Characterisation of intracellular gas bubbles in *Saccharomyces*

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I dedicate this thesis to my beloved wife Siphiwe Millicent Dithebe and my precious daughter Khumo Dithebe.

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DECLARATION

I, Khumisho Dithebe, declare that this thesis that I herewith submit for the degree in Microbiology at the University of the Free State, is my independent work and that I have not previously submitted it for qualification at another institution of higher education.

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Please note: The chapters in the thesis are prepared in manuscript format for journal submission. Consequently, repetition of some information could not be avoided.

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Chapter 1
Literature Review

1.1. Motivation

Even though the fermentation process in yeasts is one of the most extensively studied, until recently there had been no reports of intracellular CO_2 bubbles inside yeast cells. The missing link between CO_2 production and its eventual release from the cell was resolved when Swart and co-workers (2012) discovered the presence of intracellular gas bubbles, in fermenting brewer's and baker's yeasts, using various microscopy techniques. The intracellular gas bubbles were observed to accumulate and occupy a significant part of the cell, leading to the compression and deformation of cell organelles (Swart *et al*., 2013). Subsequent to the discovery, several other yeasts have been studied and the presence of intracellular gas bubbles was found to be conserved (Du Plooy, 2015; Kgotle, 2016; Saaiman, 2017). Interestingly, other cell inclusions with similar ultrastructure as the gas bubbles have been reported as either vacuole fragments (Zeiger and Mayer, 2012) or lipid droplets (Jacquier *et al*., 2011).

This research project seeks to uncover whether intracellular gas bubbles, vacuole fragments and lipid droplets are the same structures or separate inclusions. Should intracellular gas bubbles be separate inclusions, the accumulation of intracellular gas bubbles may affect cell physiology and function. The brewer's and baker's yeast are of industrial importance, thus an investigation into the effects of intracellular gas bubble formation is warranted.

1.2. Introduction

During fermentation, yeasts are capable of producing increased amounts of ethanol and carbon dioxide (CO_2) with the latter being vigorously released from the yeast cell into the surrounding medium (Van Maris *et al*., 2001). This creates an expectation that CO_2 bubbles should be present inside the yeast cells prior to release. Even though the fermentation process in yeasts is one of the most extensively studied, there have been no reports of intracellular CO_2 bubbles inside yeast cells. The lack of reports on intracellular CO_2 bubbles can be ascribed to the extensive research by Hemmingsen and co-workers (1979) who, through various supersaturation and decompression studies, reported that gas bubbles could not be formed in the cytoplasm of yeasts (Hemmingsen & Hemmingsen, 1979). These researchers suggested that the increased structuring of water inside the cells as well as the lack of water with normal nucleation properties did not allow for intracellular gas bubble formation (Hemmingsen *et al*., 1985; Hemmingsen *et al*., 1990). Furthermore, not even the protein-coated gas vesicles found in prokaryotes, such as Cyanobacteria, were expected in yeasts (Walsby, 1994).

However, the assumption that yeasts do not produce intracellular gas bubbles was against expectation since yeasts vigorously release CO_2 gas during fermentation. It is, however, not clear what happens to the CO_2 between production via the alcoholic fermentation pathway and its eventual release from the cell. Furthermore, only a small portion of CO_2 is converted to carbonic acid (H_2CO_3) in the presence of water at neutral pH (Kern, 1960; Wojtowicz, 1995). Since the yeast cytoplasm has a neutral pH (Breeuwer & Abee, 2000), it is expected that most of the CO_2 should be present as a

gas inside fermenting yeast cells. In addition to this, yeasts cells, which are usually cultivated at 30 °C, have an internal pressure of 2.1MPa (Vella *et al*., 2012). According to the pressure-temperature phase diagrams, CO_2 is present as a gas under these conditions.

A paradigm shift emanated when Swart and co-workers (2012) discovered intracellular gas bubbles in the brewer's (*Saccharomyces pastorianus*) and baker's (*Saccharomyces cerevisiae*) yeasts. They reported intracellular gas bubbles observed as light scattering granules using light microscopy (LM) in both the brewer's and baker's yeasts. Further analyses of these granules were performed using nano scanning Auger microscopy (NanoSAM) and transmission electron microscopy (TEM). Using NanoSAM, Swart and co-workers observed a maze of coalescing intracellular gas bubbles in fermenting brewer's yeast. Transmission electron microscopy was used to confirm the LM and NanoSAM observations. This resulted in the observation of non-enveloped electron transparent structures, which had the same size and shape as the bubbles observed with NanoSAM. The observations of gas bubbles in yeasts using LM and TEM were similar to the observations of protein-coated gas vesicles in blue-green algae using LM and TEM (Bowen & Jensen, 1965). Furthermore, to trace CO_2 inside the brewer's yeast, Swart and co-workers supplemented the fermentable growth medium with zinc in the form of $ZnSO_4.7H_2O$ (Swart *et al*., 2012). They reported that zinc accumulated at the periphery of the gas bubbles. Since H_2CO_3 is expected to be produced at the periphery of CO_2 gas bubbles due to the reaction of CO_2 and the surrounding water, they ascribed this accumulation to the reaction of zinc with H_2CO_3, leading to the production of

insoluble or weakly soluble metal bicarbonate at neutral cytoplasmic pH. It was later reported that the intracellular gas bubbles compress and deform cell organelles in fermenting brewer's yeast (Swart *et al*., 2013). This discovery of intracellular gas bubbles proposed to resolve the missing link between intracellular CO_2 production via the alcoholic fermentation pathway in yeasts and its eventual release from the cells (Swart *et al*., 2012). Since the initial report, other yeasts have been studied and the occurrence of intracellular gas bubbles was found to be conserved in yeasts (Du Plooy, 2015; Kgotle, 2016; Saaiman, 2017).

1.3. Intracellular gas bubbles, vacuole fragments and lipid droplets

Intracellular gas bubbles are characterised as non-enveloped electron transparent structures when observed with TEM (Swart *et al*., 2012). This lack of membranes suggests that they are not true organelles, since organelles are known to be membrane bound (Wiederhold *et al*., 2010). Interestingly, other non-enveloped electron transparent structures have been reported differently. Zeiger and Mayer (2012) reported non-enveloped electron transparent structures as vacuole fragments, while Jacquier and co-workers (2011) labelled similar structures as lipid droplets. The observation of these cell inclusions without membranes is peculiar since vacuoles are known to be membrane bound (Wiederhold *et al*., 2009), while lipid droplets are enclosed by a phospholipid monolayer (Walther & Farese Jr, 2009). It is interesting to note that Zeiger and Mayer (2012) observed vacuole membranes when they stained the cells with FM4-64, a vacuole membrane-specific fluorescent probe. Swart *et al* (2012) used zinc to determine the composition of the gas bubbles, however, it is known that yeast cells grown under

excess zinc accumulate the zinc in the vacuole (Simm *et al*., 2007). Since both inclusions have the ability to accumulate zinc, it is possible that these inclusions could be the same.

The similarities between intracellular gas bubbles, vacuole fragments and lipid droplets are not only limited to their appearance when observed with TEM, they also have a similar response to glucose concentrations. Swart and co-workers (2012) reported that intracellular gas bubbles accumulate in *S. cerevisiae* and *S. pastorianus* yeast cells cultivated on fermentable, high glucose containing medium, while Izawa and co-workers (2010) reported an increase in vacuole fragments in wine and *sake* yeast cells cultivated in high sugar-containing medium. Similarly, lipid droplets have been reported to accumulate at stationary phase in *S. cerevisiae* cells grown on high glucose containing medium (Chumnanpuen *et al*., 2011). Since intracellular gas bubbles, vacuole fragments and lipid droplets have similar ultrastructure and response to glucose concentration, further research is required to determine whether they are indeed different inclusions inside the cells.

1.4. Gas composition of intracellular bubbles

Following element mapping of the intracellular bubbles with NanoSAM, Swart and co-workers (2012) concluded that intracellular gas bubbles contain CO_2 gas. According to literature, a gas bubble can be made up of either one type or a mixture of gases (Blatteau *et al*., 2006). Although Lodolo and co-workers (2008) listed CO_2 as one of the yeast-derived flavour-active compounds in beer, it is known that a broad range of

aroma-active compounds are produced by the yeast during fermentation (Hiralal *et al*., 2014). These volatile compounds play a significant role in the complex flavour and aroma of fermented beverages such as beer and wine (Saerens *et al*., 2008; Rossouw *et al*., 2008). Volatile flavor compounds are divided, according to their structure, into higher alcohols, esters (ethyl esters and acetate esters), sulfur-containing compounds and carbonyl compounds (aldehydes and ketones; Kobayashi *et al*., 2008). *Saccharomyces* yeasts also produce sulfur-containing gases such as H_2S and SO_2 during alcoholic fermentation. The fact that yeasts produce a broad range of volatile compounds in fermented beverages and that gas bubbles can be made up of a mixture of gasses necessitates further research to determine the full complement of gases present inside the intracellular bubbles in the brewer's and baker's yeasts.

1.5. Stability of yeast intracellular bubbles

In an effort to determine the effects of intracellular gas bubbles on cell organelles, Swart and co-workers (2013) reported that the gas bubbles compressed and deformed cell organelles. A question that comes to mind is what stabilises the bubbles inside the cells. According to Yount (1979), the existence of gas bubbles requires at least a thin film of water around them. According to Blasco *et al.* (2011), various yeast-derived compounds such as proteins and polysaccharides contribute to foam formation and stabilisation. Furthermore, yeast-derived proteins and polypeptides contribute more to foam stabilisation than formation (Kordialik-Bogacka & Ambroziak, 2004; Blasco *et al*., 2012). Other yeast-derived compounds, such as lipids, are also released during yeast autolysis in sparkling wine (Alexandre & Guilloux-Benatier, 2006). These lipids have an impact on

the foam quality of sparkling wine. In their study to determine the influence of fatty acids on wine foaming, Gallart and co-workers (2002) reported that esterified fatty acids contribute positively to foam formation in wine. Since various yeast-derived compounds play a crucial role in foam formation and stabilisation in fermented beverages, it is imperative to determine whether these compounds also play a role in stabilisation of intracellular gas bubbles found in fermenting yeasts.

1.6. Effects of gas bubbles on organelle function

Swart and co-workers (2012) reported that intracellular gas bubbles occupy a significant part of the yeast cells. Additionally, Swart *et al*. (2013) reported that intracellular gas bubbles compress and deform intracellular organelles. This gives rise to the question: what effects does the physical interaction between intracellular gas bubbles and cell organelles have on the function of the cell organelles? Yeast cells, with internal pressure of 2.1 MPa, produce increased CO_2 during fermentation. Not only can the produced CO_2 reduce biomass yield and fermentation capacity (Aguilera *et al*., 2005), there are also several detailed mechanisms that have been proposed for the effects of pressurised CO_2 on yeast and bacterial cells (Garcia-Gonzales *et al*., 2007). The accumulation of CO_2 may result in the decrease in intracellular pH, which can culminate in the inactivation of certain key enzymes, leading to reduced growth or even death (Shimoda *et al*., 1998). Since CO_2 is a metabolic product of alcoholic fermentation, there is a possibility that elevated CO_2 content may also inhibit decarboxylation enzymes through product inhibition. Cells exposed to elevated CO_2 have been reported to have dysfunctional mitochondria as well as impaired cell growth (Vohwinkel *et al*., 2011). Carbon dioxide may also accumulate in the cell membrane where it will alter the

structure of the cell membrane and increase the membrane fluidity, this phenomenon is known as the “anaesthesia effect” (Isenchmid *et al*., 1995). Hydrostatic pressure has been reported to have adverse effects on the yeast *Saccharomyces cerevisiae* (Fernandes *et al*., 2001). The yeast intracellular organelles, including the nucleus, mitochondria, endoplasmic reticulum (ER) and vacuole were deformed or disrupted by the application of increased pressure (Shimada *et al.,* 1993). In addition, Ju and co-workers (2007) reported that elevated pressure triggers mitochondrial fission, leading to reduced ATP production inside differentiated ganglion cells. Furthermore, the increase in hydrostatic pressure resulted in the leaking out of internal substances such as amino acids and various metal cations from yeast cells (Shimada *et al.,* 1993).

1.7. Purpose of research

With all this as background, the aims of this study became:

i. To determine whether intracellular bubbles, vacuole fragments and lipid droplets are the same cell inclusions.
ii. To determine the possible gas composition of intracellular bubbles.
iii. To determine the effects that intracellular bubbles have on cell function.
iv. To determine whether intracellular bubbles play a role in cell buoyancy, cell flocculation and cell surface hydrophobicity.

