

Gas bubble formation in the cytoplasm of yeast

by

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This dissertation is dedicated to my family: my mother F-T. T. Dithebe, my aunt, L. E. May, my grandmother M. M. Lesufi, my uncle P. P. Lesufi, my grandfather L. T. Dithebe, my brother L. Modise, and my sister K. P. Lephuthi.

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Chapter 1

Introduction

1.1. Motivation

Fermentation is at the heart of some of the most important biotechnological processes. Under fermentation conditions, yeasts (referring to the baker's and brewer's yeasts) are capable of increased ethanol and carbon dioxide (CO₂) production (Van Maris *et al.*, 2001). It is therefore expected that yeast cells should be filled with CO₂ during fermentation. According to literature, CO₂ should be in a gas form when suspended in the cytoplasm of cells at neutral pH (Kern, 1960). It is however interesting that there are no reports in literature on the formation of intracellular gas bubbles in the brewer's and baker's yeasts. The lack of intracellular gas bubbles is considered a missing link since it is not clear what happens to the CO₂ between fermentation (when it is produced via the alcoholic fermentation pathway) and decompression (when it is released into the surrounding medium).

Consequently, the main driving force behind this research project was to expose the expected CO₂ gas bubble formation in the fermenting brewer's and baker's yeasts using a new nanotechnology for biology called Nano Scanning Auger Microscopy (NanoSAM), in combination with Light Microscopy (LM) and Transmission Electron Microscopy (TEM).

1.2. Introduction

Fermentation is one of the most important biotechnological processes known to man. This process is used to leaven bread and make alcoholic beverages by exploiting the fermenting capabilities of yeasts to produce ethanol and carbon dioxide (CO₂). Under fermentation conditions brewer's and baker's yeasts are capable of increased ethanol and CO₂ production (Van Maris *et al.*, 2001). The latter is observed during

the vigorous release of gas bubbles from cells. With this in mind, it is interesting that there are no direct reports in literature on the formation of intracellular gas bubbles inside fermenting brewer's and baker's yeasts. This is supported by the extensive research by Hemmingsen and co-workers on the formation of gas bubbles inside cells using various decompression and supersaturation studies (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1990). In these studies, various types of cells were exposed to a range of saturation levels of different gases and decompressed at varying rates (Hemmingsen *et al.*, 1985). According to these studies, gas bubbles are not formed in yeast and most other cells due to the increased ordering or structuring of water inside the cells, and also due to the lack of water with normal nucleation properties (Hemmingsen *et al.*, 1985; Hemmingsen *et al.*, 1990). Furthermore, not even protein-coated gas vesicles that are found in prokaryotes and provide the cells with buoyancy (Walsby, 1991; Walsby, 1994) are expected in yeasts.

According to Kern (1960) only a small portion of the CO₂ is converted to carbonic acid (H₂CO₃) in the presence of water at neutral pH; which is typical of the yeast cytoplasm (Breeuwer & Abee, 2000). Kern's work has also been confirmed by Wojtowicz in 1995; therefore most of the CO₂ should be present as a gas inside fermenting yeast vegetative cells. The production of CO₂ via the alcoholic fermentation pathway takes place in the cytoplasm (Boubekeur *et al.*, 1999) and this usually happens under pressure which sometimes reaches up to 2.1 MPa inside the vegetative cells of fermenting yeasts (Vella *et al.*, 2012). According to literature CO₂ should also be in a gas form under this pressure while cultivated at 25 °C (Blanco & Pena, 2012).

Two questions now arise: (i) Why were gas bubbles not observed inside the brewer's and baker's yeasts previously? (ii) If not present inside these yeast cells where do the gas bubbles that are released into the medium during fermentation, come from?

1.3. Gas bubbles

In order to be able to find gas bubbles inside yeasts it is important to know what a gas bubble is and what to expect inside these yeasts. Gas bubbles in aqueous medium range from symmetrical to asymmetrical spheres, which are sometimes coalescing and consist of one type or a mixture of gasses (Blatteau *et al.*, 2006). Their diameter ranges from a few nanometres to many millimetres (Arieli & Marmur, 2011). These bubbles are usually suspended when their diameter is less than 2 μm ; they are then called gas nuclei (Mahon, 2010). The bubbles separate from suspension when their diameter exceeds 2 μm ; and their existence is dependent on at least a thin enveloping skin of water (Yount, 1979). Under decompressed conditions, bigger gas bubbles develop from suspended pre-existing small gas nuclei which are usually formed under pressurized conditions (Blatteau *et al.*, 2006; Mahon, 2010; Arieli & Marmur, 2011).

The above description of gas bubbles can be further explained in practical terms using a carbonated liquid in a container as a model. When the carbonated liquid container is closed under pressure, the bubbles are compressed and suspended in solution. Water molecules form a layer, called a solvation shell, around the CO_2 molecule and this keeps the CO_2 gas in suspension. The question that arises is: how is the CO_2 gas kept in suspension? The surrounding water (H_2O) keeps the CO_2 in suspension through mainly ionic bonds. Due to charge differences, the oxygen of the

H₂O orientates towards the carbon of the CO₂ and the hydrogen of the H₂O orientates towards the oxygen of the CO₂ eventually forming a matrix (Fig. 1) (Jena & Mishra, 2005). Inside the bottle filled with carbonated soda, the gas bubbles exist as gas nuclei with a diameter expected to be less than 2 µm and cannot be seen by the naked eye. The pressure inside the bottle is usually about 0.38 MPa (Meraj, 2000) which is about five times less than the pressure inside the yeasts (2.1 MPa). When the bottle is opened, that is when the pressure is released, the gas bubbles start to separate from solution and coalesce to form bigger gas bubbles with a diameter of greater than 2 µm which are then visible to the naked eye. The formation of visible gas bubbles takes place at atmospheric pressure (1 atm = 0.1 MPa). When applying this model to yeasts, it is expected that the gas bubbles would be suspended in the cytoplasm of yeast cells due to the high internal pressure (2.1 MPa) and as the gas bubbles are released into the surrounding medium they would expand and coalesce to form bigger gas bubbles since the pressure in the medium is lower than the internal pressure of the cells.

1.4. Gas bubbles in yeast

1.4.1. Present status

The yeast *Saccharomyces* (including the brewer's and baker's yeasts) is a model that has been at the heart of many eukaryotic cell studies and also plays an important role in many food and biotechnological industrial processes such as baking and brewing (Katz Ezov *et al.*, 2006). Various microscopic techniques, such as Light Microscopy (LM) and Transmission Electron Microscopy (TEM), have been employed in the many studies of this genus but no evidence of intracellular gas bubbles was presented. It is however possible that throughout the many studies on

this yeast genus, gas bubbles could have been missed or ignored as they did not form part of the study or they could possibly have been mislabelled (Rosenberger *et al.*, 2009). It is also possible that during Transmission Electron Microscopy (TEM) analysis, gas bubbles could have been mistaken as artefacts that were created during the sample preparation such as during sectioning.

