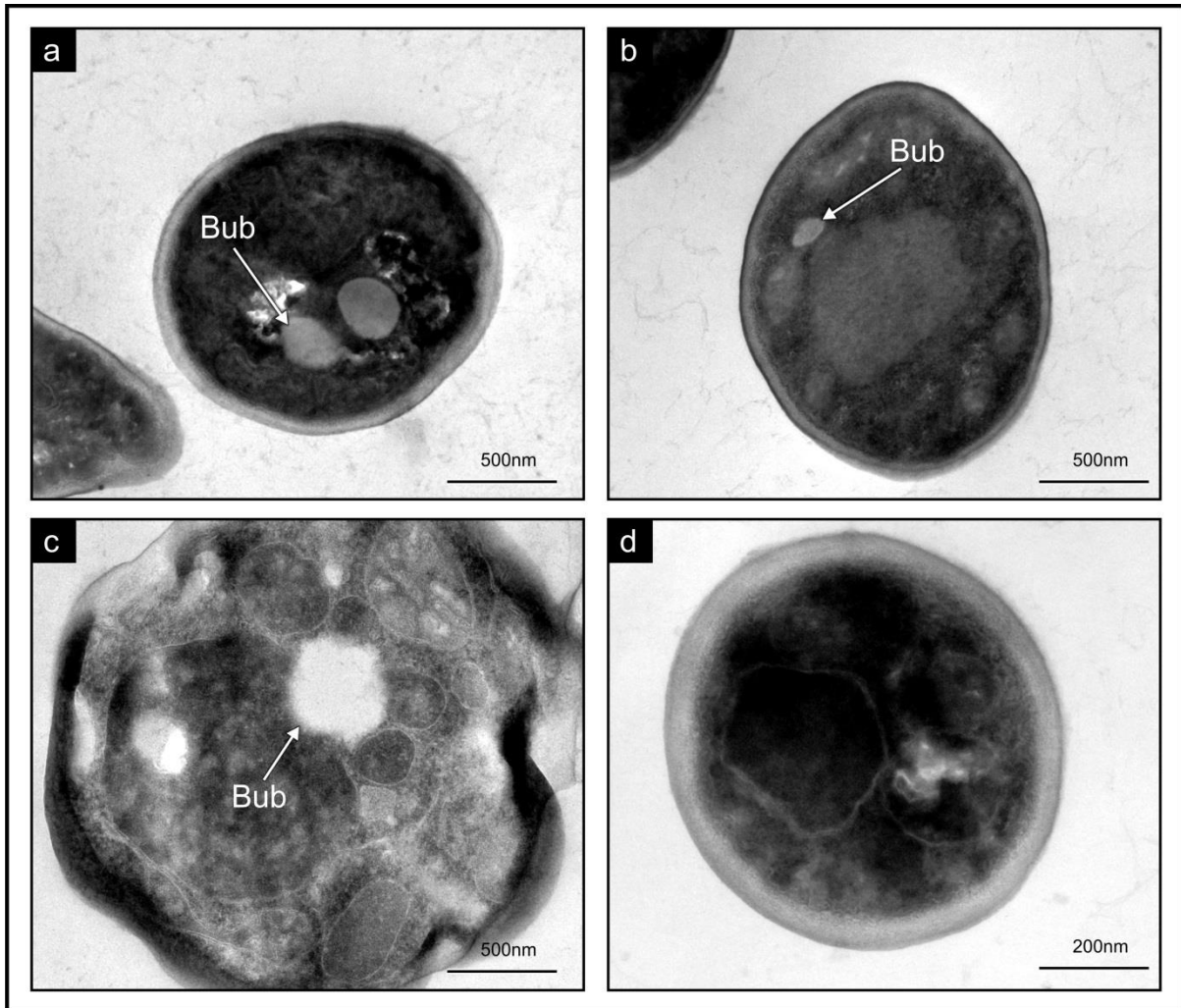


Figure 5. Transmission electron micrographs for the Crabtree-negative yeasts indicating electron transparent structures believed to be gas bubbles. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of the yeast *Kluyveromyces marxianus*. c) YM grown cells and d) YPG grown cells of the yeast *Debaryomyces hansenii*. The results indicate no significant difference in the amount of electron transparent structures formed when comparing the two media. Bub = Gas bubbles.