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Chapter 2

Gas bubbles, vacuole fragments and lipid droplets

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Abstract

The discovery of intracellular gas bubbles in fermenting *Saccharomyces cerevisiae* and *S. pastorianus* yeast cells using various microscopy techniques, is considered a paradigm shift as it provided the link between CO_2 production and eventual release from the cells. Interestingly, these intracellular gas bubbles have a similar appearance to vacuole fragments and lipid droplets when observed with transmission electron microscopy. In this study, fluorescent probes were applied to differentiate between gas bubbles, vacuole fragments and lipid droplets. The lipid content of the strains after growth on different glucose concentrations was determined. Additionally, headspace-gas chromatography was employed to analyse the gas composition of the bubbles. The study elucidates that intracellular gas bubbles, vacuole fragments and lipid droplets are separate inclusions that co-exist inside of the cells. Headspace-gas chromatography analysis confirmed that the bubbles contain CO_2 as previously reported. Despite the two yeasts having different flocculation profiles, they had similar lipid contents on high glucose, suggesting a possible role for intracellular bubbles in cell buoyant density.

2.1. Introduction

The discovery of intracellular gas bubbles in *Saccharomyces cerevisiae* and *S. pastorianus*, grown in fermentable (high glucose) medium, is considered a paradigm shift as it provided a link between CO_2 production during alcoholic fermentation and the eventual release from the cell [1]. In previous studies, intracellular gas bubbles were observed as light scattering granules using light microscopy and further analysis with nano scanning Auger microscopy (nanoSAM) revealed that the bubbles form an

interconnected maze, which occupied a significant part of fermenting yeast cells [1,2]. Transmission electron microscopy (TEM) analysis showed that the electron transparent gas bubbles were not membrane-bound [1,2]. The lack of a membrane became a distinguishing feature that suggested the bubbles are not true organelles, since organelles are known to be membrane bound [1-3]. Using nanoSAM, Swart et al. [1] observed that zinc accumulated around the gas bubbles in yeasts cultivated on fermentable medium supplemented with zinc. The researchers ascribed this accumulation to the reaction of zinc and carbonic acid, which they expected to form at the periphery of CO_2 bubbles, thereby giving an indication that the bubbles contain CO_2.

Interestingly, cultivation of *S. cerevisiae* on medium containing high glucose levels has been reported to induce vacuole fragmentation [4] and accumulation of lipid droplets [5]. Even though lipid droplets are enveloped by a phospholipid monolayer [6] and vacuoles are enclosed by a vacuolar membrane [7], these cell inclusions have been reported without the presence of a membrane [8,9] – a distinguishing feature of intracellular gas bubbles [1,2]. It is also important to note that the method of sample preparation for TEM observation may result in lipid droplets appearing as non-enveloped structures [10].

Although *Saccharomyces* yeasts are Crabtree positive (i.e. they are able to ferment glucose to ethanol under aerobic conditions) [11], cultivation of yeasts from this genus on medium with reduced glucose or a non-fermentable carbon source (both favouring an oxidative metabolism over a fermentative metabolism) results in the fusion of vacuole fragments to form a large vacuole [4,12] and mitigates accumulation of gas bubbles inside of the cells [1,13].

Given that the formation of intracellular gas bubbles, accumulation of lipid droplets and fusion of vacuole fragments are all induced or diminished under comparable glucose concentrations and that these inclusions have similar ultrastructure, this raises the question of whether these structures are the same or separate inclusions. Considering the drawback of sample preparation for TEM analysis [10] and the additional aforementioned details, an alternative method of study is required to distinguish these inclusions. In this study, fluorescent probes were applied to distinguish between intracellular bubbles, lipid droplets and vacuole fragments.

2.2. Materials and methods

2.2.1. Strains used

The following strains were used in this study: *Saccharomyces pastorianus* WS 34-70 (preserved at Cara Technology Limited, Leatherhead Enterprise Centre, Leatherhead, Surrey, UK), *S. cerevisiae* CBS 1171 NT (preserved at the Westerdijk Institute, Utrecht, Netherlands).

2.2.2. Cultivation

The yeasts cells were cultivated in yeast extract peptone dextrose (YPD) medium with the following composition: 1% yeast extract, 2% peptone and 2% or 0.2% glucose (all concentrations are in w/v). The cells were pre-cultured in 500 ml shake flasks with 100 ml YPD medium containing 2% glucose for 24 h at 30 °C on a rotary shaker at 160 rpm. Thereafter, the cells from each pre-culture were transferred to 500 ml shake flasks containing 100 ml YPD medium with either 2% or 0.2% glucose. The flasks were incubated for 48 h at 30 °C on a rotary shaker at 160 rpm.

2.2.3. Co-staining of vacuole membranes and lipid droplets

Cells were stained with the FM 4-64 dye (Life Technologies, Molecular Probes) to visualise yeast vacuolar membranes, using a modified pulse-chase procedure [14]. The cells were harvested in 2 ml microcentrifuge tubes by centrifugation at 5000 rpm for 5 minutes at room temperature (RT, HERMLE Z 326 K centrifuge, Germany). The supernatant was discarded and the cells were re-suspended in 500 µl of YPD containing either 2% or 0.2% glucose and 1 µl FM 4-64 from a 100 mM stock solution in water. The samples were incubated in the dark in a 30 °C water bath for 30 min. Thereafter, an additional 1 ml medium was added and the cells were centrifuged at 5000 rpm for 5 min at RT to remove excess stain. This was followed by two washes in 1 ml of the corresponding YPD medium and a centrifugation after each wash.

Cells were re-suspended in 1 ml corresponding YPD medium and transferred to 50 ml conical tubes where an additional 4 ml of the corresponding YPD medium was added and the samples were incubated at 30 °C for 90 min on a rotary shaker at 160 rpm. At the conclusion of the incubation, the samples were centrifuged at 5000 rpm for 5 min and re-suspended in 990 µl phosphate buffered saline (PBS). Thereafter, 10 µl BODIPY™ 493/503 (4,4-difluoro-1,3,5,7,8-pentamethyl-4-bora-3a,4a-diaza-s-indacene; Sigma-Aldrich) from a 1 mg/ml stock dissolved in dimethyl sulfoxide (DMSO) was added. This was followed by incubation in the dark for 15 min at 37 °C. The samples were centrifuged and washed with PBS to remove excess stain, and subsequently spotted onto a microscope slide. Analysis was performed with an Olympus CKX53 microscope equipped with a 100W mercury lamp coupled to a SC500 camera and micrographs from five to 10 random fields of view were taken.

2.2.4. Nile red staining

Cells were harvested by centrifugation at 5000 rpm for 5 min (HERMLE Z 362 K centrifuge, Germany), washed and re-suspended in PBS. Thereafter, 100 µl of the lipid droplet specific stain, Nile Red (Sigma-Aldrich), was added to 1000 µl of the cell suspension and incubated for 1 min. A drop of the suspension was placed on a microscope slide and the analysis was performed using a confocal laser scanning microscope (CLSM; Nikon Eclipse TE 2000E C1, Japan). Micrographs from five to 10 random fields of view were taken.

2.2.5. Total lipid extraction

Cells were harvested by centrifugation at 5000 rpm for 5 min (HERMLE Z 362 K centrifuge, Germany), transferred to pre-weighed empty petri dishes, frozen at -80 °C and then freeze-dried. Total lipid extraction was performed according to Folch et al. [15] on the freeze-dried cells. The cells were crushed and left in a 2:1 (v/v) mixture of chloroform and methanol overnight. The extracted lipids were washed twice with distilled water and the solvent was evaporated using a rotary evaporator. Thereafter, diethyl ether was added to dissolve the lipids. The mixture was then transferred to pre-weighed vials in which the solvent was evaporated using nitrogen gas. The vials were then dried at 100 °C overnight before they were weighed to determine the lipid content of the biomass (w/w). Lipid extractions of cells cultivated in YPD containing 2% or 0.2% glucose were performed in triplicate and the Student *t*-test was applied to compare mean lipid content between the different glucose concentrations for each strain wherein a *p*-value less than or equal to 0.05 denoted statistical significance.

2.2.6. Headspace-gas chromatography (GC)

To determine the gas content of the bubbles, cultivation was done in the same medium as the one used by Swart et al. [1]. The yeast cells were cultivated in 500 ml shake flasks with 100 ml of highly fermentable Yeast Malt (YM) medium (1% glucose, 0.3% yeast extract, 0.3% malt extract, and 0.5% peptone) for 48 h at 30 °C on a rotary shaker at 160 rpm. Cells were harvested by centrifugation at 5000 rpm for 10 min (HERMLE Z 362 K centrifuge, Germany) and the supernatant was discarded. The cells were re-suspended in 4 ml PBS and the yeast suspension was transferred to a gas chromatography (GC) vial containing 0.2 – 0.5 µm glass beads (6 ml). The cells were broken by vortexing for 5 min and the headspaces of the vials were sampled with a gas tight syringe to determine the gasses released from the broken cells. To obtain the samples, the vials were over-pressured by injecting 5 ml of helium with the 50 ml gas tight syringe. A 10 ml sample was then withdrawn with the same syringe and injected into a manual sampling valve with a 2.5 ml sample loop. The loop and syringe were flushed with helium between injections.

The analysis was conducted using a Shimadzu gas chromatograph (Japan), fitted with a Restek ShinCarbon ST 80/100 packed column (length 2 m, inner diameter 0.53 m) and a helium barrier plasma discharge detector (gas flow 80 ml/min and temperature 280 °C). Carrier gas was helium at 290 kPa head pressure. Initial oven temperature was 40 °C held for 2 min, then raised at 25 °C/min to 250 °C held for 2 min. The CO_2 peak was initially defined with standard gas mixes from Air Liquide (South Africa) containing either 1 or 25% CO_2 in nitrogen.

To quantify the amount of CO_2 detected, the detector response factors for oxygen (O_2), nitrogen (N_2) and CO_2 were determined from a standard mixture. The relative response factors were obtained by setting N_2 to 1 and adjusting those for O_2 and CO_2 accordingly. The effective response factor for air was calculated by assuming that the air peak contained 79% N_2 and 21% O_2. Sample response factors were normalized using the previously obtained response factors for O_2, N_2 and CO_2 and expressed as relative percentages.

2.3. Results and discussion

2.3.1. Fluorescence microscopy of cell inclusions

In order to distinguish between gas bubbles, vacuoles and lipid droplets, dual staining was performed with a vacuole membrane specific probe, FM4-64, and BODIPY™ as the lipid droplet-specific probe. This was carried out in order to identify gas bubbles, which would be identified as structures that do not fluoresce. The dual staining revealed the presence of vacuoles (red fluorescence) and lipid droplets (green fluorescence) inside of the cells (Fig 1).

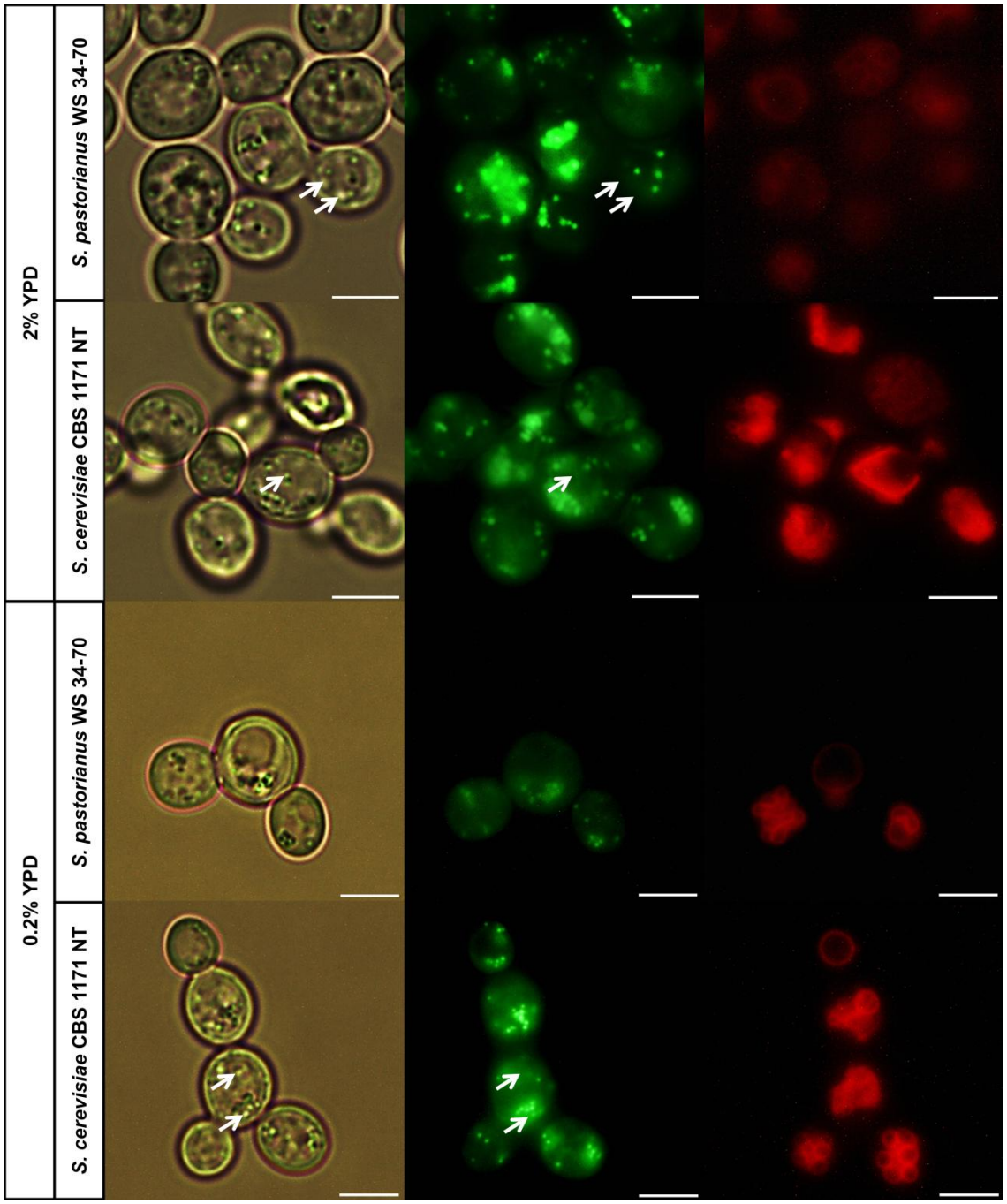

Fig 1. Dual staining of yeasts using FM4-64 (red fluorescence) and BODIPY^{TM} (green fluorescence). Red fluorescent vacuole fragments that are larger than the light scattering granules are present in the cells. Green fluorescent lipid droplets that correspond to the light scattering granules with unstained gas bubbles (arrows). Scale bar 5µm.