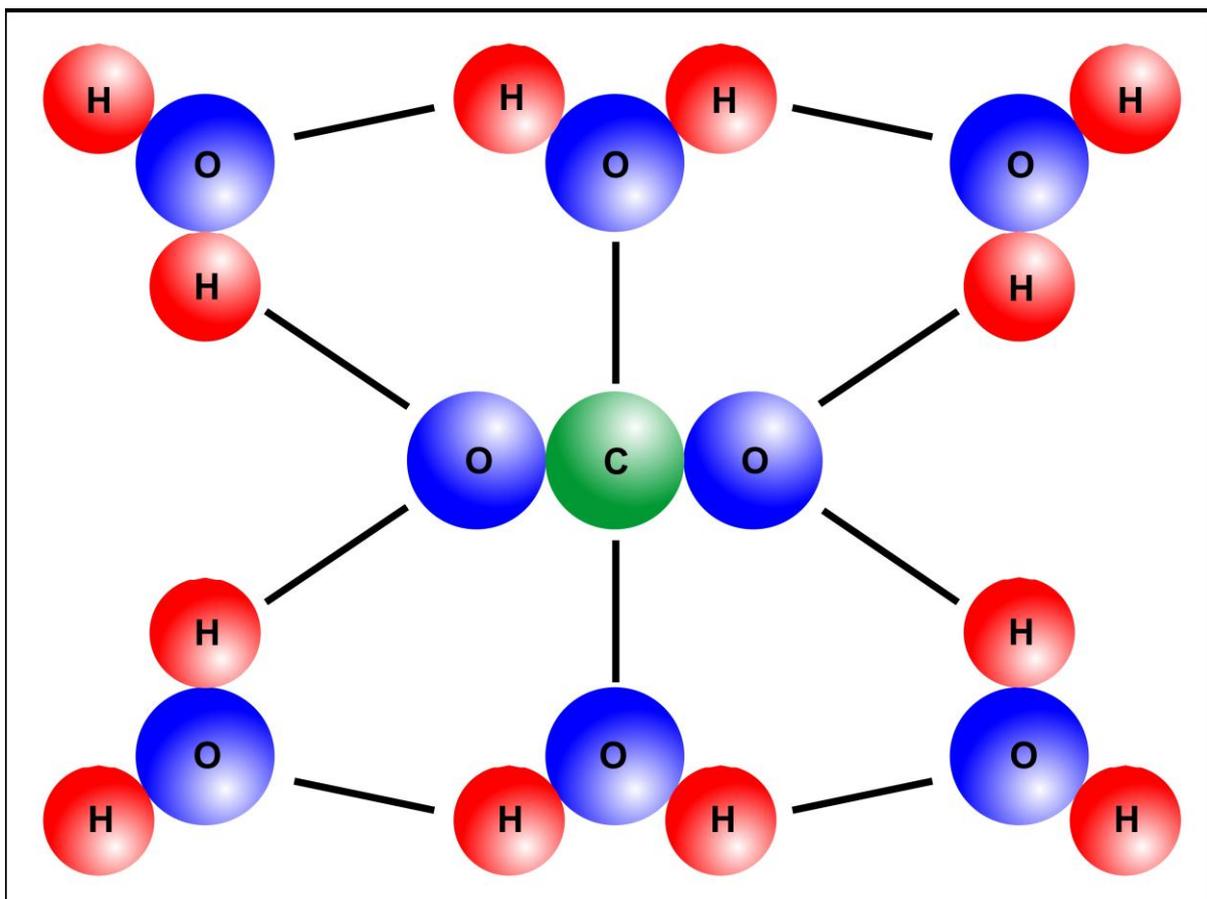


Fig. 1. A simplified matrix illustrating a solvation shell around carbon dioxide (CO₂) to keep it in suspension. Ionic bonds exist between the oxygen (blue) of the water (H₂O) and the carbon (green) of the CO₂ as well as between the hydrogen (red) of the H₂O and the oxygen of the CO₂.

Strikingly, the first evidence of gas bubbles in yeast was presented in 2010 when Swart *et al.* implicated CO₂ in the ascus of the distantly related fermenting yeast

Nadsonia which was treated with fluconazole. Fluconazole also inhibits mitochondrial activity (respiration) and as such enhances fermentation. This resulted in enhanced CO₂ production. The CO₂ was observed at a depth of 945 nm inside the ascus after etching with NanoSAM at 27 nm/min for 35 min using the Ar⁺ gun. After element mapping was performed, a C:O concentration ratio of exactly 1:2 exposed the presence of CO₂ in the ascus with carbon density drastically decreasing as etching continued into the ascus (Fig. 2) (Kock *et al.*, 2011).

1.4.2. Methods to expose gas bubbles in yeasts

In order to identify gas bubbles inside the brewer's and baker's yeasts, a good strategy should be to apply the same techniques as those which were used to identify CO₂ gas inside the ascus of the yeast *Nadsonia* as well as gas vesicles inside the blue-green algae (Cyanobacteria). In the latter case, various microscopic techniques, which include LM and TEM, have been used (Walsby, 1974).

1.4.2.1. Light Microscopy (LM)

Gas vesicles inside blue-green algae were observed using LM. The basic principle of LM is that light from a light source is focused onto the specimen by a condenser lens and the transmitted or reflected light is captured by the objective lens and focused into the eye piece lens to provide an enlarged image of the specimen (Paddock, 2010). Gas vesicles are observed as bright refractile granules inside blue-green algae using LM (Bowen & Jensen, 1965). The gas vesicles are able to scatter light because they have a lower refractive index than the surrounding cytoplasm (Waaland *et al.*, 1970), and this allows them to be easily distinguished within the cells (Walsby, 1974). Since yeast should produce CO₂ gas in the cytoplasm during

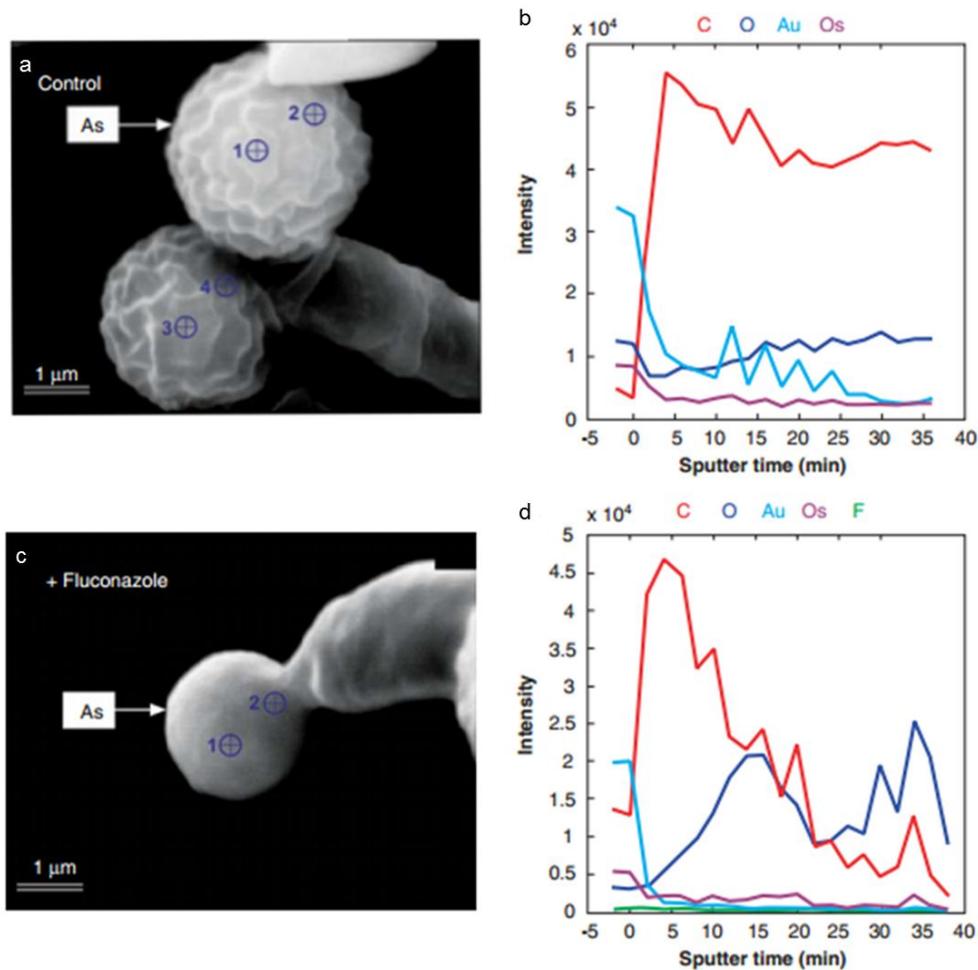


Fig. 2. Element analysis of fluconazole-treated and untreated *Nadsonia* asci. The untreated ascus with a fully developed ascospore (a) had a larger amount of carbon (C) compared to oxygen (O) as Ar^+ etching proceeds into the ascus and ascospore (b). The fluconazole-treated ascus (c) showed a C:O ratio of exactly 1:2 (d) after etching for 35 min. The intensity graphs (b) and (d) were obtained from target 3 in (a) and target 2 in (c), respectively (taken with permission from Kock *et al.*, 2011).

fermentation, it is expected that gas bubbles could be observed in this way inside the cells using LM. The bubbles should be observed as refractile granules as they are likely to have a lower refractive index as compared to the cytoplasm surrounding them. If this proves to be true, the LM observations would need to be confirmed by

other microscopic techniques, such as TEM, as it has been the case with gas vesicles in blue-green algae.