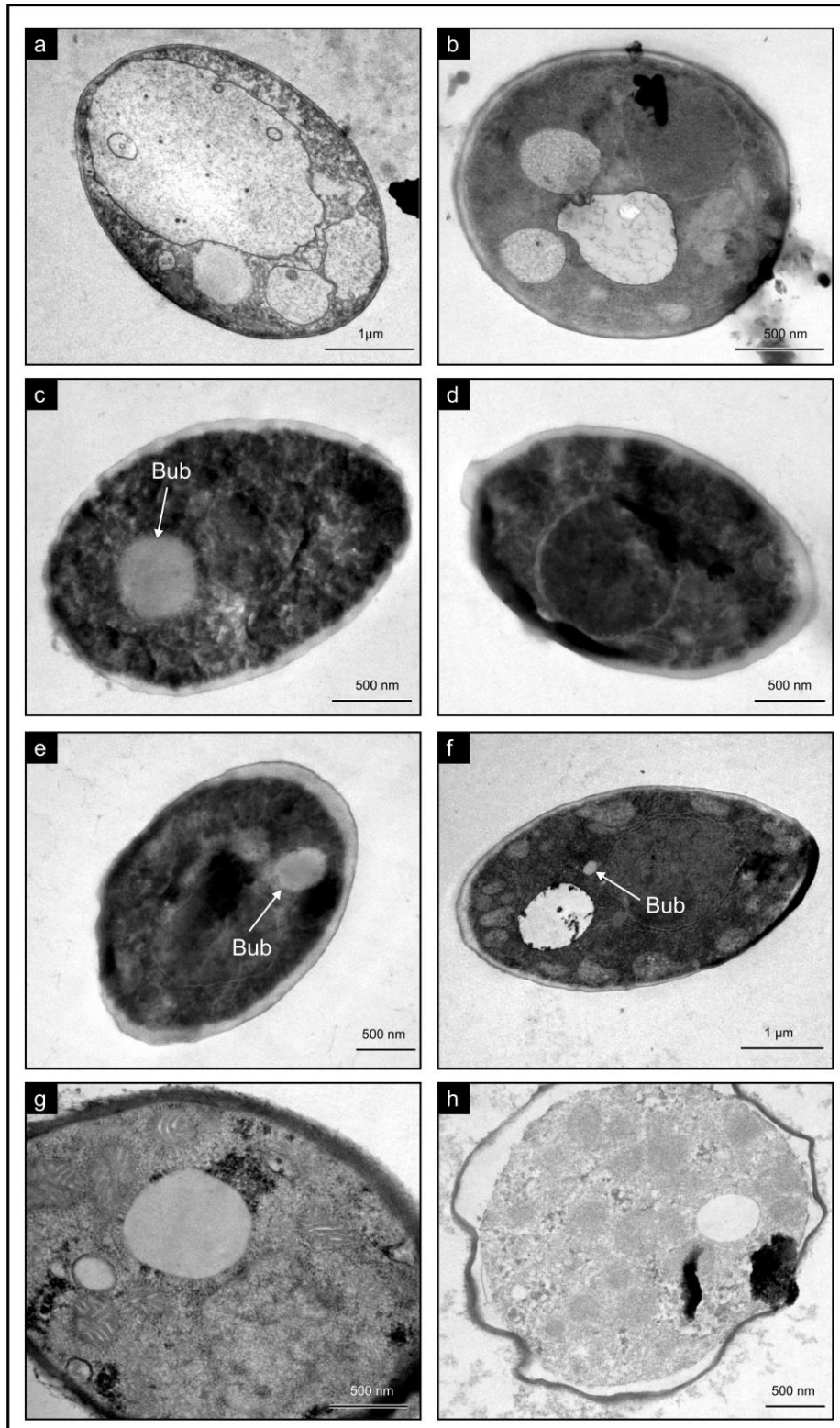


Figure 6. Transmission electron microscopy images for the strictly respiring yeasts indicating electron transparent structures believed to be gas bubbles. a) Glucose yeast malt (YM) grown cells and b) Yeast peptone glycerol broth (YPG) grown cells of the yeast *Lipomyces starkeyi*. c) YM grown cells and d) YPG grown cells of the yeast *Pichia membranifaciens*. e) YM grown cells and f) YPG grown cells of the yeast *Yarrowia lipolytica*. g) YM grown cells and h) YPG grown cells of the yeast *Rhodotorula glutinis*. Bub = Gas bubbles.

2.4.3. Gas bubble verification by NanoSAM

As the final verification step of the presence of gas bubbles, NanoSAM was used to analyse the yeast cells. The results indicated that the Crabtree-positive yeasts cells contained a maze of CO₂-bubbles (Fig. 7a, b). This is what was also observed with *Saccharomyces*, where these bubbles appeared to be coalescing to form bigger bubbles (Swart *et al.*, 2012). The analysis on Crabtree-negative yeasts indicated the same CO₂-bubbles in the yeast cells, however in these yeasts there was a decrease in the number of bubbles present (Fig. 7c, d). This was expected since these cells produce less CO₂ through respiration; therefore less bubbles would be produced. The four respiring yeasts were observed to contain less bubble-like structures in their cells (Fig. 8), which is an indication of decreased of CO₂ production through respiration. These observations were expected because similar to Crabtree-negative yeasts grown under oxic conditions, respiration is the primary source of CO₂ and as a result less CO₂ would be formed, indicating less gas bubbles.