The red fluorescent vacuoles observed in cells cultivated in high glucose and low glucose media appear to be larger than the light scattering granules observed with light microscopy, which indicates that vacuole fragments and light scattering granules are separate inclusions. Cells cultivated on high glucose medium contained more light scattering granules, and had a large number of green fluorescing lipid droplets that are the same size as the light scattering granules. There were light scattering granules that did not stain and this suggests that these granules are gas bubbles. This observation indicates that the light scattering granules that were concluded to be gas bubbles by Swart et al. [1,2] actually consist of a mixture of gas bubbles and lipid droplets.

Considering that Swart et al. [1,2] and Potter et al. [12] observed a large number of gas bubbles using nanoSAM, it is possible that the low number of unstained light scattering granules (i.e. gas bubbles) observed in this study may be a result of increased membrane permeability caused by DMSO (vehicle for the stains) leading to the gases escaping from the cells [16]. The cells cultivated on low glucose medium contained very few light scattering granules and had large vacuoles. Similar to cells grown on high glucose, there were light scattering granules that stained green and those that did not stain. The observation confirms that gas bubbles and lipid droplets are separate inclusions that can co-exist inside of the cells.

2.3.2. Intracellular bubbles and lipid content

In order to further confirm that the light scattering granules were not only lipid droplets, the total lipid content of the cells were determined. It was expected that the cells

cultivated at high glucose (containing more light scattering granules) would have higher lipid content than cells grown at low glucose (containing less light scattering granules) if all the light scattering granules were in fact lipid droplets. Interestingly, the lipid content on the two different glucose concentrations differed between the two yeasts (Fig 2). The *S. cerevisiae* strain had a significantly ($p < 0.05$) higher total lipid content in cells cultivated at high glucose (7.27 ± 1.83%) vs low glucose levels (3.98 ± 0.64%). Meanwhile, the *S. pastorianus* strain exhibited a different profile between the two conditions with higher total lipid content in cells cultivated at low glucose (9.51 ± 1.44%) vs high glucose levels (7.16 ± 1.85), but there was no statistical significance between the conditions ($p > 0.05$). This indicates that the increase in light scattering granules observed in *S. pastorianus* cells cultivated at high glucose vs low glucose is not due to the formation of more lipid droplets. This observation was further confirmed by Nile red staining (Fig 3).

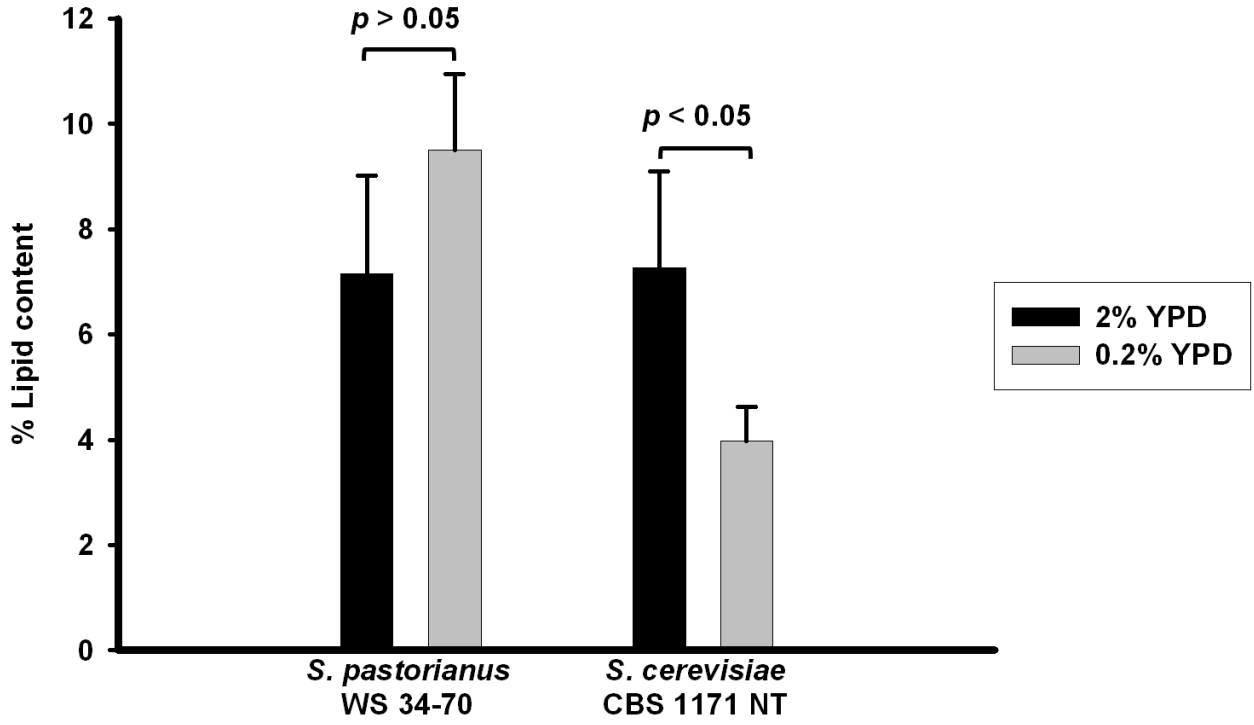

Fig 2. Total lipid content of the analysed strains. The *Saccharomyces pastorianus* strain shows no statistical significance in lipid content on low glucose medium vs high glucose medium. On the other hand, the *S. cerevisiae* strain shows significantly higher lipid content on high glucose vs low glucose medium.

It is important to note that while *S. pastorianus* is a lager yeast that settles at the bottom of the fermentation vessel and *S. cerevisiae* is an ale yeast that adheres to air bubbles and floats to the top of a fermentation vessel [17], both strains had a similar total lipid content in cells cultivated in a high glucose medium. Of interest is the fact that high lipid content results in increased cell buoyancy [18], which may be crucial for

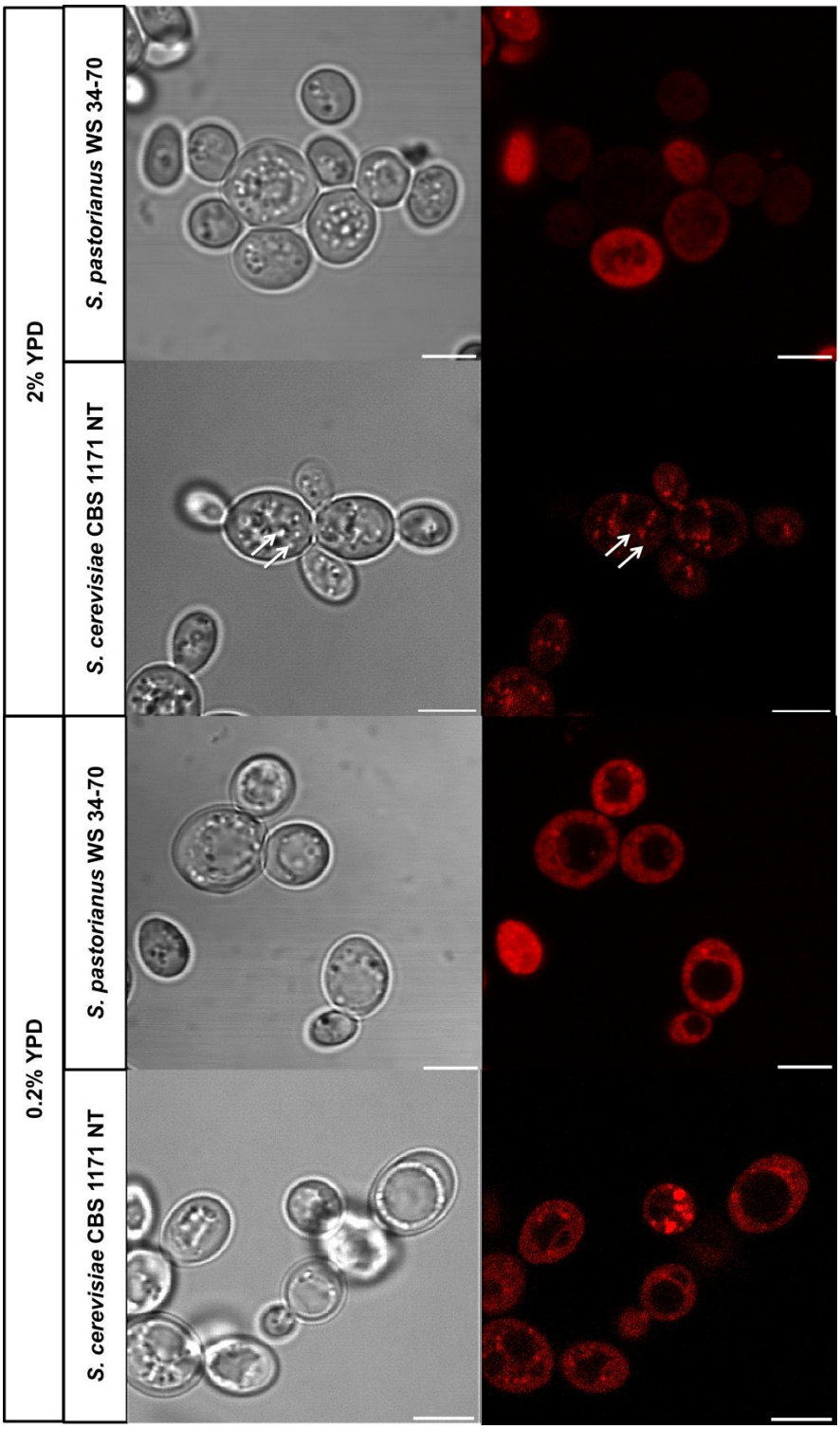

Fig 3. Nile Red staining of the yeast strains. More unstained light scattering granules, thought to be gas bubbles, can be seen in all strains cultivated on 2% glucose compared to 0.2% glucose. Scale bar 5µm.

flotation in top fermenting yeast. The fact that *S. cerevisiae* strains have a cell surface that is more hydrophobic and less negatively charged than *S. pastorianus* strains may explain why *S. cerevisiae* strains adhere to air bubbles and float to the top while *S. pastorianus* strains settle at the bottom [19].

2.3.3. Gas composition of intracellular bubbles

Swart et al. [1] reported that intracellular gas bubbles contain CO_2. Since the *Saccharomyces* yeasts also produce other gases during alcoholic fermentation, the same medium as Swart and co-workers was used to determine the presence of other gases within the bubbles. Headspace-gas chromatography analysis revealed the presence of CO_2 (Fig 4), confirming the report by Swart and co-workers. Even though *S. pastorianus* has been reported to produce more H_2S and intracellular SO_2 than *S. cerevisiae* [20], these gases were not detected in this analysis. To quantify the CO_2, a gas normalisation calculation was done and results given in relative percentage. This was done since the GC does not account for undetected gases which could be present in low quantities. The relative CO_2 contents were calculated to be 0.012% in the PBS control, 17.64% in the *S. pastorianus* sample and 24.08% in the *S. cerevisiae* sample.

The inability to detect the sulfur-containing gases may be due to the low concentrations of these gases as the headspace sampling technique has a low sensitivity [21]. Considering that *S. cerevisiae* has been reported to possess a SO_2 efflux pump [22], it is also possible that the SO_2 may have escaped via this efflux pump during cell harvesting which will in turn result in low H_2S levels due to SO_2 being an intermediate in H_2S production [23].