1.4.2.2. Transmission Electron Microscopy (TEM)

Bowen & Jensen (1965) investigated the structure of gas vesicles in blue-green algae using TEM. The principle on which TEM is based is that an electron beam is focused on an ultra-thin electron-dense specimen. As the beam interacts with the specimen some of the electrons are scattered while some pass through the specimen to form a 2-dimensional (2-D) image on a phosphorescent screen. The image can alternatively be recorded using a digital camera (Sorzano *et al.*, 2007; Paddock, 2010). The gas vesicles in blue-green algae were observed to be packed arrays of cylindrical electron transparent structures which are bound by membranes (Bowen & Jensen, 1965). It is expected that if gas bubbles are present inside yeasts, these structures may be observed as electron transparent spheres thereby enabling them to be identified from the electron micrograph. Intracellular gas bubbles would not be expected to be bound by membranes due to their rapid production and also due to the fact that the lipid matrix of biological membranes cannot act as a barrier for CO₂ diffusion (Missner *et al.*, 2008). The use of TEM may therefore serve as a confirmation of gas bubbles inside yeasts.

1.4.2.3. Nano Scanning Auger Microscopy (NanoSAM)

In order to further verify the existence of gas bubbles in the brewer's and baker's yeasts, the imaging technique called Nano Scanning Auger Microscopy (NanoSAM) should be applied - especially since CO₂ gas could be shown inside fluconazole-treated asci of the yeast *Nadsonia*.

Nano Scanning Auger Microscopy has three integral parts which are: (i) Scanning Electron Microscopy (SEM) which is used to visualize the specimen before and after etching, (ii) an Argon (Ar^+) gun that is used to bombard the specimen with Ar^+ ions resulting in the removal of 27 nm thick layers of the specimen per minute to reveal the underlying features, and (iii) a nano probe that is used to bombard the specimen in order to excite Auger electrons necessary to determine the element composition of the specimen according to the Auger profiles of the different elements (Swart *et al.*, 2010).

1.5. Purpose of research

The purpose of this study was to expose the missing link, i.e. the presence of gas bubbles in the cytoplasm of the fermenting yeasts, *Saccharomyces pastorianus* (brewer's yeast) and *Saccharomyces cerevisiae* (baker's yeast), using various microscopic techniques.

1. In order to enhance CO_2 production, the yeast cells will be grown in a fermentable medium, such as Glucose Yeast Malt broth, where increased CO_2 production is expected. These Crabtree-positive yeasts will also be cultivated in a non-fermentable medium which favours respiration, such as Yeast Peptone Glycerol broth, where a decrease in CO_2 production is expected.
2. The microscopy techniques which will be used to investigate the presence of gas bubbles in these yeast cells grown in both fermentable and non-fermentable media include:
 - 2.1. Light Microscopy (LM) to determine the presence of light scattering granules inside the cells.

- 2.2. Transmission Electron Microscopy (TEM) to study the ultrastructure of the gas bubbles and to determine the presence of membranes around them.
- 2.3. Nano Scanning Auger Microscopy (NanoSAM) to determine the 3-D ultrastructure and gas composition of the gas bubbles, if present.

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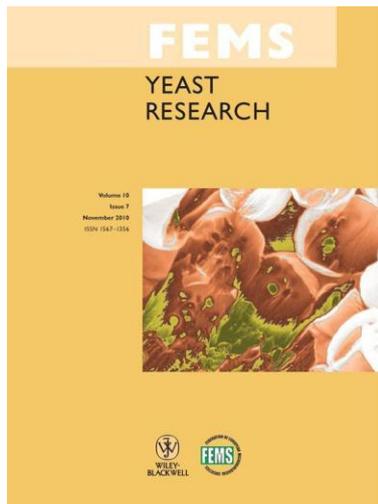
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Chapter 2

Discovery: Gas bubble formation in the cytoplasm of a fermenting yeast



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- (ii) Promoted by BiotechVisions and Global Medical Discovery (GMD – selected from 20 000 articles).
- (iii) Displayed on the cover of all 2013 issues of *FEMS Yeast Research*.
- (iv) Presented as a video lecture on Vimeo (<http://vimeo.com/61521401>).

This chapter has been formatted to the style of *FEMS Yeast Research*.

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Biosensor architectomics reveal novel drugs and their metabolic intervention.
Swart, C. W., **Dithebe, K.**, Van Wyk, P. W. J. & Kock, J. L. F.
- The 18th Biennial Conference of the South African Society of Microbiology, 24-27 November 2013, Bela-Bela, South Africa (<http://sasm2013.co.za>). *Gas bubbles exposed inside fermenting yeasts.* **Dithebe, K.**, Swart, C. W., Pohl, C. H., Swart, H. C., Coetsee, E., Van Wyk, P. W. J., Swarts, J., Lodolo, E. J. & Kock, J. L. F.

2.1. Abstract

Current paradigms assume that gas bubbles cannot be formed within the brewer's and baker's yeasts although these workhorses of the baking and brewing industries vigorously produce and release carbon dioxide (CO₂) gas. We show that these yeasts produce gas bubbles that fill a significant part of the cell. The missing link between intracellular CO₂ production by glycolysis and eventual CO₂ release from these cells has therefore been resolved. The brewer's and baker's yeasts may serve as model to study CO₂ behaviour under pressurized conditions that may impact on fermentation biotechnology.

2.2. Introduction

The fermentation process is at the heart of some of the most important biotechnological processes. This is demonstrated by the production of leavened breads and alcoholic beverages where the fermenting capabilities of yeasts are exploited to produce ethanol and carbon dioxide (CO₂). These conditions lead to cells capable of increased ethanol and CO₂ production (Van Maris *et al.*, 2001).

Since the brewer's and baker's yeasts vigorously release CO₂ gas into the surrounding medium during fermentation, the expectation is that these cells would be filled with gas bubbles. It is also expected that only a small portion of the produced CO₂ will be converted to carbonic acid at neutral pH (Kern, 1960), which is typical of the cell cytoplasm. Therefore most of the CO₂ should be present as a gas. However, no sign of CO₂ bubbles has been reported inside cells even though the yeast fermentation process is well established. The lack of efforts to search for CO₂ bubbles inside cells probably stems from the extensive work of Hemmingsen and co-

workers (Hemmingsen & Hemmingsen, 1979; Hemmingsen *et al.*, 1990), whose research suggested that gas bubbles do not form within most types of cells even during gas supersaturation. This is ascribed to cytoplasmic resistance to gas bubble formation due to the increased structuring of water. Not even protein enveloped gas vesicles that provide buoyancy to the Cyanobacteria (Walsby, 1994), have been reported within yeast cells.

The lack of reports on intracellular gas bubbles is considered to be the missing link since it is not clear what happens to the CO₂ gas between fermentation, when it is produced via the alcoholic pathway, and decompression when it is released into the surrounding medium. With this as background, the main aim of this study became to search for the elusive gas bubbles in fermenting brewer's and baker's yeast cells using various microscopy techniques.

2.3. Materials and Methods

2.3.1. Strains used

The brewer's yeast, *Saccharomyces pastorianus* WS 34-70 (preserved at Cara Technology Limited, Leatherhead Enterprise Centre, Randalls Road, Leatherhead, Surrey, KT22 7RY, UK) and the baker's yeast, *Saccharomyces cerevisiae* CBS 1171 NT (preserved at the Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, The Netherlands) were cultivated in 500 mL conical flasks containing 100 mL media. Cells were grown to stationary phase at 25 °C while shaking at 160 rpm in fermentable and non-fermentable media (Sherman, 2002). Fermentable media used was 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). The non-fermentable medium used, favoring

respiration, was Yeast Peptone Glycerol broth (30 mL L⁻¹ glycerol, 10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone). The brewer's yeast was analysed by Light Microscopy (LM), Scanning Electron Microscopy (SEM), Nano Scanning Auger Microscopy (NanoSAM) and Transmission Electron Microscopy (TEM), while the baker's yeast was analysed by LM and TEM as described by Swart *et al.* (2010) and Kock *et al.* (2011). In addition, a *Microcystis aeruginosa* culture was subjected to TEM analysis as described by Swart *et al.* (2010).

2.3.2. Light Microscopy (LM)

Samples were drawn from cultures in growth- and stationary phases and subjected to LM (Axioplan, Zeiss, Germany) coupled to a Colourview Soft Digital Imaging System (Münster, Germany) to test purity and observe granular appearance. Experiments were performed in at least triplicate and reproducible results were obtained.