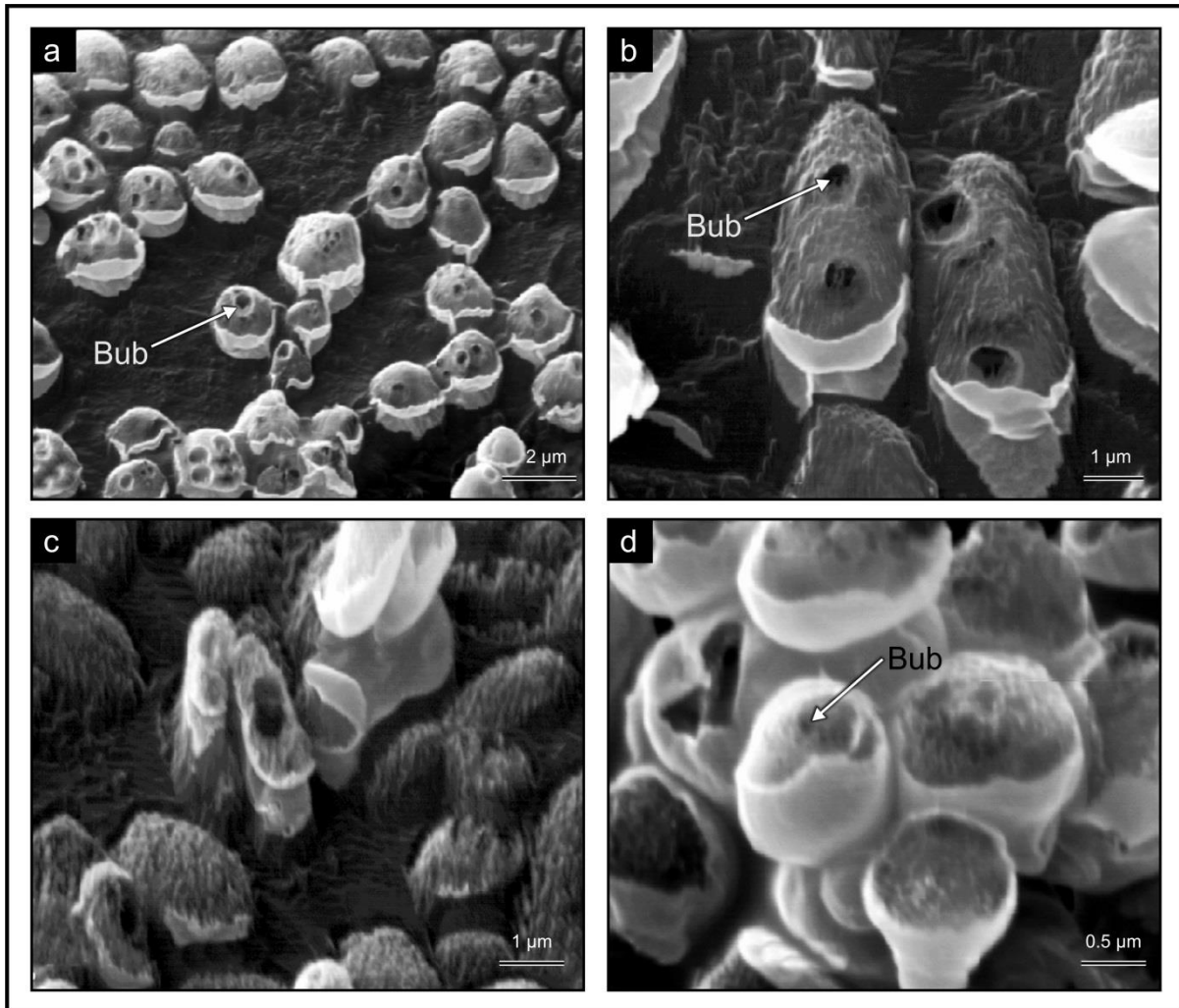


Figure 7. Nano scanning Auger microscopy images for fermenting yeasts grown on glucose yeast malt (YM) media indicating the presence of carbon dioxide-bubbles inside the yeast cells. a) *Torulaspora globosa*, b) *Zygosaccharomyces bailli*, c) *Kluyveromyces marxianus* and d) *Debaryomyces hansenii*. The two Crabtree-positive yeasts (a,b) indicated large numbers of gas bubbles while the Crabtree-negative yeasts (c,d) contained less numbers of gas bubbles. The yeasts were grown on fermentable media under oxic conditions, therefore it is expected that the Crabtree-positive yeasts would form more gas bubbles when compared with the Crabtree-negative yeasts. Bub = Gas bubbles.