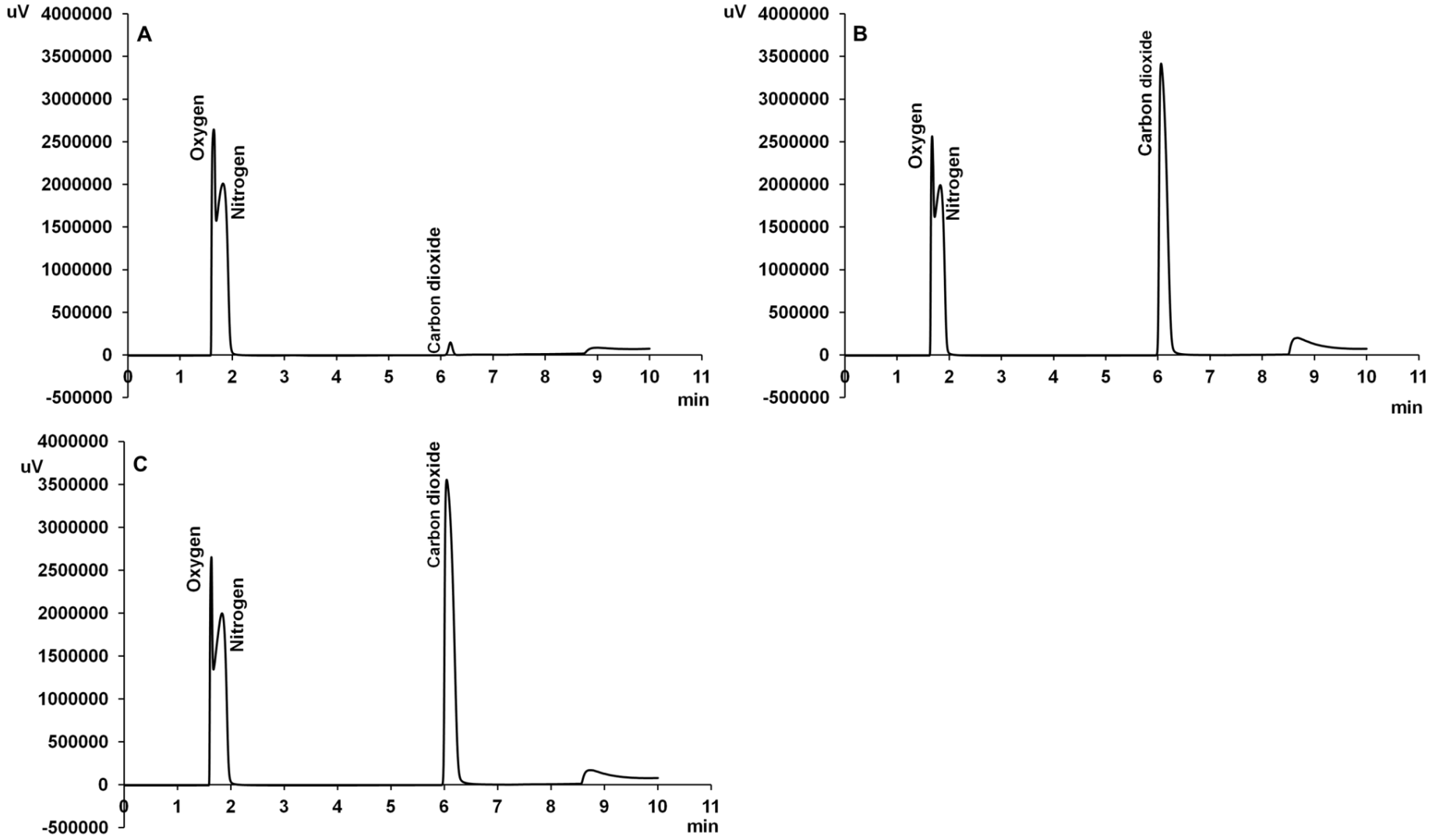

Fig 4. Gas chromatograms depicting gas content of the sampled headspace. The presence of CO_2 (peak at retention time ~ 6 min) can be seen in all vials. (A) PBS control, (B) *S. pastorianus* WS 34-70, and (C) *S. cerevisiae* 1171NT.

Alternatively, the lack of detection for sulfur-containing gases may be ascribed to the helium used in the detector. Gras et al. [24] showed that argon ionization mode is more sensitive than helium ionization and is thus better suited for analysis of sulfur containing gases. These findings emphasise the need for techniques that are more sensitive in order to determine the full gas complement of the intracellular bubbles. Interestingly, Peng et al. [25] successfully applied Raman spectroscopy to study the accumulation of ethanol in aerobically fermenting yeasts. This technique may be better suited to study the gas composition of the bubbles inside of the cells as it is non-destructive and does not require any pretreatment such as cell harvesting, washing and crushing.

2.4. Conclusions

It is near impossible to distinguish between gas bubbles and lipid droplets in fermenting cells using standard light microscopy, and novel techniques are required to ensure the inclusions are properly identified. The results presented in this study indicate that intracellular gas bubbles, vacuole fragments and lipid droplets are separate inclusions that can co-exist inside the cells, and that the inclusions are better resolved using specific staining techniques. Considering that fermenting cells have been reported to be less dense than respiring cell [26], and that bottom-fermentng *S. pastorianus* and top-fermenting *S. cerevisiae* have similar lipid content on high glucose, the role of gas accumulation on cell buoyant density needs to be investigated further.

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Conflict of interest. None declared.

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Chapter 3

The effects of intracellular bubbles on cell function

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Abstract

The discovery of intracellular gas bubbles and the subsequent observation of intracellular bubbles compressing other cell organelles, warrant the investigation into the effects of intracellular gas bubbles on cell function. Taking into consideration that all the previous analyses were performed in batch cultures, in which the media composition and cellular growth rates are continuously changing, it is possible that the effects of intracellular gas bubbles may be masked by these factors. To properly understand the influence of intracellular gas bubbles, chemostat cultivation is better suited as the conditions are kept constant throughout the cultivation. Thus, chemostat cultures were used to study the effects of intracellular gas bubbles under three modes of CO_2 production namely, respiration, respiro-fermentation and anaerobic fermentation. Membrane integrity, mitochondrial activity, cell surface hydrophobicity, flocculation and buoyant densities of the cells were assessed. The results indicate that even though intracellular gas bubbles may not have a negative impact on metabolic activity, they can potentially play a role in lowering buoyant cell density.

3.1. Introduction

The yeast fermentation process is well established. However, until recently no sign of intracellular bubbles had been reported. A paradigm shift emanated when the presence of intracellular bubbles was reported in the baker's and brewer's yeasts using various microscopy techniques (Swart *et al*., 2012). In previous studies using nano scanning Auger microscopy (NanoSAM), intracellular bubbles were observed to form a maze of coalescing bubble-like holes that occupied a significant part of the yeast cells (Swart *et al*., 2012; 2013; Potter *et al*., 2018). The lack of membranes

around bubbles analysed by transmission electron microscopy (TEM, Swart *et al*., 2012, 2013; Potter *et al*., 2018), became a distinguishing feature of the intracellular bubbles, since organelles are membrane bound (Wiederhold *et al*., 2010).

Swart and co-workers (2012) reported that supplementation of fermentable medium with zinc resulted in the accumulation of zinc around the bubbles, this lead them to conclude that the bubbles contain CO_2. Further analysis with TEM revealed that the intracellular bubbles compress and deform cell organelles (Swart *et al*., 2013). Considering that yeast cells have an internal pressure of 2.1 MPa (Vella *et al*., 2011) and that pressurised CO_2 has been reported to have adverse effects on microorganisms with several mechanisms of action being reported (Garcia-Gonzales *et* al., 2007), it is possible that the physical interaction of intracellular gas bubbles and cell organelles may influence cell function.

It is important to take into account that the discovery and subsequent analysis of intracellular bubble formation were conducted on yeast cells in stationary phase (Swart *et al*., 2012; 2013; Potter *et al*., 2018). It has previously been reported that fermenting yeast cells have a lower buoyant density than respiring yeast cells at stationary phase (Allen *et al*., 2006). Of significance is the fact that lipid droplets, which accumulate at stationary phase in yeast cells cultivated on fermentable medium (Chumnanpuen *et al*., 2011), have been reported to influence the buoyant density of microbial cells (Bracero *et al*., 2014). In the previous chapter, we reported that fermenting *Saccharomyces cerevisiae* and *S. pastorianus*, both with the ability to accumulate gas bubbles, had similar total lipid content despite being a top fermenter and a bottom fermenter, respectively. This suggests that factors other than lipid content may influence cell buoyant density.

Of further interest is the fact that the discovery and subsequent studies of intracellular gas bubbles have all been done in batch cultures (Swart *et al*., 2012; 2013; Potter *et al*., 2018). It is important to note that in batch cultivation, the composition of the medium and growth rate of the yeast are continuously changing (Ziv *et al*., 2013). It is possible that, when studying the effect of bubble formation, factors such as nutrient depletion and product (including ethanol) accumulation may also influence cell physiology and function, thus masking the effects of bubble formation (Hoskisson and Hobbs, 2005). In order to properly study the effect of intracellular bubble accumulation, continuous chemostat cultivation in which fresh medium is continuously added to the culture while yeast cells and spent medium as well as metabolic products are continuously removed to keep the culture volume constant, while maintaining a specific growth rate were used (Ziv *et al*., 2013). This circumvents the aforementioned drawbacks that are associated with batch cultures.

Considering that *Saccharomyces* yeasts are Crabtree positive i.e. they are able to ferment glucose to ethanol under aerobic conditions (De Deken, 1966), three modes of CO_2 production, namely respiration, respiro-fermentation and anaerobic fermentation were selected to study the effects of bubble formation.

3.2. Materials and methods

3.2.1. Strains used

The following strains were used in this study: *Saccharomyces pastorianus* WS 34-70 (Cara technology Limited, Leatherhead Enterprise Centre, Leatherhead, Surrey, UK), *S. cerevisiae* CBS 1171 NT (Westerdijk Institute, Utrecht, Netherlands).

3.2.2. Chemostat cultivation

Yeast cells from a 48 h yeast extract peptone dextrose (YPD) plate (10 g/l yeast extract, 20 g/l peptone, 20 g/l glucose and 18 g/l agar) were used to prepare an inoculum in 500 ml shake flask containing 100 ml synthetic medium (pH 5.5) with the following composition: 10 g/l glucose, 1 g/l KH_2PO_4, 2.5 g/l $(NH_4)_2SO_4$, 0.25 g/l $MgSO_4 \cdot 7H_2O$, 0.02 g/l $CaCl_2 \cdot 2H_2O$, 1ml/l trace element (du Preez and van der Walt, 1983), 10 ml/l amino acid (de Kock et al., 2000; Pronk, 2002) and 1 ml/l vitamin (Schulze, 1995) stock solutions and 0.20 ml/l Dow Corning® silicone antifoam (USA). Cells were cultivated at 30 °C for 24 h on a rotary shaker at 160 rpm.

Cells were inoculated into a Fermac 360 bioreactor (Electrolab, United Kingdom) with synthetic medium and a working volume of 600 ml. Cells were cultivated at 30 °C and the pH was maintained at 5.5 by automatic addition of 3 M KOH or 3 N H_2SO_4. Stirrer speed and gas flow were set at 400 rpm and 600 ml/l, respectively. Dissolved oxygen was monitored with an oxygen probe. Cells were grown at D = 0.107 h^{-1} and D = 0.31 h^{-1} aerobically and anaerobically until steady state. For anaerobic cultivation, both the feed bottle and the bioreactor were bubbled with nitrogen gas (Air Liquide, South Africa). Steady state was confirmed after at least 3 residence times by a constant optical density (OD) at 690 nm with a Photolab S6 photometer (WTW, Germany). The samples were kept on ice to prevent any further metabolic activity.

3.2.3. Adenylate kinase release assay

The Toxilight® assay was performed according to the manufacturer's instruction to assess the release of adenylate kinase from the cells with impaired membranes. The

samples were centrifuged at 5000 rpm for 2 minutes (HERMLE Z 362 K centrifuge, Germany). Thereafter 20 µl of the supernatant was transferred to a white-walled 96-well plate, followed by the addition of 100 µl of the Toxilight® reagent (Lonza, USA). The plate was incubated in the dark for 5 min at room temperature and the luminescence measured using the Fluoroskan Ascent FL microplate reader (Thermo-Scientific, United States).

3.2.4. Reactive oxygen species (ROS) assay

The samples were diluted to an OD_{690nm} of 0.3 in phosphate buffered saline (PBS) and the accumulation of reactive oxygen species (ROS) was measured by adding 1 µl of the fluorophore 2',7-dichlorofluorescin diacetate (DCFHDA, 1 µg/ml; Sigma-Aldrich, South Africa) to 999 µl of the diluted sample. The cells were incubated in the dark for 30 min at room temperature. Following a wash step in PBS, 100 µl of the cells was then transferred to a black-walled 96-well microtitre plate. The fluorchrome was excited at 485 nm and the subsequent emission read at 535 nm using the Fluoroskan Ascent FL microplate reader (Thermo-Scientific, United States).