2.3.3. Nano Scanning Auger Microscopy (NanoSAM)

2.3.3.1. Cell analysis

Cells were harvested after 48 h by centrifugation at 1450 g for 5 min, and prepared for NanoSAM in SEM mode linked to Argon (Ar⁺) etching as described by Swart *et al.* (2010) and Kock *et al.* (2011). The cells were first fixed with 0.1 M (pH 7) sodium phosphate-buffered 3 % glutardialdehyde (Merck, Darmstadt, Germany) for 3 h and then for 1 h in similarly buffered 1 % osmium tetroxide (Merck, Darmstadt, Germany). The cells were rinsed after each step with the same buffer for 5 min, once after the glutardialdehyde fixation step and twice after the osmium tetroxide fixation step. The fixed cells were then subjected to a sequence of dehydration steps using

50 %, 70 % and 95 % ethanol for 20 min per step. These were followed by two 100 % ethanol dehydration steps with both steps performed for 1 h each. Thereafter the cells were critical point dried, mounted on metal stubs and coated with gold to make it electron conductive. Samples were then examined with a PHI 700 Nanoprobe (Japan) equipped with SEM and Scanning Auger Microscopy (SAM) facilities. For the SEM and SAM analyses the field emission electron gun used was set at: 2.34 A filament current; 4 kV extractor voltage and 238.1 μ A extractor current. A 20 kV, 10 nA electron beam was obtained with these settings for the Auger analyses and SEM imaging. The electron beam had a diameter of 12 nm. The electron gun unit had an upper pressure of 8.8E-10 Torr and the pressure of the main chamber was 2.29E-10 Torr. Aperture A was used for all the measurements. For SEM the Field of View (FOV) was 8 μ m and the number of frames was 4. Ten (10) cycles per survey, 1 eV per step and 20 ms per step were used to obtain the Auger point analyses. The Ar⁺ ion sputtering gun, which the Nanoprobe was also equipped with, was set at: 2 kV beam voltage, 2 μ A ion beam current and a 1 x 1 mm raster area, giving a sputter rate of about 27 nm.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.

2.3.3.2. CO₂ tracking with zinc

To trace CO₂ bubbles inside cells, the metal salt zinc sulphate (ZnSO₄.7H₂O) (Merck, Germany) was added to the culture media, containing brewer's yeasts in fermenting mode, to a final concentration of 2 mg L⁻¹. The yeast cells were grown in 500 mL conical flasks containing 100 mL Glucose Yeast Malt broth. Cells were

grown for 48 h while shaking at 160 rpm, where after cells were prepared as above for NanoSAM analysis.

2.3.4. Transmission Electron Microscopy (TEM)

Cells were harvested by centrifugation at 1450 g for 5 min, washed with equal volumes of de-ionised water and prepared for TEM according to Swart *et al.* (2010). The cells were first fixed with 0.1 M (pH 7) sodium phosphate-buffered glutardialdehyde (3 %) for 3 h and then for 1 h in similarly buffered osmium tetroxide (1 %). The cells were rinsed with the same buffer for 5 min after each fixation, once after the glutardialdehyde step and twice after the osmium tetroxide step. The cells were then dehydrated in a series of acetone concentrations of 50 %, 70 %, 95 % (20 min for each step) and twice in 100 % acetone (1 h for each step). The dehydrated cells were then embedded in epoxy resin and polymerised at 70 °C for 8 h in special moulds. A Leica Ultracut UM7 microtome was used to prepare 60 nm thick sections with a glass knife. A double staining technique was performed using uranyl acetate (Merck, Darmstadt, Germany) followed by lead citrate (Merck, Darmstadt, Germany), each step was performed for 10 min and the sections rinsed after each staining. The sections were viewed using a TEM [FEI (Philips) CM 100, Netherlands].

In addition, Transmission Electron Microscopy was performed as described above on a *Microcystis aeruginosa* culture by Prof P. W. J. van Wyk (Centre for Microscopy, University of the Free State).

2.4. Results and Discussion

2.4.1. Gas bubbles exposed by NanoSAM

With this unique new application of nanotechnology we could demonstrate the presence of a maze of coalescing bubbles that filled a significant part of fermenting cells (Fig. 1a; Supplementary Information, Movie S1). A significant increase in the number and size of cross sectioned bubbles were observed on surfaces of fermenting yeasts after Ar⁺ etching (Fig. 1a,b; Supplementary Information, Table S1).

2.4.2. Gas bubble verification by TEM

To verify these striking results by an independent and non-related technique, we used the same TEM method with which we visualized protein coated gas vesicles in a cyanobacterium (Fig. S1a), to show the naked cross sectioned bubbles in brewer's yeast (Fig. S1b-d). The TEM-observed bubbles were of similar size to the bubbles observed in NanoSAM-SEM Ar⁺-etched cells (Fig. 1a,b; Table S1). A similar trend in bubble formation was found in the model baker's yeast, *S. cerevisiae*, when grown in fermentable and non-fermentable media (Table S1). The table shows a significant increase in the number ($P < 0.0001$, $n = 50$; Two tailed t-Test) and size ($P < 0.05$, $n = 50$; Two tailed t-Test) of bubbles in the brewer's and baker's yeasts respectively when cultivated in fermentable medium (increased CO₂ production) compared to when grown in a non-fermentable medium (decreased CO₂ production).

2.4.3. Gas bubble verification by LM

We could identify bubbles as granules in both the baker's and brewer's yeasts by using LM directly on living cells (Fig. S1e,f) thereby verifying both the NanoSAM- and TEM-demonstrated bubble formation in cells. This is reminiscent of gas vesicles

observed by LM in the Cyanobacteria (Walsby, 1994). These results imply a wide distribution of the CO₂ bubble formation phenomenon in fermenting *Saccharomyces* species. We found increased bubble production in young as well as older fermenting cells, suggesting that bubble production and fermentation were not strictly linked to cell age (Movie S1).