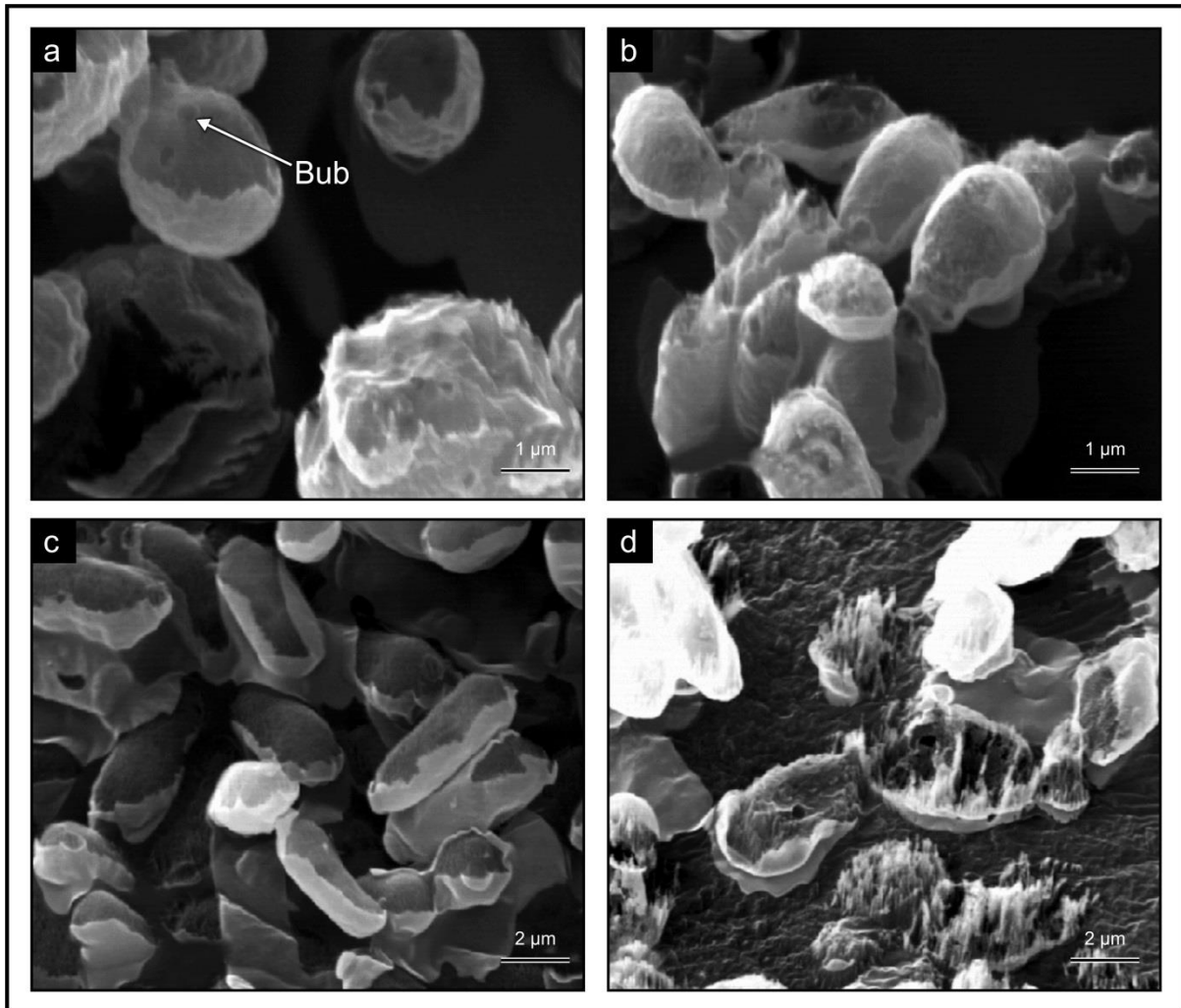


Figure 8. Nano scanning Auger microscope images for the strictly respiring yeasts indicating bubble-like structures believed to be gas bubbles, however these yeasts produced less to no gas bubbles in their cytoplasm. a) *Lipomyces starkeyi*, b) *Pichia membranifaciens*, c) *Yarrowia lipolytica* and d) *Rhodotorula glutinis*. The respiring yeasts contained less numbers of gas bubbles, which is similar to what was observed with the Crabtree-negative yeasts, therefore it is expected that they would form less gas bubbles when compared with the Crabtree-positive yeasts. Bub = Gas bubbles.

2.5. Conclusions

Yeast metabolism is very important in understanding the functioning of yeast cells and it has been shown that fermentation and respiration are the main sources of CO₂ production in yeasts. The discovery by Swart and co-workers (2012) showed that CO₂ produced during fermentation can accumulate inside yeast cells in the form of gas bubbles. This information led to the conceptualization of this study, to investigate gas bubble formation in all types of yeasts i.e. Crabtree-positive, Crabtree-negative and strictly respiring yeasts. A similar strategy to that of Swart and co-workers (2012) was used to investigate gas bubble formation in two Crabtree-positive, two Crabtree-negative and four strictly respiring yeasts.

From the results obtained, it can be concluded that all yeasts used in this study can form gas bubbles, however this is affected by the mode of CO₂ production. The Crabtree-positive yeasts were observed to produce more gas bubbles when compared to the other yeasts. In addition, Crabtree-positive yeasts produced large numbers of gas bubbles when cultivated on YM fermentable media, while on YPG non-fermentable media little to no gas bubbles were formed. Furthermore, the Crabtree-negative yeasts produced less gas bubbles on both YM and YPG media compared to Crabtree-positive yeasts, which was expected since these yeasts were grown under oxic conditions where they can only respire. Similar to the Crabtree-negative yeasts, the strictly respiring yeasts produced less gas bubbles on both fermentable and non-fermentable media and again this was expected because of the inability of these yeasts to ferment. From the results it can be deduced that gas bubble formation seems to be conserved in the yeast domain. The next step in this research would be a detailed analysis of the contents of these gas bubbles as well

as the mechanism by which these bubbles are accumulated and released into the environment.

2.6. Acknowledgements

The author would like to thank the National Research Foundation (NRF) [Thuthuka Funding TTK14042466569] and the University of the Free State, South Africa for financial support.

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Chapter 3

Characterization of gas bubbles in fermenting yeasts

3.1. Abstract

The discovery of gas bubbles by Swart and co-workers (2012) was the missing link between the production of carbon dioxide (CO₂) inside yeasts and its eventual release into the surrounding environment, where they found that these gas bubbles do contain CO₂ (Swart *et al.*, 2012). However this piece of information raised even more questions about the gas composition of these gas bubbles. It is possible that other gases can accumulate inside gas bubbles, since the initial description of a gas bubbles states that these bubbles can contain one type or a mixture of gases (Dean, 1944; Blatteau *et al.*, 2006). In addition it has been observed that during the beer brewing process volatile sulphur compounds can be produced, which provide the beer with off-flavours and bad odour (Anderson & Howard, 1974; Suomalainen & Lehtonen, 1978). As a consequence, this study aims to investigate the gas composition of intracellular gas bubbles in three different Crabtree-positive yeasts. Nano scanning Auger microscopy (NanoSAM) detected elements such as carbon (C), oxygen (O) and nitrogen (N) while with time-of-flight secondary ion mass spectrometry (ToF-SIMS) different sulphur compounds were detected with variable intensities. Although the results obtained should be verified and extended with more specific techniques that can provide more information about the exact production point of these detected compounds.

3.2. Introduction

Hemmingsen and co-workers suggested that gas bubble formation cannot occur in the cytoplasm of microorganisms even under high gas supersaturation (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1990). However in 2012, gas bubble formation was exposed in the highly fermentative yeast *Saccharomyces*. During this study *S. cerevisiae* and *S. pastorianus* were subjected to various imaging techniques that revealed the presence of intracellular inclusions that were later concluded to be gas bubbles (Swart *et al.*, 2012).

During this study two important characteristics of these gas bubbles were identified. Firstly, these gas bubbles lack surrounding membranes, which is an indication that they are not true cell organelles (Swart *et al.*, 2012). Secondly, carbon dioxide (CO₂) was identified as the gas contained within these gas bubbles and this was carried out using the element mapping function of nano scanning Auger microscopy (NanoSAM). For this experiment it was assumed that if CO₂ is present inside these gas bubbles, carbonic acid should be produced in high quantities at the boundary/periphery of the gas bubble. According to the reaction of CO₂ in the bubbles with the water in the cytoplasm of the cell, carbonic acid will be formed. To test this hypothesis zinc (Zn⁺) was added to the growth media in the form of the compound zinc sulphate heptahydrate (ZnSO₄ · 7H₂O). Zinc ions reacted with the carbonic acid forming an insoluble metal bicarbonate that was then detected by NanoSAM. Nano scanning Auger microscopy element map images showed the elements detected, with zinc being observed to have galvanized the gas bubbles. This then led to the conclusion that these gas bubbles contain CO₂ (Swart *et al.*, 2013).