3.2.5. Propidium iodide staining

The samples were diluted to an OD_{690nm} of 0.3 in PBS to obtain a volume of 5 ml, this was followed by centrifugation at 5000 rpm for 10 min (HERMLE Z 362 K centrifuge, Germany). The propidium iodide (PI) staining was performed by re-suspending the cells in 999 µl of PBS, followed by the addition of 1 µl of the PI stain (Life Technologies, USA). The cells were incubated in the dark for 30 min at room temperature. The fluorochrome was excited at 485 nm and the emission read at 635

nm using the Fluoroskan Ascent FL microplate reader (Thermo-Scientific, United States).

3.2.6. Mitochondrial activity assay

The mitochondrial activity was determined using XTT (2,3-bis (2-ethoxy-4-nitro-sulfophenyl)-5-[(phenylamino)carbonyl]-2H-tetrazolium hydroxide; Sigma-Aldrich, South Africa). A volume of 54 µl XTT, activated with 1 mM menadione (Sigma-Aldrich, South Africa), was added to 100 µl of the diluted sample in a black- walled flat-bottom 96-well microtitre plate. The plate was wrapped with foil and incubated at 37 °C for 2.5 h. The Biochrom EZ Read 800 spectrophotometer was used to measure the OD of the samples at 492 nm.

3.2.7. Cells surface hydrophobicity (CSH) assay

Cell surface hydrophobicity (CSH) was determined using microbial adhesion to hydrocarbon (MATH) according to the protocol used by Ells *et al*. (2014). Samples were diluted to an OD of 0.1 (A_0) at 525nm (OD_{525nm}), thereafter 5 ml was transferred to three glass test tubes where 1 ml of xylene was added to each test tube. The test tubes were left to equilibrate for 10 min in a water bath at 37 °C. Following this they were vortexed for 30 s and returned to the water bath where the xylene and aqueous phases were left to separate for 30 min. The aqueous phases of the samples were then transferred to clean test tubes and traces of xylene removed by bubbling air into the samples. The samples were mixed by vortex (5 s) to ensure that no aggregates were formed and the absorbance was measured at 525nm (A_t). Cell surface hydrophobicity was calculated as a percentage of cells that adhered to the xylene

using the equation: % CSH = (1 – A_t/A_0) x 100 (Hsu *et al*., 2015). The higher the percentage, the more hydrophobic the cells are.

3.2.8. Flocculation assay

The flocculation assay was performed according to American Society for Brewing Chemists (2013) flocculation test. Following sample collection, two 15 ml conical centrifuge tubes were marked “A” and “B” with each tube filled with 10 ml of the sample. For tube A, cells were harvested by centrifugation at 630 xg for 2.5 min (HERMLE Z 362 K centrifuge, Germany) and the supernatant was discarded. The pellet was re-suspended by adding 9.9 ml sterile distilled water and 0.5 M EDTA (0.1 ml) followed by withdrawing and expelling the sample 10 times with a pipette and then vortexing for 15 s. The cell suspension (1 ml) was then diluted in 9 ml sterile distilled water and the absorbance of the diluted sample was measured at 605 nm.

For tube B, cells were harvested by centrifugation at 680 xg for 2.5 min. The cells were re-suspended by adding 10 ml washing solution (0.51 g/l calcium sulfate) followed by withdrawing and expelling as well as vortexing the sample as previously described. The tube was centrifuged for 2.5 min at 630 xg. Cells were re-suspended in a solution containing 0.51 g/l calcium sulfate, 6.8 g/l sodium acetate and 4.05 g/l glacial acetic acid. The same resuspension technique was used as above, the tube was slowly inverted 5 times in 15 sec and then left to sit for 6 min where after 1 ml of the suspension was diluted in 9 ml sterile distilled water and the absorbance measured at 605 nm. Percentage flocculence was determined using the equation:

% Flocculence = ((Abs605nm Tube A – Abs605nm Tube B)/ Abs605nm Tube A) x 100.

3.2.9. Density assay

The effect of bubble formation on cell buoyancy was determined using isopycnic density centrifugation using modified protocols by Allen *et al*., (2006) and Pertoft (2000). To prepare 100% Percoll stock solution, the 23% (v/v) Percoll^{TM} (GE Healthcare) was diluted in a ratio of 9:1 in 0.1 M PBS (pH 7.2), thereafter several dilutions were made from the 100% solution with the same PBS to prepare 75%, 50% and 25% solutions used to prepare the gradient. The solutions were layered from the bottom upwards in round base centrifuge tubes with 1 ml 100% at the bottom, 2 ml 75%, 3 ml 50% and 3 ml 25% at the top. Cells were centrifuged at 5000 rpm for 2 min to obtain a 1 ml pellet which was re-suspended in 300 µl distilled water. The cells were then layered on the gradient followed by centrifugation at 400 xg at 20 °C for 1 h in a benchtop centrifuge fitted with a swinging bucket rotor and left to settle for 24 h at room temperature (Eppendorf centrifuge 5702, Germany). Test tubes were photographed to compare the positions of the cells within the gradient.

3.2.10. Statistical analysis

Student *t*–test was performed to compare the mean values between the three modes of CO_2 production for all the assays performed in each strain. A *p*-value less than or equal to 0.05 was considered to denote statistical significance.

3.3. Results

3.3.1. Influence of bubble formation on membrane integrity

Pressurised CO_2 has been reported to increase membrane permeability and leaking of certain enzyme (Garcia-Gonzales *et* al., 2007). The influence of bubble formation

on membrane permeability was assessed using the Toxilight® assay which measures the release of the enzyme adenylate kinase (AK), which is only released in cells with impaired membranes (Jacobs *et al*., 2013). Significantly higher levels of AK were detected in *S. pastorianus* cells carrying out respiro-fermentation ($p < 0.001$) and anaerobic fermentation ($p < 0.001$) compared to those that are respiring (Figure 1). In the *S. cerevisiae* strain, a significantly higher level of AK was detected in cells carrying out respiro-fermentation compared to those carrying out anaerobic fermentation ($p = 0.05$).There was however no significant difference in comparison to respiring cells ($p = 0.10$).

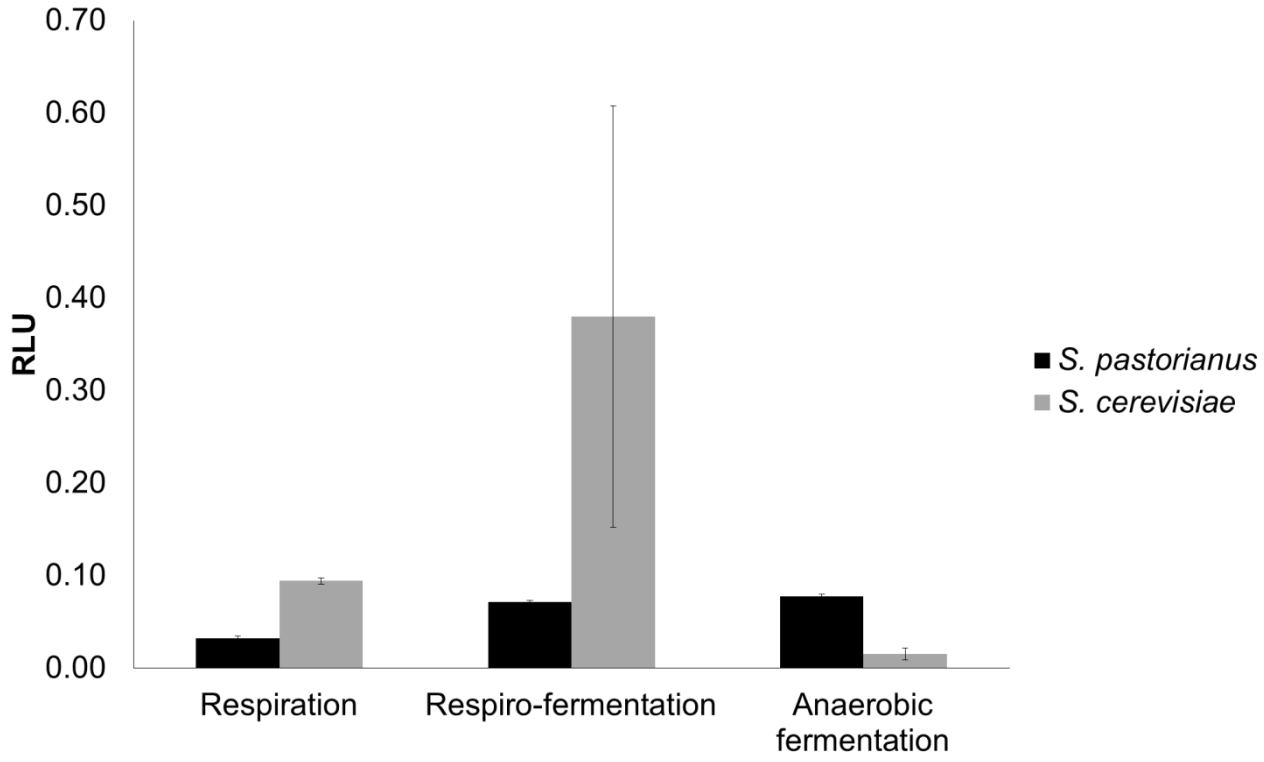

Figure 1 Detection of adenylate kinase (AK) enzyme released by cells under different CO_2 production modes. Fermenting *S. pastorianus* cells release more AK than respiring cells, whereas respiro-fermenting *S. cerevisiae* cells release more of the enzyme than the other conditions.

Significantly higher ($p < 0.001$) PI accumulation was observed in respiro-fermenting compared to respiring cells in both strains (Figure 2). Since PI is able to permeate damaged or leaky membranes (Davey and Hexley, 2011), taken together with the AK results, these findings suggests that the membranes of fermenting (respiro-fermenting and anaerobically fermenting) cells of the *S. pastorianus* strain and the respiring cells of the *S. cerevisiae* strain are more permeable.

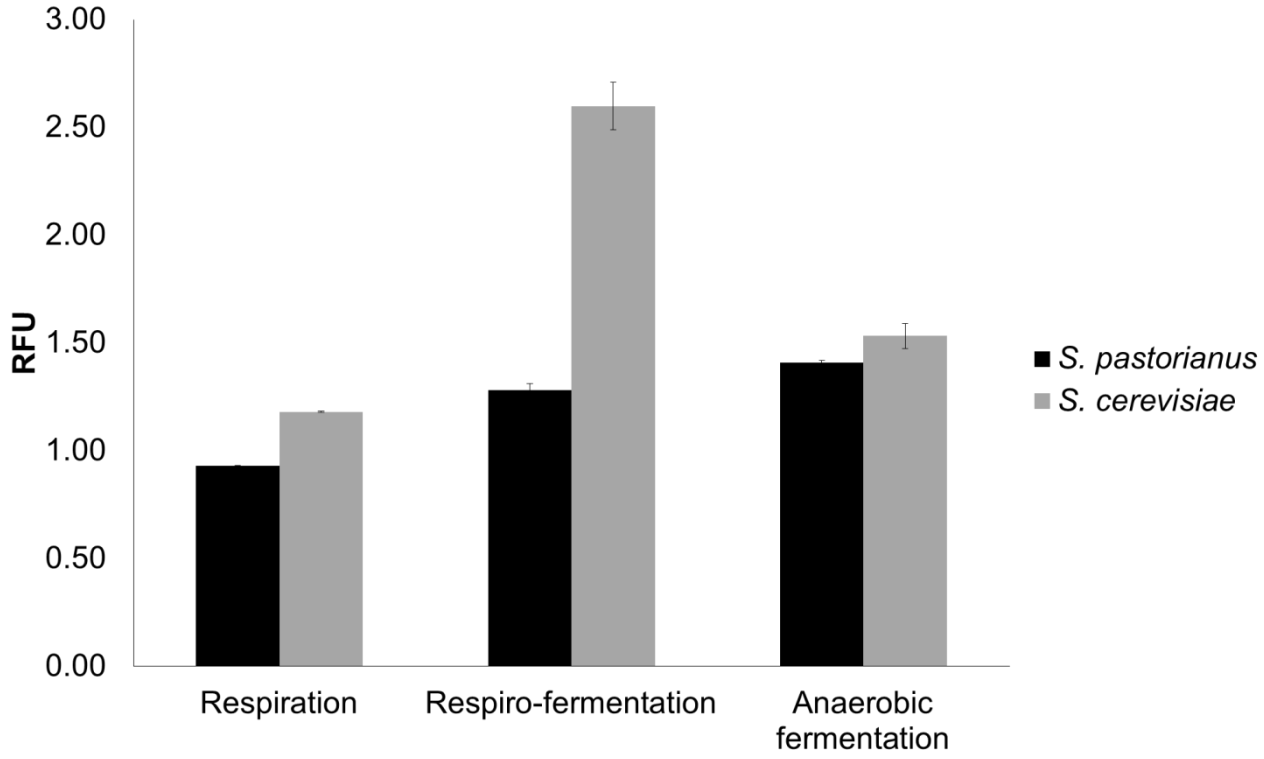

Figure 2 Accumulation of propidium iodide (PI) by cells under the different modes of CO_2 production. Fermenting (respiro-fermenting and anaerobically fermenting) cells are more permeable to PI than respiring cells in both strains.