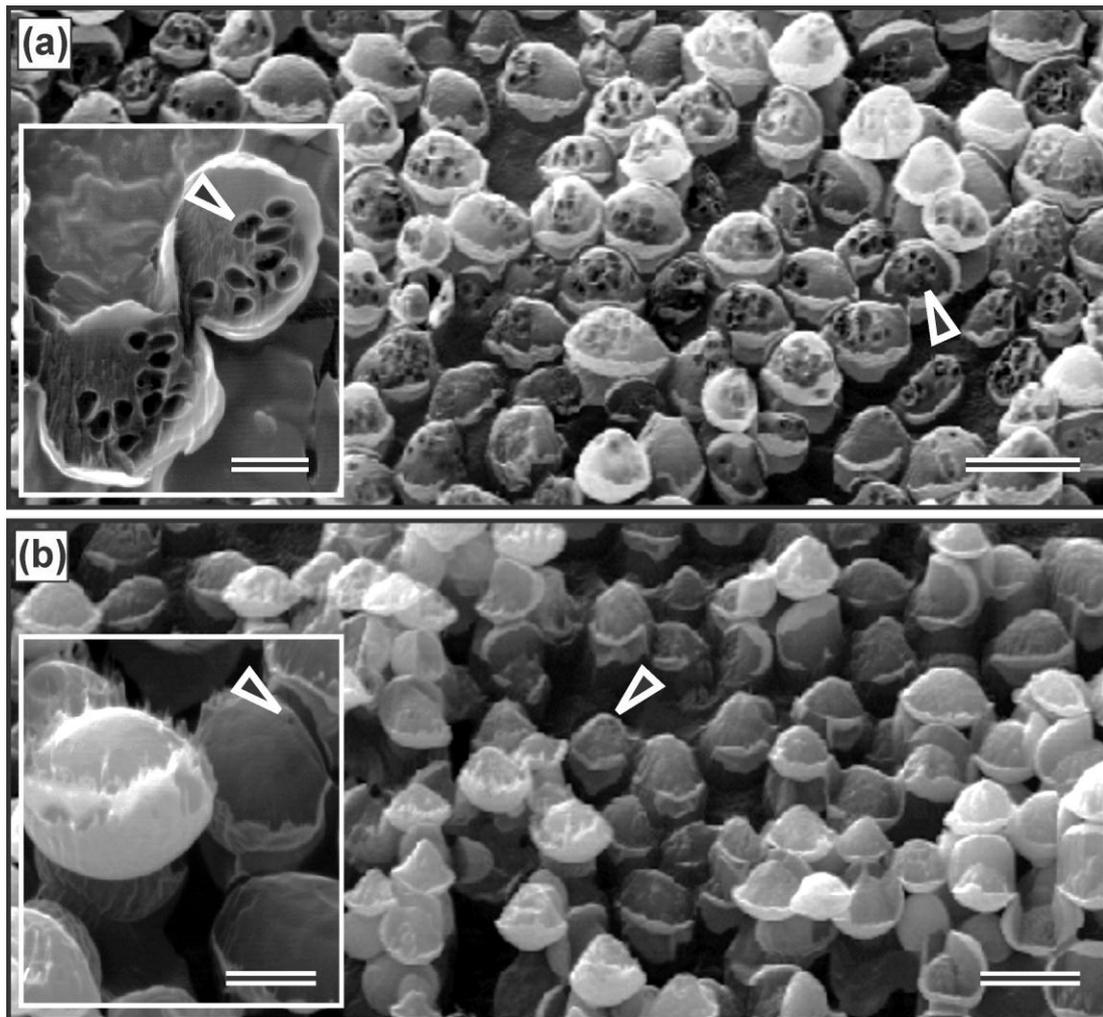


Figure 1 Bubbles (▶) within brewer's yeasts observed by Nano Scanning Auger Microscopy (NanoSAM) Argon (Ar⁺)-etched Scanning Electron Microscopy (SEM). (a) Fermenting cells with increased bubble formation. Insert: Cross sectioned bubbles on etched surface. (b) Respiring yeasts with decreased bubble formation. Insert: Cross sectioned bubbles on etched surface. Scale bars, (a, b) = 6 μm; (a, b) inserts = 2 μm.

2.4.4. CO₂ tracking in gas bubbles by NanoSAM

As expected, we observed "galvanized" bubbles inside brewer's yeasts after the addition of zinc in the form of ZnSO₄·7H₂O to the growth medium. This suggested that zinc reacted with carbonic acid (H₂CO₃) to form insoluble or weakly soluble metal bicarbonate at neutral pH in the cytoplasm (Ryan & Baumann, 1978). Carbonic acid should be produced at higher concentrations nearer to the boundary of the bubble if CO₂ were present inside it, due to the reaction $\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}_2\text{CO}_3$ in the cytoplasm (Fig. S1g,h). This mechanism may sequester metals to protect cells against toxicity when an excess of metal is present (Eide, 2006).

2.5. Conclusions

Based on the above and the fact that the observed empty bubbles were not collapsed by reported high intracellular osmotic pressure that may reach 2.1 MPa (Vella *et al.*, 2012), we conclude that these intracellular bubble-like holes are gas bubbles containing CO₂. We suggest that intracellular CO₂ may eventually be secreted by pressure through the yeast cell wall to affect pressure homeostasis. This in turn should result in vigorous bubble release under diminished pressure (decompression) of the external environment, resulting in their coalescence and enlargement to visible bubbles of millimeter and centimeter size as is generally experienced in products of fermentation such as leavened bread, traditional beer and champagne. Internal cell pressure is probably needed in these yeasts to keep bubble size at a minimum in order to decrease any adverse effects on cell function. Alternatively, these bubbles may be in non-pressurized transit as they are shipped out of the cell upon production.

An important question that needs to be answered is what stabilizes the bubbles in the cell? According to Blasco and co-workers (2011), there are several cellular components of yeasts that are involved in foam formation and stabilization in fermented beverages. These surface active compounds may also be responsible for bubble stabilization inside the cell. Additionally, the potential meliorating effects of zinc and other inorganic ions on intracellular CO₂ gas bubble structures warrants further investigation. The effects, control and kinetics of intracellular gas bubbles at molecular and cellular levels as well as their impact on biotechnological processes should now be assessed. More generally, the yeast-bubble phenomenon may serve as a model that will provide a better understanding of the origins and effects of CO₂ in biology, food, medicine, physics as well as the environment. We expect that this work will advance research on gas exchange in prokaryotic and eukaryotic cells and for example, diving mammals where gas bubbles are formed in tissues under elevated pressurized conditions (Hooker *et al.*, 2012).

2.6. Acknowledgements

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

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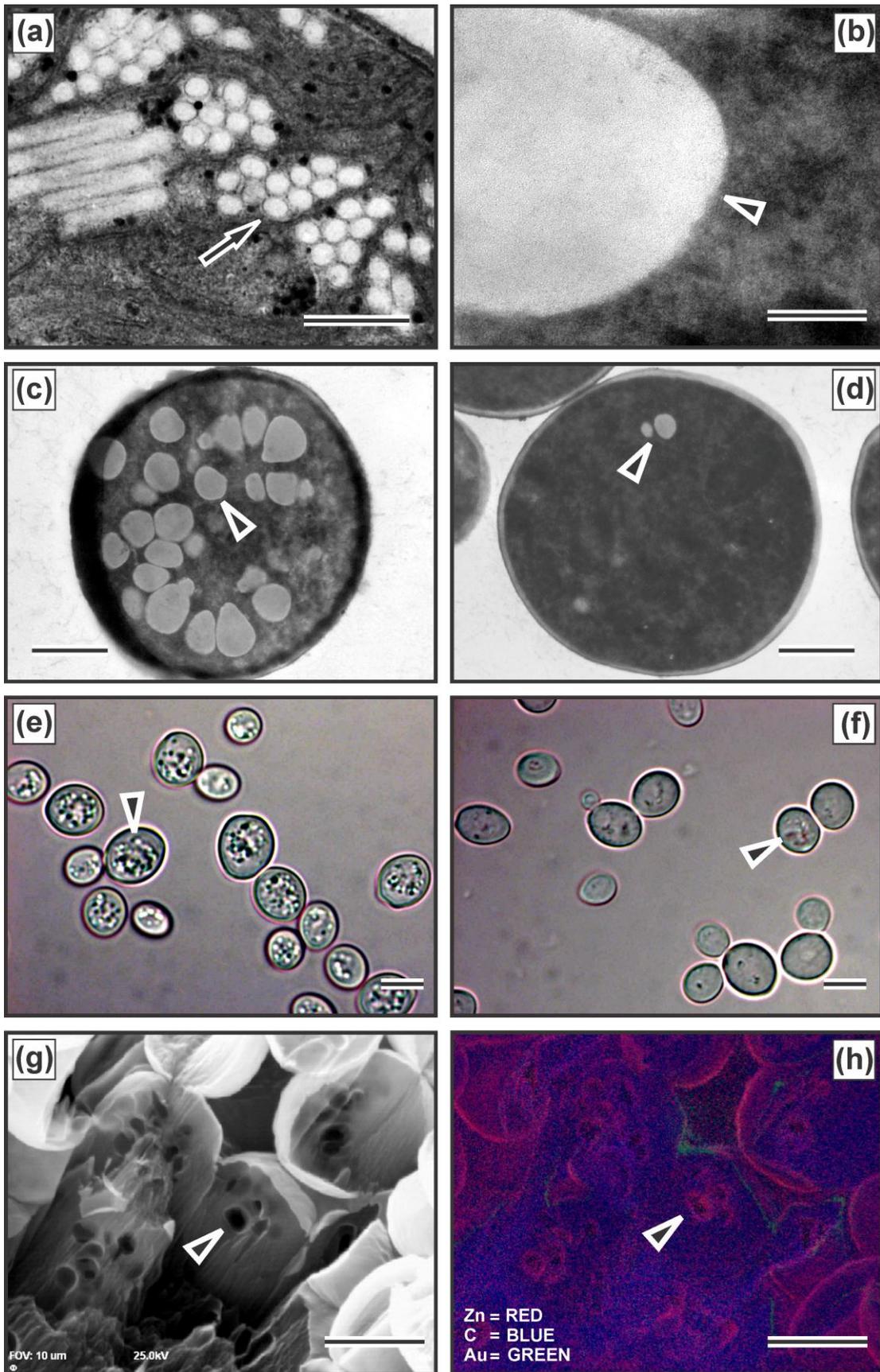
2.8. Supplementary Information