The initial description of a gas bubble states that gas bubbles can contain one type or a mixture of gases (Blatteau *et al.*, 2006). Therefore this implies that other gases can be accumulated into a gas bubble similar to CO₂ (Dean, 1944; Blatteau *et al.*, 2006). Furthermore, the brewing industry has reported that yeast cells can produce different kinds of compounds during the beer brewing process, such as fatty acid esters, aroma compounds and volatile compounds. The most important of these being volatile sulphur compounds that provides the beer with off-flavours and bad odour (Anderson & Howard, 1974; Suomalainen & Lehtonen, 1978). Consequently, the aim of this study is the characterization of these gas bubbles, with emphasis on the types of sulphur compounds that can be detected in the selected yeasts samples.

3.3. Materials and Methods

3.3.1. Strains used and Cultivation

The yeasts used in this study (Table 1) were preserved in the UNESCO MIRCEN Culture Collection of the Department of Microbial, Biochemical and Food Biotechnology, University of the Free State, South Africa, 9300. These yeasts were chosen according to their mode of CO₂ production.

Table 1. A list of the Crabtree-positive yeast strains used in the experiments.

Yeast Names	Strain Number	Mode of carbon dioxide production
<i>Schizosaccharomyces pombe</i>	CBS 356, UOFS Y-2174 T	Crabtree-positive
<i>Torulaspota globosa</i>	CY 130 8/95, UOFS Y-0847	Crabtree-positive
<i>Zygosaccharomyces bailli</i>	IGC 4245, UOFS Y-1535	Crabtree-positive

The three yeasts were maintained on glucose yeast malt (YM) agar plates (Wickerham, 1951) for 48 h at 30 °C. The inoculum was prepared by inoculating 100 ml of YM media in 500 ml conical flasks with a loopful of the cells. The inoculum was incubated at 30 °C for 24 h, while shaking at 160 rpm. Experimental flasks were then prepared by inoculating 500 ml conical flasks, containing 100 ml YM media, with 1 ml of the inoculum. The flasks were incubated for 48 h at 30 °C, while shaking at 160 rpm and after 48 h, samples were drawn for analysis. The samples were analysed with NanoSAM as described by Swart *et al.* (2010) and time-of-flight secondary ion mass spectrometry (ToF-SIMS) (Malm *et al.*, 2009; Passarelli & Winograd, 2011).

3.3.2. Nano scanning Auger microscopy (NanoSAM)

This was performed according to the procedure in Chapter 2, 2.3.3.

3.3.3. Time-of-flight secondary ion mass spectrometry (ToF-SIMS)

Cells were harvested after 48 h of incubation using centrifugation at 1450 g for 5 min. Then these cells were subjected to fixation with 3 % glutaraldehyde (Merck, Darmstadt, Germany) buffered with 0.1 M (pH 7) sodium phosphate buffer followed

by a second fixation with a buffered solution of 1 % osmium tetroxide for 1 h. After each fixation step the cells were rinsed twice with sodium phosphate buffer for 5 min. The next step is dehydration of the fixed cells with a series of ethanol concentrations; 50 %, 70 %, 95 %, for 20 min per step. Then followed two 100 % ethanol steps, each performed for 1 h. Thereafter, the cells were critically point dried and mounted on metal stubs. For ToF-SIMS the stubs were not coated with gold because this machine is equipped with its own flood gun to provide conductive contact for charge compensation. The sample analysis was performed in a ToF-SIMS IV instrument (ION-TOF GmbH, Münster, Germany) equipped with a bismuth (Bi^+) cluster primary ion source. Using 30 keV Bi^+ primary ions, a negative ion spectrum was recorded with electron flooding for charge compensation. During the analysis, data was acquired in 512 x 512 pixels over an analysis area of 20 x 20 μm within the sputtered area of 100 x 100 μm . The sputtering was carried out for 10 sec producing 125 scans for each sputtering session with the rate being 62.93 sec/scan. The mass spectrum with specific peaks of interest was extracted from the raw data after acquisition.

3.4. Results and Discussion

3.4.1. NanoSAM element analysis for Crabtree-positive yeasts

The targeted etching function of NanoSAM was used for the analysis of the three Crabtree-positive yeasts. The points where gas bubbles were observed to appear were targeted for the analysis (Fig. 1a, c, e). The readings from these points are represented in a spectrum that indicates the elements found at that specific point on the cell. From the results obtained, five elements were detected and these results

were observed with each of the three yeasts. The points where gas bubbles were appearing was observed to contain Carbon (C), Oxygen (O), Nitrogen (N), Osmium (Os) and Gold (Au) (Fig. 1b, d, f). There was no significant difference in the intensity of these detected elements between the three yeasts. Furthermore, the detected elements were to be expected since C, O, N are basic building blocks of the cell and the samples were initially fixed with osmium tetroxide (OsO_4) and coated with gold before analysis.