3.3.2. Influence of bubble formation on ROS production and mitochondrial activity

Intracellular bubbles have been shown to compress and deform cell organelles, such as the mitochondria, which could potentially influence the function of the mitochondria (Swart *et al*., 2013). Impairment of the mitochondria in yeasts has been reported to result in the in the production of reactive oxygen species (ROS), with the increase in ROS being linked to membrane damage. For this reason, a ROS assay was performed to assess the production of ROS under different modes of CO_2 production. A similar profile in ROS production was observed for both the *S. pastorianus* and the *S. cerevisiae* strains (Figure 3), i.e. significantly higher ($p < 0.001$) ROS production was observed in cells carrying out respiro-fermentation and anaerobic fermentation than in respiring cells.

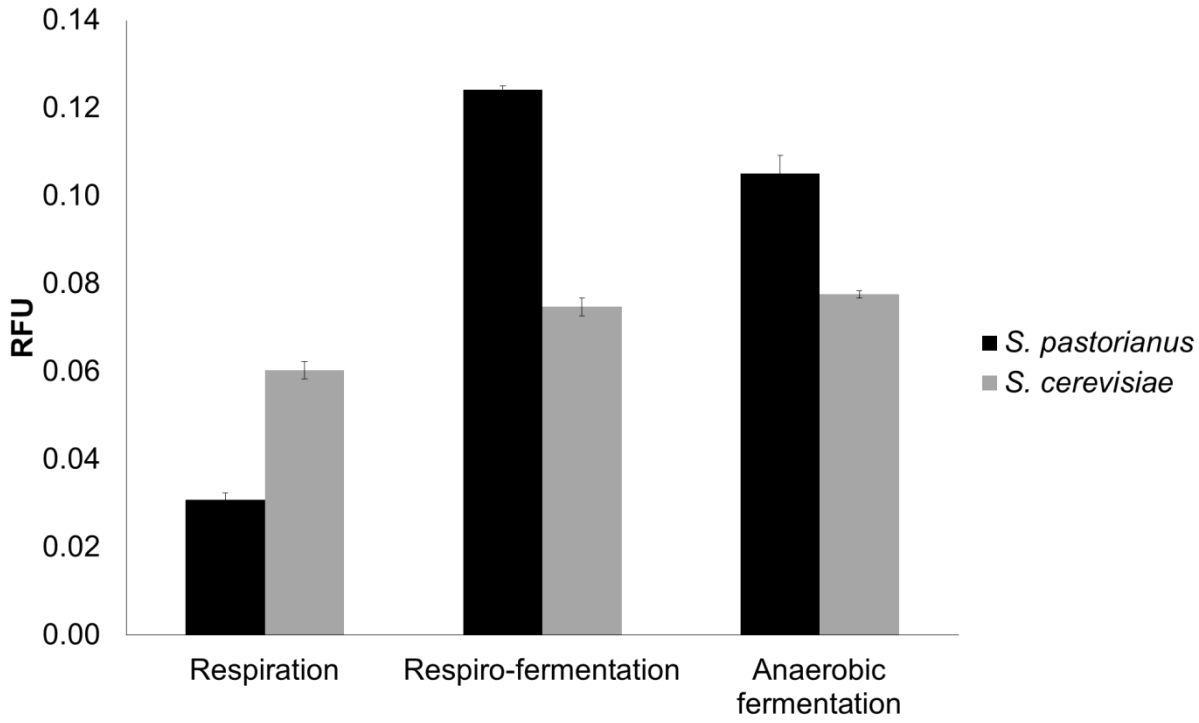

Figure 3 Production of ROS by cells under different modes of CO_2 production. Fermenting cells produce more ROS than respiring cells in both strains.

It was postulated that the accumulation of ROS could be ascribed to the compression of the mitochondria by the intracellular bubbles as previously reported (Swart *et al*., 2013). To understand the effect of intracellular gas bubble formation and the resultant ROS production, an XTT assay was applied to measure mitochondrial activity. The assay is used to measure mitochondrial activity by measuring the formazan produced as a result of XTT cleavage by mitochondrial succinate dehydrogenase in metabolically active cells (Roehm *et al*., 1991; Kregiel, 2012). A similar trend of significantly increased ($p < 0.001$) mitochondrial activity in the respiro-fermenting and the anaerobically fermenting cells compared to the respiring cells was observed for both strains (Figure 4). Since this trend is similar to that observed for ROS production (Figure 3), it was concluded that the accumulated ROS does not have a negative impact on mitochondrial activity, but may be as a result of increased mitochondrial activity.

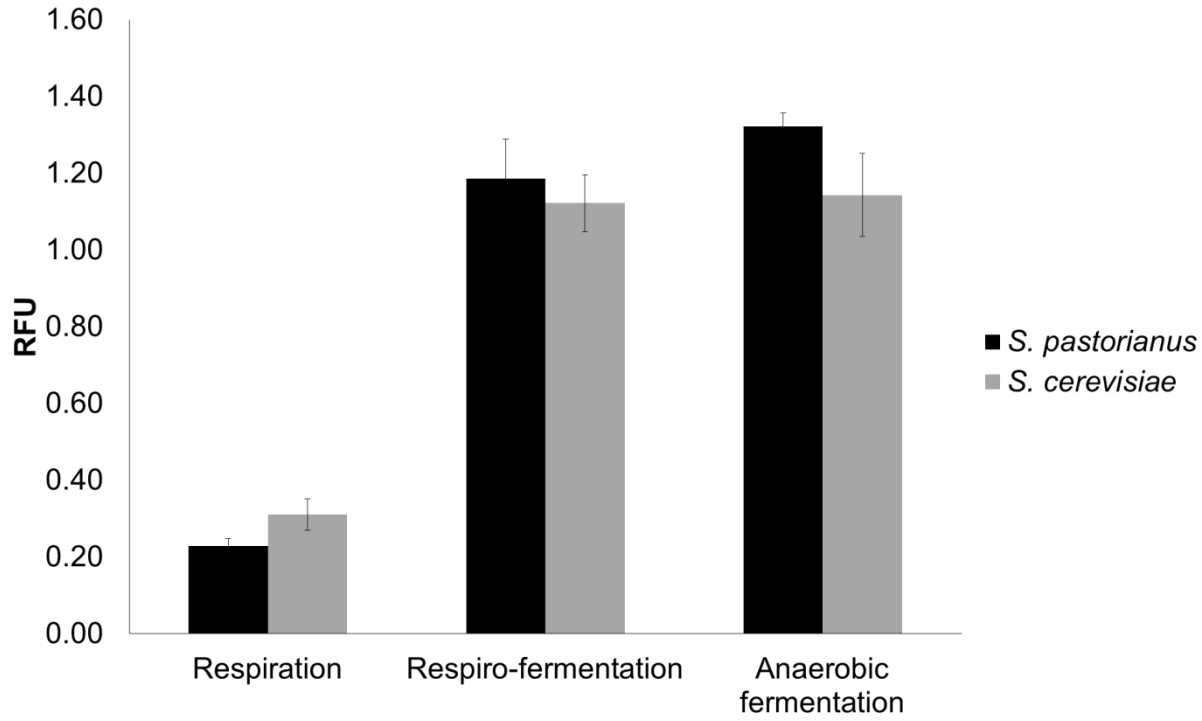

Figure 4 Mitochondrial activity of cells under different modes of CO_2 production. Fermenting cells have higher mitochondrial activity than respiring cells in both strains.

3.3.3. Influence of bubble formation on flocculation, cell surface hydrophobicity and buoyant density

Increased mitochondrial activity has previously been linked to flocculation (Strauss *et al*., 2007). Consequently, a flocculation assay was performed to assess the difference in flocculation amongst the different modes of CO_2 production (Figure 5). For the *S. pastorianus* strain, respiring and anaerobically fermenting cells had significantly higher flocculation ability than respiro-fermenting cells (p = 0.03 and p = 0.02, respectively). There was however no significant difference between respiro-fermenting cells and the respiring cells (p = 0.35). In the *S. cerevisiae* strain, the respiring cells were significantly more flocculant than the respiro-fermenting (p <

0.001) and anaerobically fermenting cells ($p < 0.001$). These results do not coincide with the observed increased mitochondrial activity observed in both respiro-fermenting and anaerobically fermenting cells for both strains.

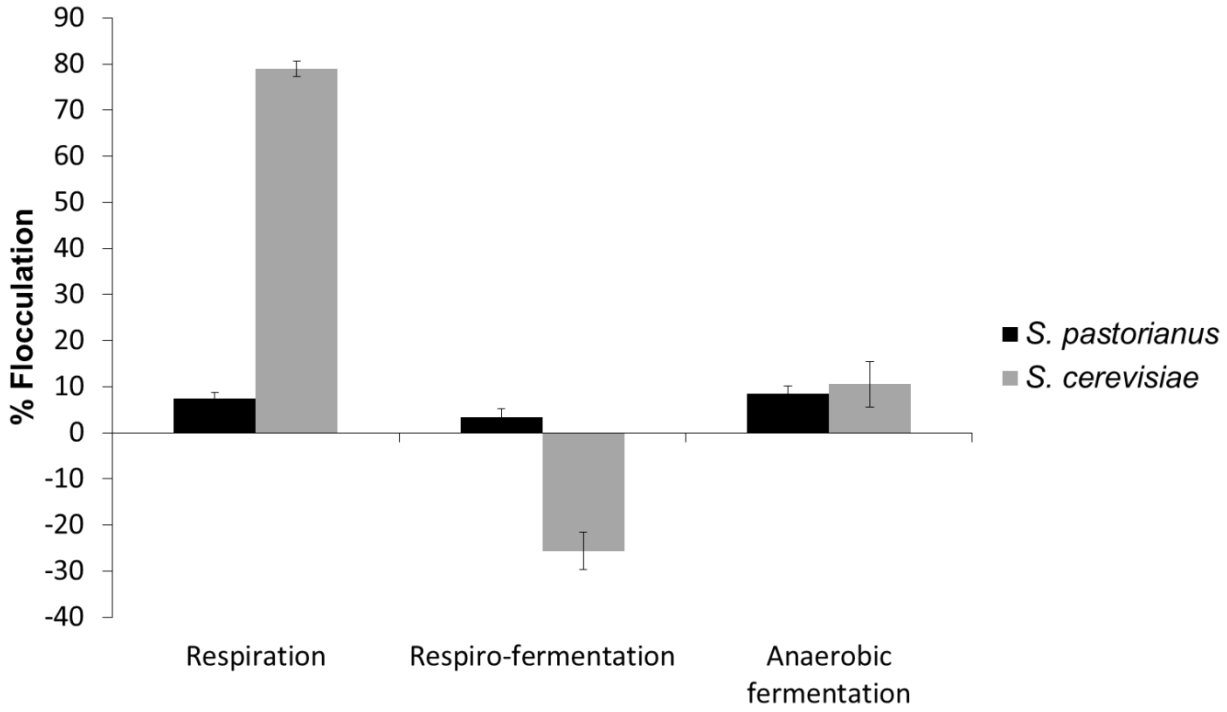

Figure 5 Flocculation profiles of cells under different modes of CO_2 production. The flocculation profile is strain dependent with respiring *S. cerevisiae* cells being more flocculent than the rest.

The difference in cell surface hydrophobicity (CSH) was assessed under the different modes of CO_2 production (Figure 6). The anaerobically fermenting cells of the *S. pastorianus* strain had a significantly higher CSH than the respiro-fermenting cells ($p = 0.03$) with no significant difference when compared to respiring cells ($p = 0.09$). Interestingly, the respiro-fermenting and anaerobically fermenting cells were observed to have a significantly higher CSH than respiring ($p = 0.02$ and $p = 0.01$, respectively) cells in the *S. cerevisiae* strain.