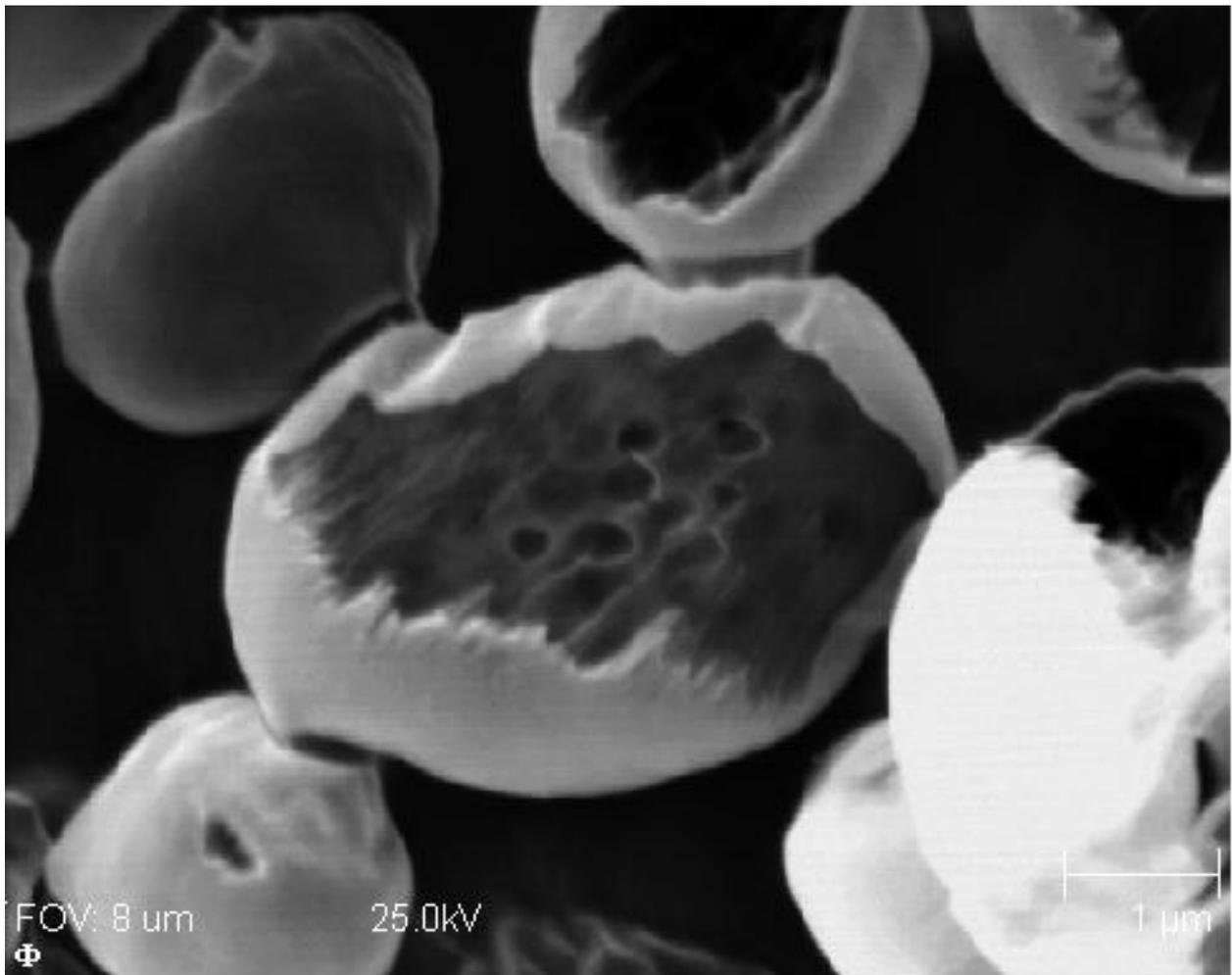
Table S1. Transmission Electron Microscopy (TEM) of yeast cells: Cell size (diameter) and gas bubble (Bub) number and diameter dimensions inside brewer's and baker's yeasts grown in fermentable and non-fermentable media.

	Fermentable media	Non-fermentable media
Brewer's yeast		
Bub number	4.8 ± 2.7	0.8 ± 1.4
Bub Size (nm)	547 ± 228	392 ± 139
Cell size (µm)	(3.6 – 4.8) x (3.7 – 5.3)	
Baker's yeast		
Bub number	7.8 ± 4.4	1.0 ± 1.3
Bub size (nm)	564 ± 116	278 ± 120
Cell size (µm)	(3.2 - 4.8) x (3.6 - 6.1)	

The dimensions of 50 yeast cells (brewer's and baker's yeasts) grown in fermentable medium were determined by Light Microscopy (LM).

Fig. S1. Bubble (►) and gas vesicle (→) analyses in the brewer's yeast and cyanobacterium, *Microcystis* respectively. (a) Transmission Electron Microscopy (TEM) micrograph of enveloped gas vesicles (→) in *Microcystis*. (b) Transmission Electron Microscopy enlargement of a gas bubble inside yeasts showing no enveloped membranes or protein coats. (c) Transmission Electron Microscopy micrograph of yeasts grown in fermentable medium showing many naked gas bubbles inside cells. (d) Transmission Electron Microscopy micrograph showing only a few naked bubbles when yeasts were grown in non-fermentable medium. (e) Light Microscopy micrograph of yeasts showing many light scattering granular cell inclusions when growing in fermentable medium. (f) Light Microscopy micrograph of yeasts showing only a few granules when grown in non-fermentable medium. (g) Nano Scanning Auger Microscopy (NanoSAM) (in Scanning Electron Microscopy (SEM) mode) micrographs showing the structure of carbon dioxide (CO₂)-induced bubbles inside yeasts after exposure to zinc sulphate (ZnSO₄). (h) Corresponding NanoSAM (in Scanning Auger Microscopy (SAM) mode) micrograph showing zinc accumulated (galvanized) linings (indicated in red) around the bubbles. Scale bars, (a) = 500 nm; (b) = 100 nm; (c,d) = 1 μm; (e,f,g,h) = 3 μm.





Movie S1. Nano Scanning Auger Microscope–Scanning Electron Microscopy (NanoSAM-SEM) video showing sequential Argon (Ar^+)-etching through a dense maze of gas bubbles inside fermenting brewer’s yeast cells of different ages i.e. older mother cell (center) with attached younger daughter cells (Please see DVD at the back of M.Sc. dissertation).

Chapter 3

Intracellular gas bubbles deform organelles in fermenting brewing yeasts

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This Chapter has been formatted to the style of the Journal of the Institute of Brewing & Distilling.

Parts of this Chapter have been presented at a Conference:

- The 18th Biennial Conference of the South African Society of Microbiology, 24-27 November 2013, Bela-Bela, South Africa (<http://sasm2013.co.za>). *Gas bubbles exposed inside fermenting yeasts*. **Dithebe K.**, Swart C. W., Pohl C. H., Swart H. C., Coetsee E., Van Wyk P. W. J., Swarts J., Lodolo E. J. & Kock J. L. F.

Please note: Implications of finding on practical brewing (page 44) have been prepared by Prof E. J. Lodolo and have been included for the sake of completeness.

3.1. Abstract

We reported previously the presence of large numbers of gas bubbles inside fermenting brewing yeasts. Here we report that these bubbles deform organelles. The mechanical effects on metabolism and coding functions of yeasts, when organelles including the nucleus are deformed and severely contorted, should now be addressed. The implications of this finding on practical brewing conditions require consideration during optimisation of fermentation parameters to prevent carbon dioxide (CO₂) toxicity effects on fermentation performance and flavour formation.

3.2. Introduction

We recently exposed the formation of gas bubbles in the cytoplasm of a fermenting brewing yeast using Auger-architectomics i.e. Nano Scanning Auger Microscopy (NanoSAM) linked to Scanning Electron Microscopy (SEM) and Argon (Ar⁺) nano-mining (1). This is considered a paradigm shift since naked gas bubbles are not expected inside any type of cell due to cytoplasmic resistance to gas bubble formation as a result of increased structuring of water (1).