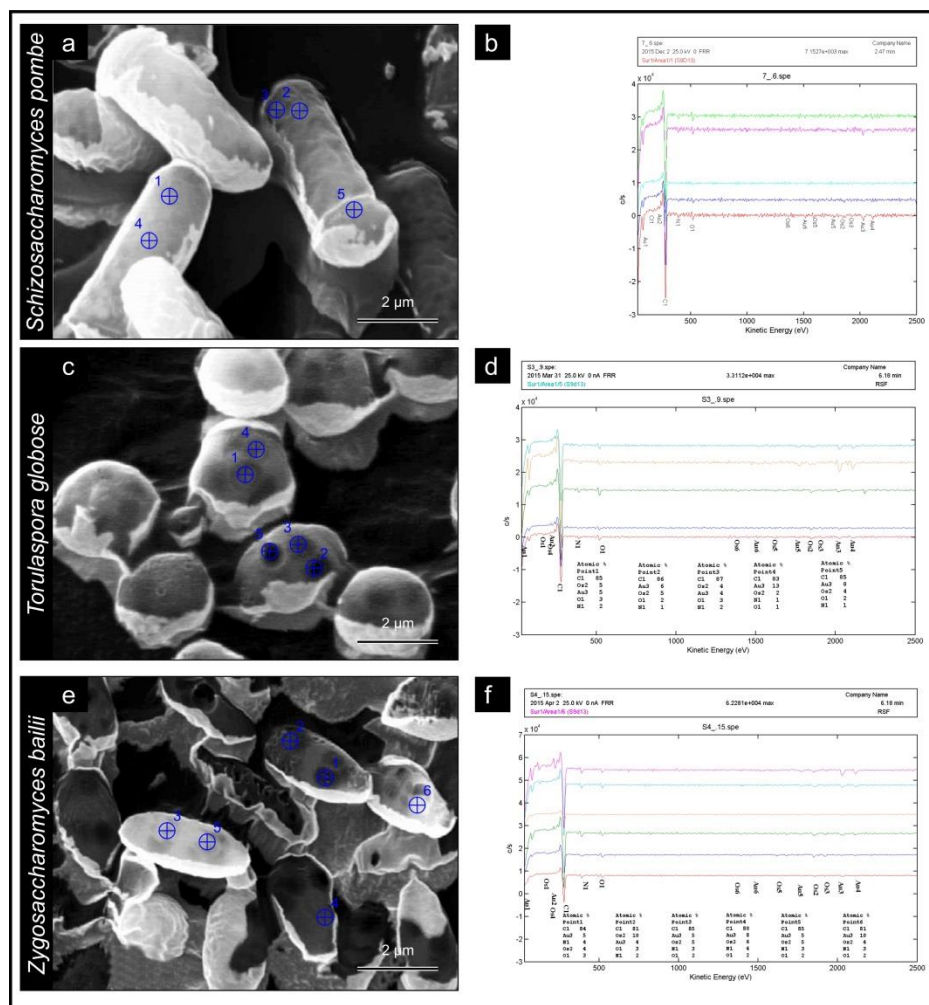
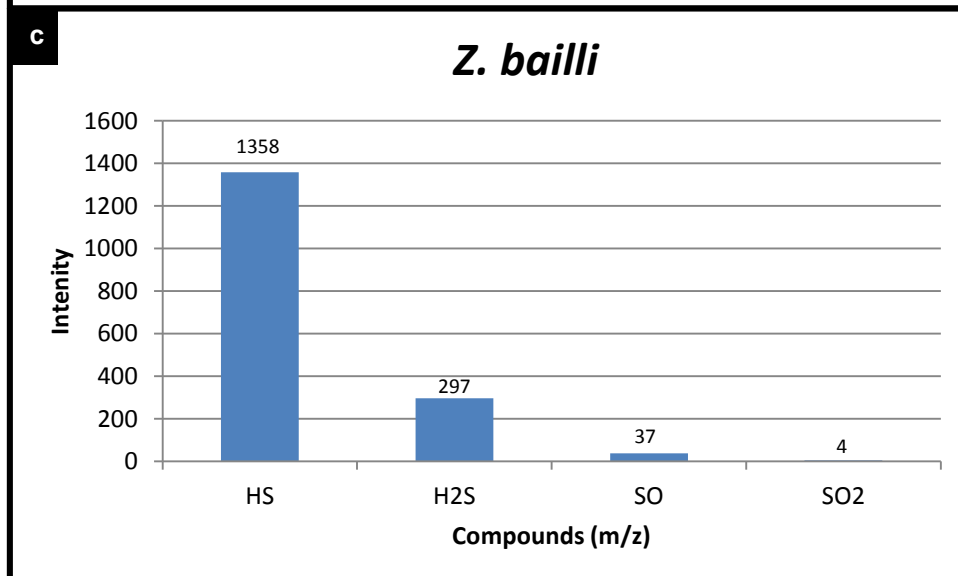
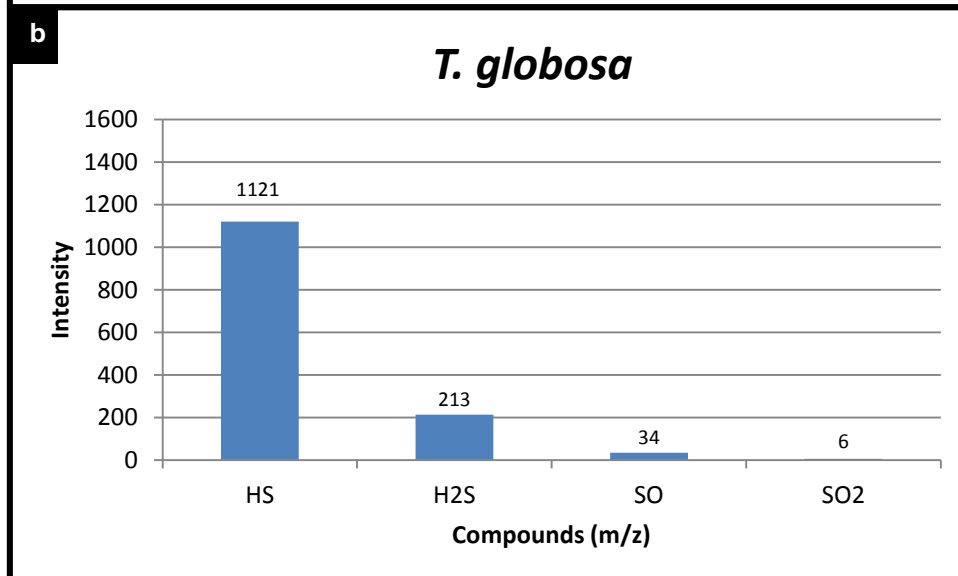
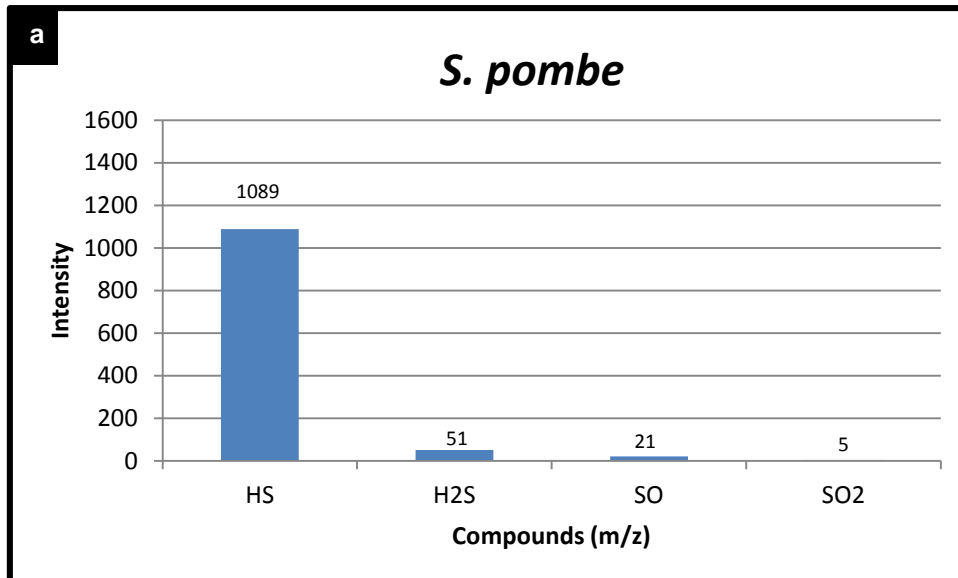