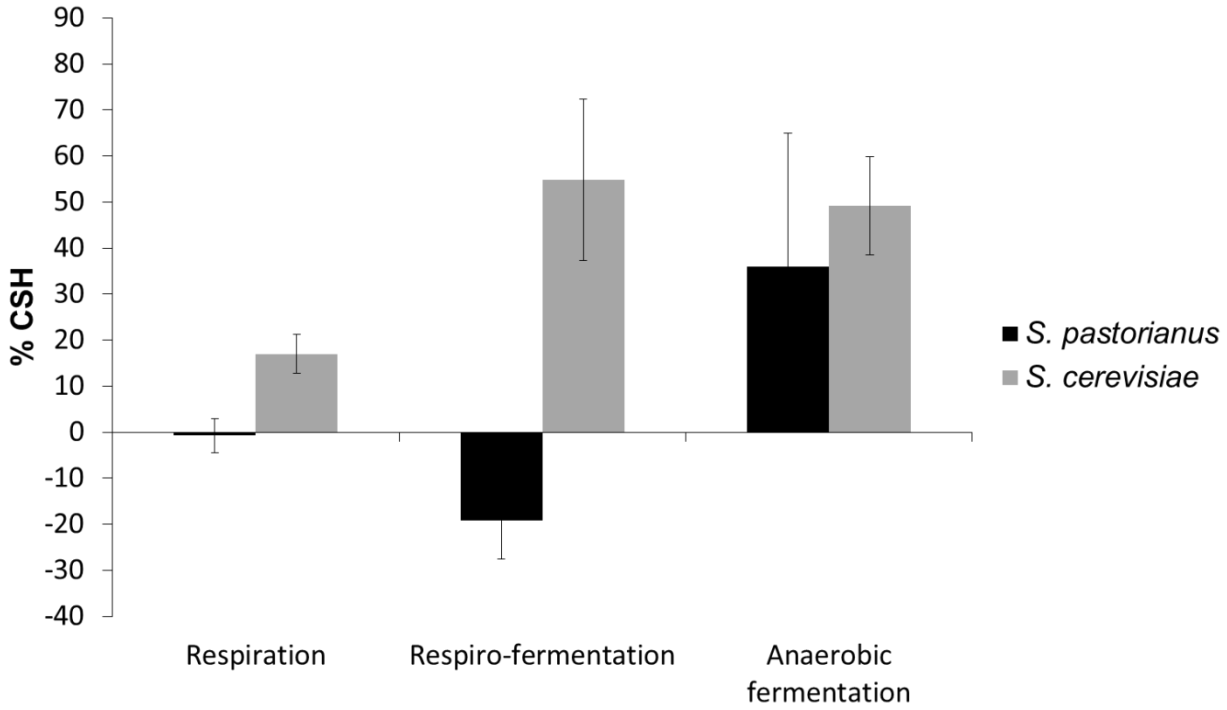

Figure 6 Cell surface hydrophobicity (CSH) profiles of cells under different CO_2 production. The CSH profile is strain dependent, with *S. cerevisiae* showing higher hydrophobicity than *S. pastorianus*.

Since both *S. pastorianus* and *S. cerevisiae* are able to accumulate gas bubbles and produce the same amount of lipids under aerobic fermentation (previous chapter results), the buoyant densities of the yeast cells cultivated under the different conditions were determined using discontinuous Percoll™ density gradients with the highest density at the bottom and the lowest density at the top (Figure 7). It was observed that the *S. pastorianus* cells carrying out respiro-fermentation and anaerobic fermentation formed a band higher in the tube than cells cultivated under respiratory conditions. Since the layers of the Percoll™ density gradients were packed with the highest density at the bottom and the lowest density at the top, this suggests that the respiring cells have a higher buoyant density than the respiro-fermenting and anaerobically cells. Similarly, the *S. cerevisiae* strain,

showed a decrease in density across the different conditions with respiring cells having a higher density than the respiro-fermenting and the anaerobically fermenting cells.

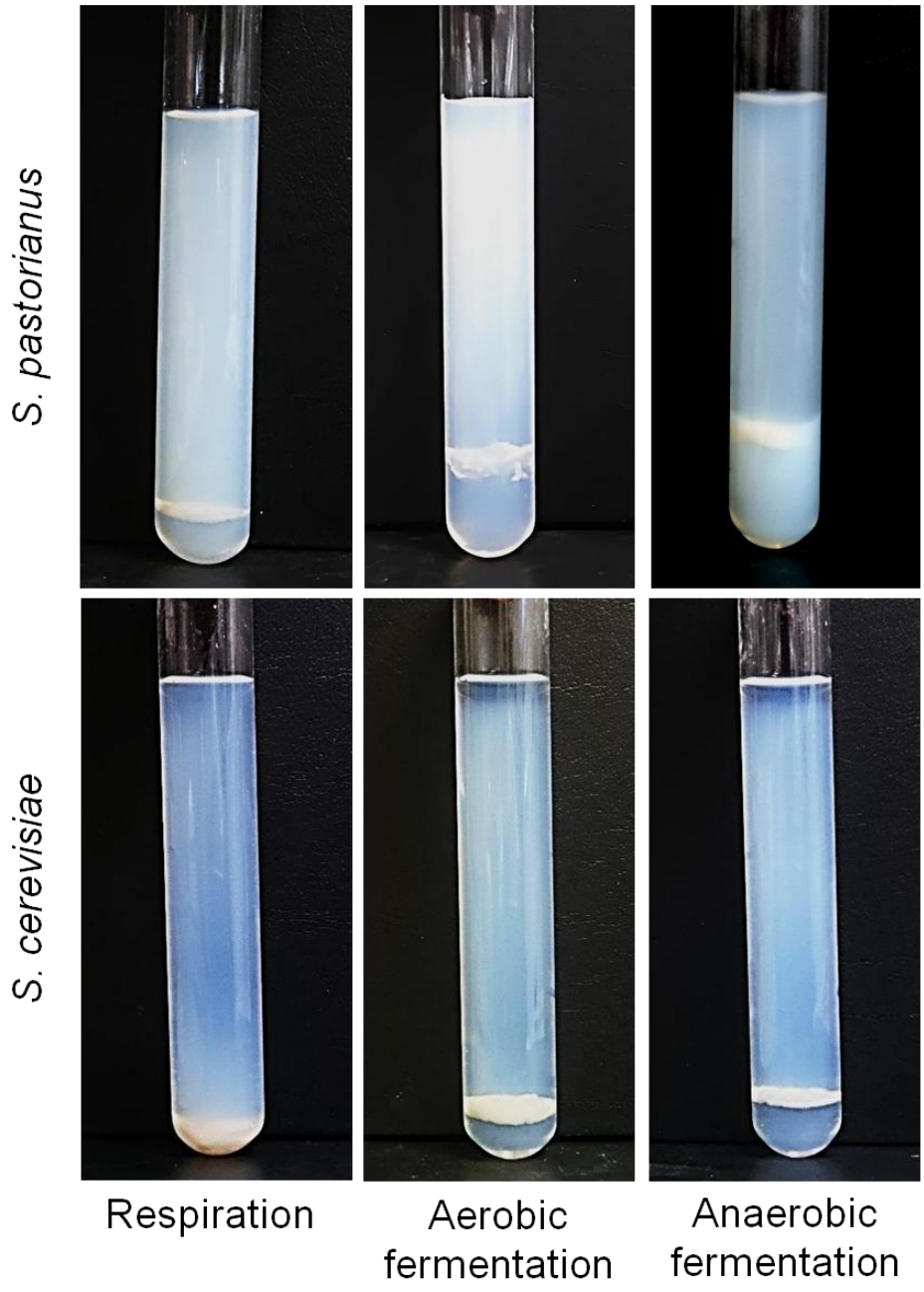

Figure 7 Buoyant density profiles of cells under different modes of CO_2 production. Fermenting cells have lower buoyant density than respiring cells in both strains.

3.4. Discussion

The release of AK and the accumulation of PI have previously been applied to assess membrane integrity (Ogundeji *et al*., 2016). The detection of the AK enzyme has been applied to monitor autolysis in the brewing industry (Driscoll *et* al., 2002; Cameron-Clarke *et al*., 2003). Findings from this study indicate that the membranes of the respiro-fermenting and anaerobically fermenting *S. pastorianus* cells are more permeable than the respiring cells while the respiro-fermenting cells of the *S. cerevisiae* strains are more permeable than the respiring and the anaerobically fermenting cells. In glucose-limited chemostat cultures, CO_2 the production increases with the increase in dilution rates (Sierkstra *et al*., 1992). Since higher levels of AK were detected in fermenting cells at high dilution rates, it is possible that the accumulation of intracellular bubbles may lead to increased membrane permeability. Alternatively, the accumulation of intracellular bubbles may result in cells bursting thus releasing the enzyme.

The mitochondria play a major role in the production of ROS in microbial cells (Kowaltowski *et al*., 2009). Considering that increased CO_2 production did not negatively impact the mitochondrial activity of fermenting cells, it is possible that the high mitochondrial activity of fermenting cells may be responsible for the increased ROS production. For the *S. pastorianus* strain, there appears to be a correlation between membrane permeability, ROS production and mitochondrial activity. Interestingly, the accumulation of ethanol has previously been reported to induce ROS production (Pérez-Gallardo *et al*., 2013). Additionally, a positive correlation between ethanol production and membrane permeability has been observed (Landolfo *et al*., 2008). This suggests that the increased membrane permeability may

also be due to ROS production. Since ethanol is not expected to accumulate to toxic levels in continuous chemostat cultures at the evaluated dilution rates due to the continuous removal of metabolic products, it is possible that increased ROS production in fermenting cells is a consequence of intracellular ethanol accumulation (Peng *et al*., 2010). The findings coincide with previous studies which have shown that in yeasts, the production of ROS is higher in fermenting cells than in respiring cells (Barros *et al*., 2004; Oliveira *et al*., 2008).

Even though previous studies have linked mitochondrial activity to flocculation (Strauss *et al*., 2007), no correlation was observed between high mitochondrial activity and flocculation in this study. This observation suggests that the link between flocculation and mitochondrial activity is strain dependent. Since the high dilution rate used for fermentative growth coincides with high growth rates, it is possible that the high mitochondrial activity observed in fermenting cells may be a consequence of high growth rate. The mitochondria have been reported to be the site of 3-hydroxy oxylipins which have been suggested to increase CSH in *S. cerevisiae* (Kock *et al*., 2000). This potentially explains why a correlation between high mitochondrial activity and CSH was observed in the *S. cerevisiae* strain. The findings in this study coincide with previous reports that the cell surface of *S. cerevisiae* strains is more hydrophobic than the *S. pastorianus* strains (Amory *et al*., 1988). Interestingly, a previous study by Rinatala and co-workers (2009) reported higher transcriptional responses for mitochondrial translational and import machineries in anaerobic than in aerobic glucose-limited chemostat cultures in *S. cerevisiae*. This may explain why increased mitochondrial activity was observed in anaerobically fermenting cells, in both strains, in this study.

The buoyant density results obtained in this study coincides with previous reports that fermenting cells have a lower buoyant density than respiring cells (Allen *et al*., 2006). In general, *S. pastorianus* appears to be less dense than *S. cerevisiae*. In the previous chapter it was reported that aerobically fermenting cells of the *S. cerevisiae* strain have significantly higher lipid content than respiring cells. As such, the lower buoyant density observed for in this strain may be due to the high lipid content which has been shown to influence buoyancy in microbial cells (Bracero *et al*., 2014). The low density and high CSH observed in the *S. cerevisiae* strains may allow cells to adhere to air bubbles thus allowing the cells to float to the top of the fermentation vessel. Considering that in the previous chapter the respiring cells of the *S. pastorianus* strain produced more lipids than aerobically fermenting cells, albeit not statistically significant, this suggests that gas bubble accumulation may play a role in lowering buoyant density in *S. pastorianus*. The low density may allow the cells to stay in suspension during fermentation while the higher CSH observed in anaerobically fermenting cells may allow cells to adhere to each other to form flocs thus allowing the cells to settle at the bottom of the fermentation vessel.

To conclude, the formation of intracellular gas bubbles appears not to have a negative effect on mitochondrial activity but does appear to influence buoyant density, particularly in *S. pastorianus*, thus necessitating further investigations to determine whether this observation is strain dependent. The use of chemostat cultures was shown to be useful as the secondary effects of batch cultivation, such as accumulation of toxic products (e.g. ethanol), are alleviated, allowing us to understand the effects of intracellular bubble formation. Considering that fermentation was carried out at a higher dilution rate than respiration, it would be

interesting to study the influence of gas bubble accumulation at a dilution rate using medium with high enough glucose to allow for fermentation at low dilution rates. This will provide an indication of whether the increased mitochondrial activity and consequence increased ROS production was not due to the high growth rate of the fermenting cells. The positive correlation between ethanol and ROS production that has been reported suggests that the effects of intracellular gas bubble formation should be studied using an alcohol dehydrogenase deletion mutant in order to fully understand this phenomenon. Since yeast cells in chemostat cultivations are actively growing and thus never reach stationary phase, the lipid content of the cells under the three difference modes of CO_2 production needs to be investigated. This will shed even more light on the role of gas bubble accumulation on cell buoyant density, more especially in the *S. pastorianus* strain.