Gas bubbles were first implicated at a depth of 945 nm inside sexual cells (asci) of yeast (2). This was after mining with Ar⁺ at 27 nm/min, for 35 min, in a fluconazole-treated ascus of the fermenting yeast *Nadsonia*. Here respiration was strongly inhibited by fluconazole. Using Auger-architectomics (2, 3), we mapped the 3-dimensional (3-D) structure and element building blocks of the bubble-like structures. We show a C:O concentration ratio of exactly C:O = 1:2 at a depth of 945 nm indicating the presence of CO₂ inside the bubble, while carbon intensity decreased drastically towards the centre of the bubble, as Ar⁺ mining continued into

this structure. This exposed a less dense central area as part of the bubble architecture (Figure 2D from Ref. 2).

This observation played a key role in the discovery of gas bubbles in yeasts (1). When fermentation was favoured in the baker's and brewer's yeasts (1, 4), a drastic increase in gas bubbles was observed inside the cytoplasm of yeasts using this nanotechnology. This was verified by Light Microscopy (LM) and Transmission Electron Microscopy (TEM) analysis (1). Strikingly, it was found that a large portion of fermenting yeast cells consists of gas bubbles leaving only limited space for organelles.

The aim of this study became to search for organelles between these bubbles and assess their influence on organelle structure in the cytoplasm of fermenting brewing yeasts.

3.3. Materials and Methods

3.3.1. Strains used

Here, the same brewer's yeast used in a previous study (1), *Saccharomyces pastorianus* WS 34-70 (preserved at Cara Technology Limited, Leatherhead Enterprise Centre, Leatherhead, Surrey, UK) was cultivated in 500 mL conical flasks containing 100 mL fermentable media and analysed by LM, TEM and NanoSAM linked to Ar⁺ etching and SEM as described before (1).

3.3.2. Nano Scanning Auger Microscopy (NanoSAM)

This was performed according to the procedure in 2.3.3.1.

3.3.3. Transmission Electron Microscopy (TEM)

3.3.3.1. Organelle analysis

This was performed according to the procedure in 2.3.4.

3.3.3.2. Enhancement of membrane structure

To enhance membrane visibility, the cells were fixed with 2 % potassium permanganate (KMnO₄, SAARCHEM, Krugersdorp, South Africa) (w/v) dissolved in distilled water for 2 h at room temperature. The cells were then rinsed several times with distilled water. The dehydration, embedding in epoxy, preparation of sections as well as the staining procedure was similar to the procedure described in 2.3.4.

3.4. Results and Discussion

3.4.1. Presence of gas bubbles shown by NanoSAM

With this nanotechnology, we could confirm the presence of gas bubbles that occupied a large portion of the fermenting yeast cells (Fig. 1; insert). The coalescing inter-connectivity of the gas bubbles can also be seen in the insert.

3.4.2. Effects of gas bubbles shown by TEM

Strikingly, those areas analysed in cells containing bubbles clearly showed indentations of membranes surrounding organelles when in contact with bubbles (Fig. 1). Organelle 1 (Org 1) could not positively be identified (most likely a mitochondrion) while Org 2 was identified as a part of the Golgi apparatus. Organelle 1 was deformed at the contact point with gas bubbles and both organelles in turn deformed the membrane of the nucleus (Nu). We concluded that this phenomenon was due to direct and indirect gas bubble compression exerted on organelles.

3.4.3. Membrane visibility

The use of KMnO_4 to enhance membrane visibility confirmed the lack of membranes surrounding the gas bubbles and further verified that the gas bubbles do indeed deform the organelles (Fig. 2). This suggests that the skin (surface active compounds – see Chapter 2, page 33) surrounding the gas bubbles is able to withstand tension as it does not disintegrate when it presses against organelle membranes, such as the nuclear membrane. The nuclear membrane prevents the stabilized gas bubbles from penetrating the nucleus.

3.5. Conclusions

This work demonstrates for the first time the physical effects of gas bubbles on organelles. The effects of bubble compression on organelle structure and function need attention, especially in cells that are filled with gas (1). The influence of such compressions or deformations on the potential mechano-sensitive nature of nuclei, mitochondria and other life giving organelles should now be addressed in all cell types that are able to produce gas (5, 6). This could lead to new insights into the regulation of organelle function. It is expected that these bubbles not only influence cell metabolism physically, but also contribute to the high pressure formed inside fermenting yeast cells, in addition to turgor pressure (7).

To prevent CO_2 toxicity effects on fermentation performance and flavour formation, the implications of this finding should be considered during optimisation of fermentation parameters. The main aim of the brewer is to balance glycolytic flux by managing cell growth (nutrient pull) and fermentation speed (temperature and pitching rate dependent), however, increased glycolytic flux through pyruvate results in increased CO_2 formation (8). Therefore, future brewery optimisation studies need

to be cognisant of fermentation management aspects such as wort zinc concentrations, sufficient fermenter head space to prevent foaming into the CO₂ collection mains, relevant CO₂ collection strategies and potential CO₂ collection capacity constraints. The discovery of CO₂ nano-bubbles in the yeast cytoplasm (1) and its contorting effects on organelles can provide brewers with insights into the ramifications of fermenter back pressure causing super-saturation such that nano-bubbles may not be released. The potential impacts of these CO₂ nano-bubbles on yeast growth, fermentation progression, flocculation and final product quality can now form part of future investigations.

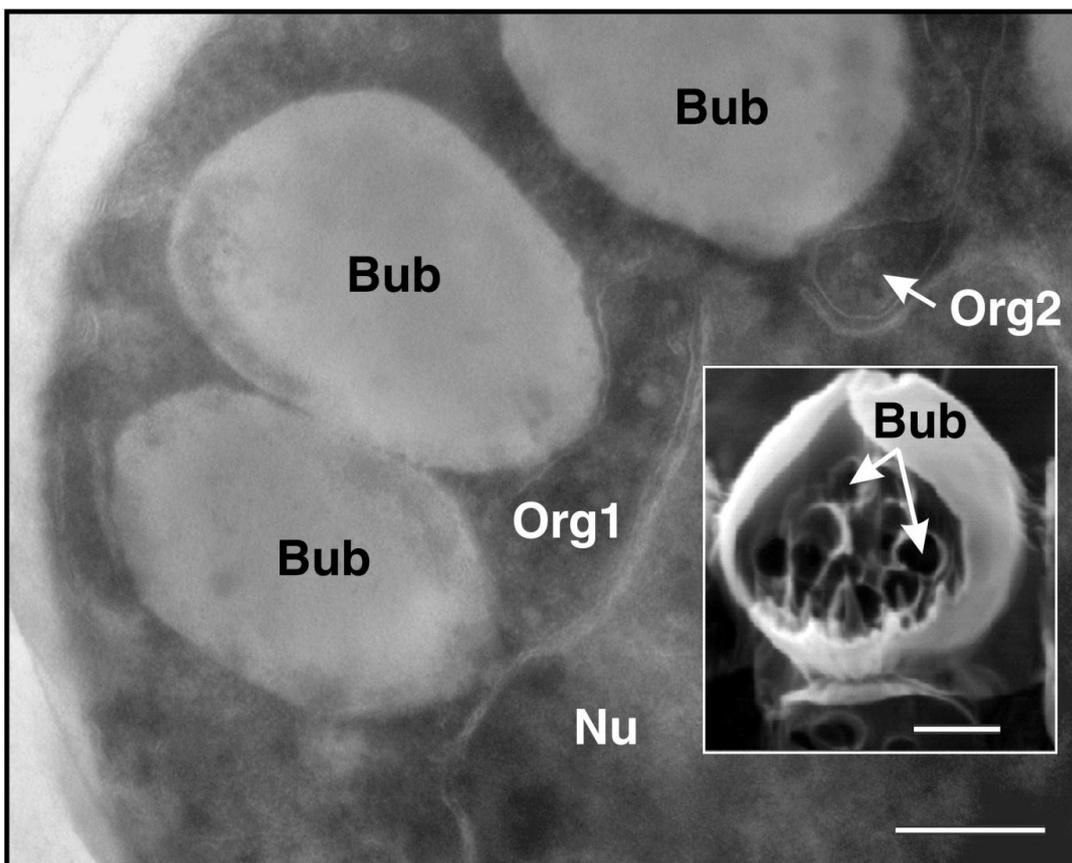


Figure 1. Intracellular gas bubbles deform organelles in fermenting brewing yeasts. Bub: gas bubbles; Org 1&2: organelles; Nu: nucleus. Insert: cell almost filled with gas bubbles as exposed by Auger-architectomics in Scanning Electron Microscopy (SEM) mode. Scale bar, 200 nm. Scale bar of insert, 1 μ m.