Figure 1. Nano scanning Auger microscopy (NanoSAM) targeted etching mode results for the Crabtree-positive yeasts. a) *Schizosaccharomyces pombe*, c) *Torulaspora globosa*, e) *Zygosaccharomyces bailli*, indicating the specific points where the analysis was performed, which are the points where gas bubbles were beginning to appear. While the spectra indicate the detected elements such as; oxygen, nitrogen, carbon, osmium and gold and at what intensity were they detected. b) *Schizosaccharomyces pombe*, d) *Torulaspora globosa*, f) *Zygosaccharomyces bailli*.

3.4.2. Sulphur compound detection by ToF-SIMS in Crabtree-positive yeasts

The ToF-SIMS generated spectrum was used to create bar graphs with specific peaks that were monitored. The initial graphs included all the selected peaks while the second graphs focuses only on the sulphur compounds detected. With the three Crabtree-positive yeasts analysed it was observed that they all produced similar sets of results. The initial bar graphs indicated C and O having the highest intensity while the other compounds were not intensely detected. These bar graphs (Fig. 3) indicate the intensity of the sulphur ions relative to the other sulphur compounds not relative to the whole spectrum that was generated from the analysis. Hydrogen sulphide ion (HS^-) was the sulphur compound with the highest intensity when specifically focusing on the sulphur compounds graphs (Fig. 3). These observations were similar with all the three yeasts, with a slight increase in the intensity of the sulphur compounds in the yeast, *Zygosaccharomyces bailli* (Fig. 3c). From the results obtained all three of the yeasts did indicate sulphur compound detection, however further investigations need to be done for more specific information.



3.5. Conclusions

The initial description states that a gas bubble may contain one type or a mixture of gases; with the implication being that most gases may accumulate into a gas bubble, whether as a single gas or a combination of gases. In a study by Swart and co-workers (2012) CO₂ was identified as the gas inside the gas bubbles observed in the highly fermentative yeast *Saccharomyces*. From these findings other questions were raised about the nature of the gas composition of gas bubbles in other fermenting yeasts such as *Schizosaccharomyces*, *Zygosaccharomyces* and *Torulaspota*. Nano scanning Auger microscopy was able to detect five elements on the specific points on the samples where the analysis was performed. The detected elements were expected since C, O and N are found in all cells while Os and Au were used in the sample preparation process. It is possible that the gas accumulating inside these gas bubbles was released prior to analysis; therefore the analysis could have been performed on cell matter rather than the gas in the gas bubble. This could have occurred due to the thin layer that is surrounding the gas bubble being removed by the etching of the microscope leading to the dispersal of the gas before the actual analysis begins. Although this is a precision issue of the microscope that can be circumvented only when the exact position of a gas bubble is known.

Furthermore, ToF-SIMS detected sulphur compounds in the same three yeasts with slight differences in the intensity of the compounds. The Hydrogen sulphide ion was detected in larger quantities in the samples as compared to the other sulphur compounds. However since the analysis was not specifically performed on a point where a gas bubble was observed, it cannot be said that these compounds were detected specifically inside a gas bubble. Time-of-flight secondary ion mass

spectrometry is a surface analytical technique providing chemical information regarding the outermost part of the sample (Sjövall *et al.*, 2008). Accordingly, a longer sputtering session would have improved the quality of our results assuming that the deeper the sputtering occurs the more likely to encounter gas bubbles. In addition, this technique includes high vacuum prerequisites which demands proper sample preparations. Furthermore, it has been stated in literature that for vacuum compatibility, chemical fixations for sample preparation must be averted or used under very clean and careful conditions (Chandra, 2008; Sjövall *et al.*, 2008; Malm *et al.*, 2009). Therefore for improved results our samples can be prepared using cryofixation or glutaraldehyde only fixation considering that samples fixed with osmium tetroxide (OsO_4) tend to be dominated with peaks of different osmium oxide species (Malm *et al.*, 2009). With the focus on the brewing industry, the determination of the gas composition of these gas bubbles may demonstrate an alternative way for the accumulation of sulphur compounds in the beer production process. This could give new insight on how to regulate and eventually prevent the production of these specific compounds. However more specific techniques need to be employed to investigate this further, with the focus being on the sulphur compounds found in gas bubbles specifically.

