Evolutionary engineering of Wickerhamomyces subpelliculosus and Kazachstania gamospora for baking

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Evolutionary engineering of *Wickerhamomyces subpelliculosus* and *Kazachstania gamospora* for baking

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Abstract

The conventional baker's yeast, *Saccharomyces cerevisiae*, is an indispensable baking workhorse of all times. Its monopoly coupled to its major drawbacks such as streamlined carbon substrate utilisation base and a poor ability to withstand a number of baking associated stresses prompt the need to search for alternative yeasts to leaven bread in the era of increasingly complex consumer lifestyles. Our previous work identified the inefficient baking attributes of *Wickerhamomyces subpelliculosus* and *Kazachstania gamospora* as well as preliminarily observations of improving fermentative capacity of potential alternative baker's yeasts using evolutionary engineering. Here we report the characterisation and improvement in baking traits in five out of six independently evolved lines incubated for longer time and passaged for at least 60 cycles relative to their parental strains as

well as the conventional baker's yeast. In addition, evolved clones produced bread with a higher loaf volume when compared to bread baked with either ancestral strain or the control conventional baker's yeast. Remarkably, our approach improved the yeasts' ability to withstand baking associated stresses, a key baking trait exhibited poorly in both the conventional baker's yeast and their ancestral strains. *W. subpelliculosus* evolved the best characteristics attractive for alternative baker's yeasts as compared to the evolved *K. gamospora* strains. These results demonstrate the robustness of evolutionary engineering in development of alternative baker's yeasts.

1 Introduction

The conventional baker's yeast, *Saccharomyces cerevisiae*, remains the baking workhorse of all times. Baker's yeast is a key ingredient in baking that serves three functions; production of CO_2, dough maturation, and development of flavour of bread and other farinaceous products (Attfield, 1997, Kariluoto *et al.*, 2004). In addition, to its baking attributes, *S. cerevisiae* was the first yeast to be domesticated (Fay & Benavides, 2005), the first eukaryote to be completely sequenced (Goffeau *et al.*, 1996), the first model organism (Botstein *et al.*, 1997), the first genetically modified food producing organism (Aldhous, 1990), and it is the most studied yeast (Liti *et al.*, 2009). This list shows why baker's yeast is the yeast of choice and probably the main reason behind its millennia-long monopoly of not only the baking industry, but also the wine and beer industries (Gibson *et al.*, 2017). The primary role of a baker's yeast lies in its ability to rapidly ferment sugars (mainly maltose) found in flour production (Randez-Gil *et al.*, 1999) and subsequent accumulation of CO_2. Yeast determines the overall quality of the bread beyond just gas production, such as bread aroma, texture, crumb structure and overall appearance of the bread. However, the conventional baker's yeast has challenges impacting the overall bread quality, which therefore affects its continued use in modern consumer-driven markets.

Firstly, *Saccharomyces cerevisiae* has a stream-lined utilisation of carbon sources (Ostergaard *et al.*, 2000), produces a low diversity of secondary metabolites, and has poor ability to resist baking associated stresses as compared to recently described nonconventional yeasts (Hernández-López & Vargas-Albores, 2003, Struyf *et al.*, 2017). Incomplete sugar fermentation leads to an unhealthy diet in our modern lifestyles where reduction of intake of sugars remains the cornerstone to fight obesity and diabetes. The production of less diverse secondary metabolites leads to poor development of

bread flavour (Harvey *et al.*, 2010, Aslankoohi *et al.*, 2016). During baking, there are baking-associated stresses that yeast may encounter such as high osmotic stress, high oxidative stress, ethanol stress and others as well as low/high temperatures during baking and storage (Attfield, 1999). However, the conventional baker's yeast has a poor ability to withstand stressful conditions, which ultimately leads to reduced product yields. Conditions, such as high osmotic pressure associated with downstream processing during starter culture production as well as during fermentation of dough with high amounts of sugars and salts and low pH in some dough, have been well documented (Attfield, 1997, Hernández-López & Vargas-Albores, 2003, Takagi, 2017). Fermentation generates reactive oxygen species, which exert an oxidative stress to yeast (Takagi, 2017) and the conventional baker's yeasts is poor in responding to oxidative stress (Takagi, 2017). Ethanol formed during fermentation, exerts major stress to yeast cells, and acts as a membrane defacer, which the conventional baker´s yeast can withstand. These many challenges create a need to find alternative yeasts presenting robust baking traits.