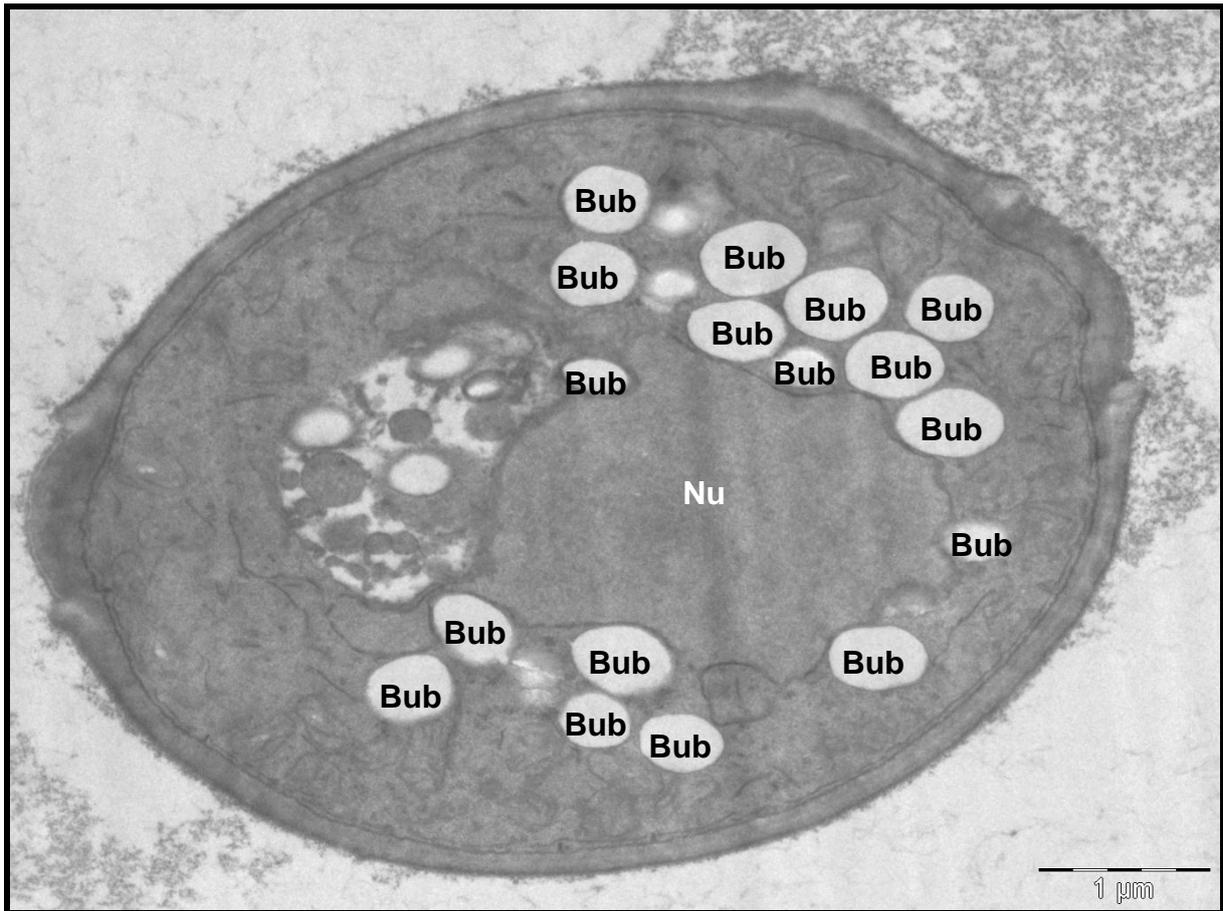


Figure 2. Intracellular gas bubbles deform organelles in fermenting brewer's yeast (potassium permanganate (KMnO_4) treatment). Bub: gas bubbles; Nu: nucleus.

3.6. Acknowledgements

The author would like to thank the National Research Foundation (NRF) and University of the Free State (UFS), South Africa for funding.

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Chapter 4

Conclusions

The Discovery

In a nutshell, the missing link concerning the formation of intracellular gas bubbles in the brewer's and baker's yeasts has been uncovered. In addition, their effect on organelle structure has been elucidated. In future the influence of growth kinetics, respiration and fermentation on the formation of gas bubbles in the cytoplasm of yeasts should be addressed. Furthermore, the effects of gas bubbles on cell organelles, metabolism as well as the life-span of yeast cells should be further investigated.

Summary/ Opsomming

Summary

It has previously been implicated in literature that intracellular gas bubbles cannot form in yeast cells even under high gas supersaturation conditions. Furthermore, not even protein-coated gas vesicles found in Cyanobacteria are expected in yeasts. The lack of intracellular gas bubbles has been attributed to the increased structuring of water and lack of water with nucleation properties. This, however, is considered a missing link since yeasts, the workhorses of the baking and brewing industry, are known to produce and vigorously release carbon dioxide (CO₂) gas during fermentation. Here we resolve the missing link between CO₂ production by glycolysis and the eventual release of CO₂ from the cells, and show that yeasts are capable of producing intracellular gas bubbles which were found to occupy a significant part of the cell. These gas bubbles do not contain a membrane that surrounds them. Furthermore, addition of zinc to the growth medium resulted in the “galvanization” of the bubbles suggesting that the gas bubbles may possibly contain CO₂. These findings should pave way for future research on CO₂ behaviour under pressurized conditions that may have an impact on fermentation biotechnology. Furthermore we show that these intracellular gas bubbles deform cell organelles such as the nucleus. The skin surrounding the gas bubbles is able to withstand tension as they do not disintegrate when they come in contact with organelle membranes. Further research should now be performed on the mechanical effects of the gas bubbles on metabolic and coding functions of yeasts as gas bubbles deform and contort cell organelles. From these findings careful consideration is required during optimization of fermentation parameters to prevent CO₂ toxicity effects on fermentation performance and flavor formation in practical brewing.

Keywords: Brewing yeast; carbon dioxide; compression; deformation; fermentation; intracellular gas bubbles; NanoSAM; organelles; yeast.

Opsomming

Volgens literatuur, kan intrasellulêre gasborrels nie in gisselle gevorm word nie, selfs nie onder hoë gassupersaturasie nie. Verder word selfs nie eens proteïenomhulde gasvesikels wat in Cyanobakterieë voorkom verwag in gisselle nie. Die afwesigheid van intrasellulêre gasborrels was toegeskryf aan die verhoogde strukturering van water en tekort aan water met kernvormingseienskappe. Die afwesigheid van gasborrels word as 'n raaisel beskou aangesien giste, die werkesels van die bak en bierbrou industrieë, bekend is vir die vrystelling van groot hoeveelhede koolstofdiksied (CO_2) tydens fermentasie. In hierdie stuk verklaar ons die raaisel aangaande die produksie van CO_2 deur glikolise en die uiteindelijke vrystelling van CO_2 vanuit die selle en wys ons dat giste oor die vermoë beskik om intrasellulêre gasborrels te vorm wat 'n groot deel van die selvolume uitmaak. Hierdie gasborrels het geen membraanomhulsel nie. Wanneer sink by die groeimedium gevoeg word vind daar "galvanisering" van die borrels plaas wat impliseer dat hierdie gasborrels CO_2 bevat. Hierdie resultate behoort die weg te baan vir toekomstige navorsing rakende die optrede van CO_2 onder druk en dit mag moontlik 'n impak hê op biotegnologiese fermentasies. Verder wys ons ook dat hierdie borrels die selorganelle, soos onder andere die kern, vervorm. Die "vel" rondom die gasborrels kan drukking weerstaan en dus disintergreer die borrels nie wanneer dit in kontak kom met die organelmembraan nie. Verdere navorsing moet nou volg om die meganiese effek van die gasborrels op die metaboliese en koderingsfunksies van die gisselle te bepaal aangesien die borrels die organelle vervorm. Na aanleiding van hierdie resultate moet die vorming van gasborrels in ag geneem word tydens die optimisering van fermentasie parameters om CO_2 toksisiteit te voorkom.

Sleutelwoorde: Brouersgis; fermentasie; gis; intrasellulêre gasborrels; kompressie; koolstofdiksied; NanoSAM; organelle; vervorming.