3.6. Acknowledgements

The author would like to thank the National Research Foundation (NRF) [Thuthuka Funding TTK14042466569] and the University of the Free State, South Africa for the financial support.

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Chapter 4

Conclusions

Main Conclusions

As a result of this study, the conserved status of gas bubbles has been confirmed in yeasts, however the amount formed depends on whether the yeast ferments, respire or both. In addition, regarding the gas composition of the Crabtree-positive fermenting yeasts, sulphur compounds were detected, although this needs to be investigated further. Moving forward the relationship between gas bubble formation and fermentation time or sugar consumption needs to be addressed. Furthermore, the point of production and the amount of the detected sulphur compounds should be further investigated.

Summary / Opsomming

Summary

Literature previously suggested that intracellular gas bubble formation cannot occur in microorganisms even under high gas supersaturation. This observation was disproved with the discovery of gas bubbles in the yeasts *Saccharomyces cerevisiae* (baking industry) and *Saccharomyces pastorianus* (Brewing industry). As a result the missing link between the excessive production of carbon dioxide (CO₂) by alcoholic fermentation inside the yeast cells and the vigorous release of this CO₂ into the surrounding environment was uncovered. Further investigations proved that CO₂ was indeed the gas contained inside these gas bubbles yet additional analysis of this gas composition was still needed.

Here we investigate the occurrence of gas bubbles in yeasts that are fermenting and non-fermenting to identify any occurring differences and conserved status of gas bubbles formation in yeasts. Gas bubbles occurring in Crabtree-positive fermenting yeasts were observed to be more than those formed in the other yeasts (Crabtree-negative and strictly respiring yeasts). Although this was observed only on fermentable media, while on non-fermentable media less or no gas bubbles were formed. Furthermore, the Crabtree-negative fermenting and strictly respiring yeasts revealed similar results on both fermentable and non-fermentable media, with little to no gas bubble formation. Gas composition analysis was also carried out on three Crabtree-positive yeasts and it indicated the occurrence of five elements that were expected to be present in our samples. Moreover detection of sulphur compounds was observed in the same three samples with varying intensities. Nonetheless further research is still needed to confirm the point of production for these compounds, using more specific and sensitive techniques. From these findings it can

be concluded that gas bubble formation is conserved in yeasts, with the amount of bubbles formed depending on the mode of CO₂ production. In addition, it is possible that other gases or compounds such as sulphur compounds can be contained inside these gas bubbles.

Keywords: Carbon dioxide, Crabtree-negative, Crabtree-positive, Fermentation, Intracellular gas bubbles, NanoSAM, Respiration, Sulphur compounds.

Opsomming

Literatuur tot dusver het voorgestel dat intrasellulêre gasborrels nie in mikroörganismes kan voorkom nie, selfs nie onder toestande van hoë gas-superversadiging nie. Hierdie waarneming is egter verkeerd bewys met die ontdekking van gasborrels in die giste *Saccharomyces cerevisiae* (bakkersbedryf) en *Saccharomyces pastorianus* (brouersbedryf). Die ontbrekende skakel tussen die uitermatige vorming van koolstofdiksied (CO_2) tydens alkoholiese fermentasie binne die gisselle en die hoë tempo van CO_2 vrystelling na die omliggende omgewing is ontdek. Verdere ondersoek het bewys dat CO_2 inderdaad die gas is wat die borrels vul, alhoewel die gassamestelling verder ondersoek moes word.

Hier vergelyk ons die teenwoordigheid van gasborrels in gis wat fermenteer met die teenwoordigheid van gasborrels in gis wat nie fermenteer nie om die status van borrelvorming in hierdie giste te bepaal. Meer gasborrels het in Crabtree-positiewe fermenterende gis gevorm wanneer hierdie gis met ander gis vergelyk is (Crabtree-negatiewe fermenterende gis en slegs-respirerende gis). Hierdie verskynsel geld egter net wanneer fermenterende gis op fermenteerbare medium gegroei is, groei op nie-fermenteerbare medium het minder of geen borrels tot gevolg gehad. Verder het die groei van Crabtree-negatiewe fermenterende gis en slegs-respirerende gis op fermenteerbare medium soortgelyke resultate as met groei op nie-fermenteerbare medium gelewer, met geen of min borrelvorming. Die samestelling van die gas in borrels van drie Crabtree-positiewe fermenterende giste is bepaal en die teenwoordigheid van vyf elemente is vasgestel, soos wat verwag is. Verder is die teenwoordigheid van swaelverbindinge in dieselfde drie monsters waargeneem maar het egter in hoeveelheid gewissel. Verdere ondersoek met meer spesifieke en

sensitiewe toerusting is egter nodig om die hoeveelheid wat gevorm word te kan bevestig. Vanuit hierdie waarnemings kan daar afgelei word dat gasborrel-vorming gekonserveerd is in gis en dat die hoeveelheid borrels wat vorm afhang van die wyse waarop CO₂ gevorm word. Bykomstig is dit moontlik dat ander gasse of verbindings, soos swaelverbindings, deel kan vorm van die inhoud van hierdie borrels.

Sleutelwoorde: Crabtree-negatief, Crabtree-positief, Fermentasie, Intracellulêre gasborrels, Koolstofdiksied, NanoSAM, Respirasie, Swaelverbindings.