3.5. Acknowledgements

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Chapter 4

General discussion and conclusions

4.1. Main discussion and conclusions

Respiro-fermenting cells have been reported to produce more intracellular gas bubbles than respiring cells (Swart *et al*., 2012). Given that intracellular gas bubbles, vacuole fragments (Zeiger and Mayer, 2012) and lipid droplets (Jacquier *et al*., 2011) have a similar ultrastructure when observed with transmission electron microscopy (TEM), the application of fluorescent probes, in this study, allowed for better resolution of these inclusions. The use of fluorescent probes was especially useful for differentiating intracellular gas bubbles and lipid droplets, which are difficult to differentiate using normal light microscopy.

Gas analysis using headspace-gas chromatography revealed the presence of CO_2 in crushed cells cultivated on highly fermentable medium. This observation confirmed previous findings by Swart and co-workers (2012). Even though other gases such as SO_2 and H_2S are produced during fermentation (Yoshida *et al*., 2008), these gases were not detected in this study. The failure to detect these gases may be ascribed to the low sensitivity of the technique (Kobayashi et al., 2008). Previous research by Gras and co-workers (2006) revealed that while helium ionization (used in this study) is able to detect CO_2, argon ionization was more sensitive and better suited for detection of sulfur compounds. This indicates the need to use more sensitive techniques to determine the full gas complement of intracellular bubbles. Cell numbers were not determined in this study as such, the amount of CO_2 produced could not be accurately determined. This suggests that better experimental design is required for accurate measurements. Interestingly, intracellular ethanol accumulation has successfully been studied using Raman spectroscopy (Peng *et al*., 2010). Given that this technique does not require any pretreatment such as cell

harvesting, washing and crushing, this technique may be better suited to study the gas composition of intracellular gas bubbles.

The discovery of intracellular gas bubbles and initial analyses have all been done in batch culture. The constant change in medium composition and yeast growth rate may mask the effects of intracellular gas bubbles on yeast cells. To circumvent this drawback, glucose-limited chemostat cultivations were applied in this study. In glucose-limited chemostat cultures of *Saccharomyces cerevisiae*, the switch from respiration to fermentation by increasing the dilution rate coincides with an increase in CO_2 production (Sierkstra *et al*., 1992). In this study, the effects of intracellular gas bubble accumulation were studied under low dilution rate (respiration) and high dilution rate (respiro-fermentation and anaerobic fermentation).

In respiring and respiro-fermenting cells, intracellular gas bubble formation positively correlates with the increase in membrane permeability, mitochondrial activity, cell surface hydrophobicity and cell buoyant density in both *S. pastorianus* and *S. cerevisiae* (Figure 1). Since cells have an internal pressure of 2.1 MPa, it is possible that the increase in membrane permeability may be due to accumulation of CO_2 in the membrane (Garcia-Gonzales *et* al., 2007). Given that CO_2 production is higher during fermentation than during respiration, cells may increase their membrane permeability to allow the CO_2 to be released. Mitochondrial activity of anaerobically fermenting *S. pastorianus* cells was similar to that of respiro-fermenting cells. This could be ascribed to the fact that high dilution rates, used for fermentative growth, coincide with higher growth rates. To eliminate the influence of

dilution rate on growth rates, gas bubble formation should be studied at low dilution rate using medium with high glucose. This will resolve whether the increased mitochondrial activity was a due to bubble formation or whether it was a consequence of high growth rate. The observation that increased CO_2 production did not have adverse effects on the metabolism of fermenting cells coincides with previous reports (Aguilera *et al*., 2005). This suggests that fermenting cells are more adapted to growing in environments with high CO_2 levels and are more resistant to their effects.

The mitochondria play a significant role in ROS production in microbial cells (Kowaltowski *et al*., 2009). Even though ROS production is often associated with respiration, higher ROS production was observed in fermenting cells than in respiring cells. This coincides with previous research which showed that in yeasts, the production of ROS is higher in fermenting cells than in respiring cells (Barros *et al*., 2004; Oliveira *et al*., 2008). Given that increased CO_2 production did not negatively affect the mitochondrial activity, the increased ROS production in fermenting cells may be a consequence of increased mitochondrial activity. Taking into consideration that there is a positive correlation between ethanol production, which induces ROS production (Perez-Gallardo *et al*., 2013), and membrane permeability. It is possible that the increased membrane permeability in fermenting cells may also be due to ethanol-induced ROS production. Since ethanol is not expected to accumulate to toxic levels in continuous chemostat cultures at the evaluated dilution rates due to the continuous removal of metabolic products, it is possible that increased ROS production in fermenting cells is a consequence of intracellular ethanol accumulation (Peng *et al*., 2010). In order to fully understand the effects on intracellular bubble

formation on ROS production, a mutant in which all alcohol dehydrogenase isozymes are deleted should be employed as the complication brought about by ethanol production will be removed (Ida *et al*., 2012). It is important to note that care needs to be exercised to ensure that the deletion mutant will have the same growth rate as the parental strain to alleviate complications that can be brought about by the deletion of all the ADH isozymes.

High mitochondrial activity in both strains did not correlate with flocculation in contrast to previous reports (Strauss *et al*., 2007). Since chemostat cultures never reach stationery phase, the fact that cells are constantly dividing in chemostat cultures may be the reason for the reduced flocculation. In both strains, anaerobically fermenting cells were more flocculant than respiro-fermenting cells at the same dilution rate. Given that the CO_2 production should be the same in respiro-fermenting and anaerobically fermenting cells at the assessed dilution rate, the differences observed in respect to flocculation suggests that other physiological factors may contribute to the increased flocculation observed under anaearobic conditions. The observed correlation between mitochondrial activity and cell surface hydrophobicity (CSH) was observed in the *S. cerevisiae* strain may be due to the increased production of oxylipins, via the mitochondria, which have previously been reported to influence CSH (Kock *et al*., 2000). The high mitochondrial activity observed in anaerobically fermenting cells coincide with findings from a previous study in which mitochondrial import and translational machinery had higher transcriptional responses under anaerobic conditions than in aerobic conditions in *S. cerevisiae*, thus suggesting an important role for mitochondria during anaerobic growth (Rantala *et al*., 2009).

The decrease in lipid content (determined in batch cultures) in respiro-fermenting cells of *S. pastorianus* correlates with the decrease in flocculation under respiro-fermentative conditions. However, these results do not coincide with the cell buoyant density. A negative correlation was observed between flocculation and lipid content in *S. cerevisiae*. There was however a positive correlation between lipid content and cell buoyant density. Given the observation that respiro-fermenting *S. cerevisiae* cells have higher lipid content than the respiring cells, it is likely that the high lipid content lowered the buoyant density as has been reported in literature (Bracero *et al*., 2014). The low buoyant density coupled to high CSH in *S. cerevisiae* explains how these cells are able to attach to air bubbles and float to the top of the fermentation vessel. The observation that respiro-fermenting cells of *S. pastorianus* (that have a lower lipid content compared respiring cells even though it is not statistically significant) had a lower buoyant density than respiring cells suggests that intracellular gas bubble formation may influence buoyant density in *S. pastorianus*. The high CSH of anaerobically fermenting *S. pastorianus* cells may play a role in allowing cell-to-cell adhesion resulting in the formation of flocs which allows the cells to settle at the bottom of the fermenter.

This study highlights the usefulness of chemostat cultivation in order to study the effects of intracellular bubble formation in yeast cells. The constant removal of spent medium and toxic products ensured that the effects of intracellular gas bubbles were not masked or enhanced by the presence of toxic products. It is however important to consider using the same dilution rate, and by extension growth rate, to ensure that the observed effects are not due to higher growth rate in fermenting cells than in respiring cells.

To our knowledge, this is the first study that investigated the influence of bubble formation under three different CO_2 production conditions on cell physiology, flocculation ability, CSH and buoyant density.

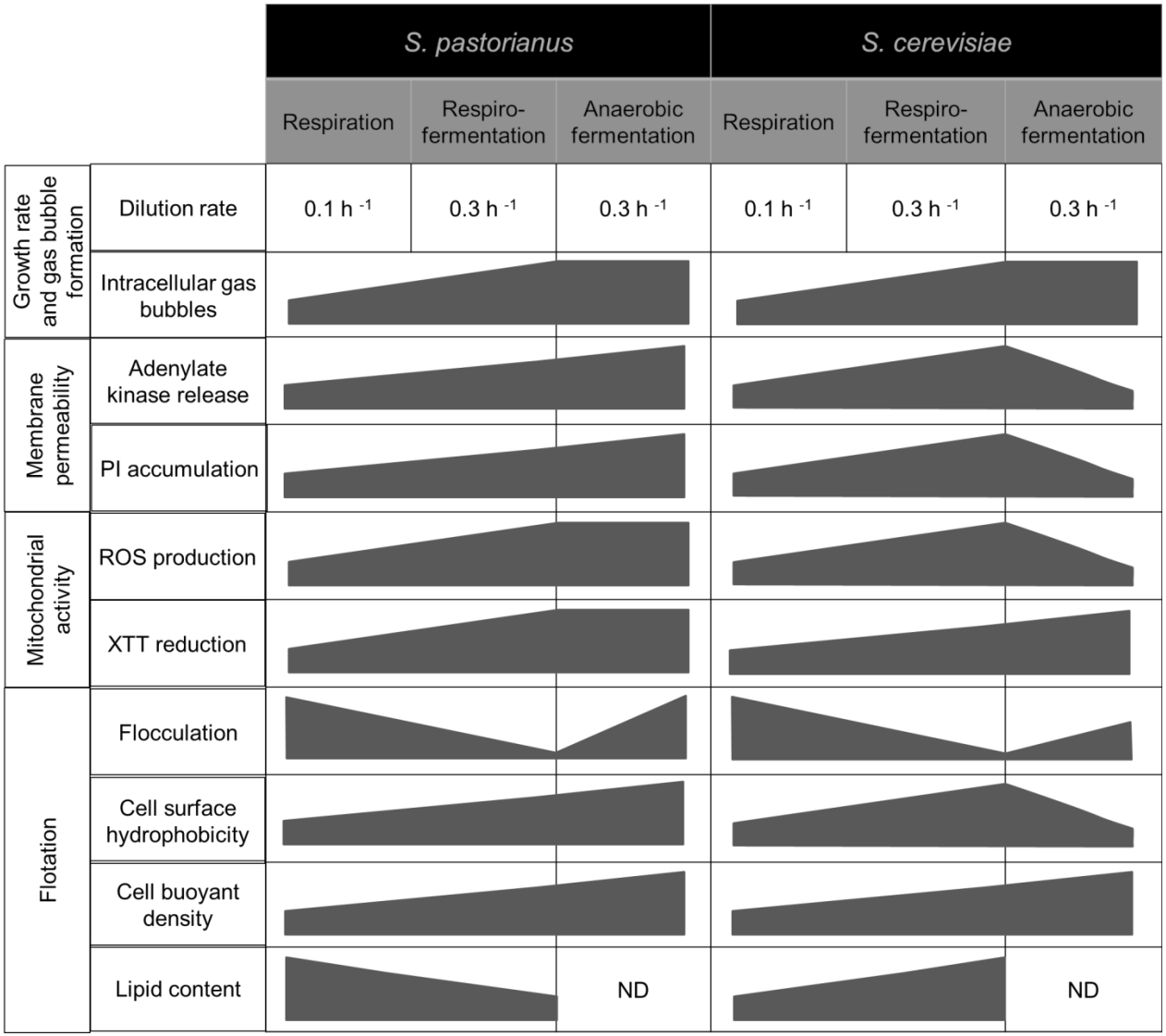

Figure 1 Influence of CO_2 production on membrane permeability, mitochondrial activity and yeast flotation.

4.2. References

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Summary

The yeast fermentation process is well established. However, until recently no sign of intracellular gas bubbles had been reported. A paradigm shift emanated when intracellular gas bubbles were discover using various microscopy techniques thereby resolving the missing link between CO_2 production and eventual release from the cells. The lack of membranes around the gas bubbles served as a distinguishing feature of the bubbles. However, there had been other non-enveloped structure that had been reported as either lipid droplets or vacuole fragments. This study, using organelle-specific fluorescent probes, shows that intracellular gas bubbles, lipid droplets and vacuole fragments are separate inclusions that can co-exist in the cell. Considering that intracellular gas bubbles compress and deform organelles, coupled to the fact that yeast cells have an internal pressure of 2.1 MPa and that pressurised CO_2 has been reported to have adverse effects on microorganisms warranted an investigation into the effect of intracellular gas bubbles on cell function. This study concludes that the formation of intracellular gas bubbles did not have a negative effect on mitochondrial activity. The observation of fermenting *S. pastorianus* cells (more gas bubbles) having lower lipid content than respiring cells (few gas bubbles) indicates that the gas bubble formation plays a role in controlling the buoyant cell density of fermenting in this strain. This suggests that the influence of bubble formation may be limited and strain dependent.

Keywords:

Buoyant density, chemostat cultures, cell surface hydrophobicity, fermentation, intracellular gas bubbles, *S. pastorianus*, *S. cerevisiae*, physiology.