The ability to ferment sugars and produce CO_2 is not circumscribed to *S. cerevisiae* (Hagman *et al.*, 2013). Recent advances on these non-conventional yeasts as producers of desirable and unique aromas (Gamero Lluna & de Jong, 2013, Gamero *et al.*, 2016, Ravasio *et al.*, 2018), their resistance to many industry associated stresses (Radecka *et al.*, 2015) suggest their applicability in baking. We previously the screening of two potential non-conventional yeasts, *W. subpelliculosus* and *K. gamospora*, as alternative baking yeasts (Zhou *et al.*, 2017). These yeasts exhibited a poor fermentative capacity and poor dough leavening attributes when compared to the conventional baker's yeast. Preliminary dough leavening abilities after adaptively evolving these yeasts in flour dough highlighted serial passing in flour dough could be used to improve baking traits (Zhou *et al.*, 2017). In this study we set a longer incubation time (48 h) coupled to a relatively higher number of passaging cycles (at least 60) as compared to the parameters reported in (Zhou *et al.*, 2017) and showed that evolutionary engineering is an attractive strain improvement strategy to increase the gassing power, dough leavening and baking associated stress tolerance of alternative baker's yeasts. In brief, we report the improvement in the baking attributes of clones from 5 out of 6 of independently and parallel-evolved lines when compared to their respective ancestral strains as well as the control conventional baker's yeast. In addition, this work further reports a similar trend in improvements of baking traits based on baking trials, thus confirming our hypothesis that evolutionary engineering improves baking traits.

2 Materials and methods

2.1 Strains

The two potential alternative baker's yeasts, *W. subpelliculosus* (CBS 5552) and *K. gamospora* (CBS 10400) reported in our previous work (Zhou *et al.*, 2017) were used for evolutionary engineering experiments. A control conventional baker's yeast from Anchor Yeast as instant dry yeast (*S. cerevisiae)* sold in retail outlets was selected as a positive control.

2.2 Culture media

Yeast Peptone Maltose (YPM) media constituted of 2 % Maltose, 0.5 % Yeast extract and 1 % Peptone), at a pH of 6.2 was used to revive isolates stored at –80 ºC. Yeast peptone dextrose (YPD) media constituting of 2 % Glucose, 0.5 % Yeast extract and 1 % Peptone) adjusted to a pH of 6.2 using 1 M NaOH and 1 M HCl was used for stress tolerance tests.

2.3 Evolutionary engineering scheme

Evolution experiments were conducted by serially passaging yeast in wheat flour dough as reported previously (Zhou *et al.*, 2017) with modifications of flour dough preparations (shorter sterilisation time and lower sterilisation temperature), longer duration of incubation times and an increased number of passaging cycles. In brief, flour dough was prepared by mixing 0.6 g of wheat flour (sterilised by heating for 48 h at 70 ºC) and sterile 500 µL of 0.5 M NaCl in 2 mL Eppendorf microtubes. An isogenic colony from each of the master plates (*W. subpelliculosus* (CBS 5552) and *K. gamospora* (CBS 10400)) was grown overnight in YPM media and harvested thereafter and set to an OD_{600nm} of 1. Triplicates (parallel lineages) from each isogenic colony were then inoculated into wheat flour dough and microtubes were then incubated without shaking at 30 ºC (see Supplementary Materials, Figure S1). Parallel lines from *K. gamospora* were designated as *Kg_1*, *Kg_2*, and *Kg_3*, whereas those from *W. subpelliculosus* were designated as *Ws_1*, *Ws_2*, and *Ws_3*. After 48 h of incubation, a toothpick-full of dough was transferred into fresh dough to start another passage. This procedure was repeated for several passages (see Supplementary Materials, Figure S2) until the microtubes popped open before the set 48 h incubation time. After every passage, the tubes were cryopreserved at –80 ºC in 25 % glycerol for further studies and as starter culture in case of a mishap during evolution such as contamination. After passaging for 60 passages cells from a toothpick from each of the parallel-evolved lines were serially dilution plated out and used for characterisation

experiments. To be specific, we selected the biggest colony from each of the 6 parallel-evolved lines (*Kg_1*, *Kg_2* and *Kg_3* from *K. gamospora* evolved lines and *Ws_1*, *Ws_2* and *Ws_3* from *W. subpelliculosus* evolved lines) and characterised the change in baking attributes when compared to their ancestral strains as well as those of the conventional baker's yeast.

2.4 Confirmation of evolving strains and detection of contamination

After every 10 passages, the absence of contamination was verified by microscopy and sequencing. For sequencing, DNA extraction was carried out using ZR Soil Microbe DNA kit™ (Zymo Research, Orange, CA, USA) according to manufacturers' recommendations. A 560 – 750 bp amplicon size was amplified from ITS-5.8S rDNA using ITS1 (5'–TCCGTAGGTGAACCTGCGG–3') and ITS4 (5'–TCCTCCGCTTATTGATATGC–3'). The following PCR program: Initial denaturation: 94 °C – 4 min, cycle denaturation: 94 °C – 30 sec, primer annealing: 54 °C – 30 sec, chain extension: 72 °C – 1 min, chain elongation: 72 °C – 7 min, number of cycles: 36 cycles, and holding temperature: 4° C was run. PCR products were analysed by gel electrophoresis (using 1 % agarose gel in 1X TBE buffer) and then purified before being quantified using an ND- 1000 spectrophotometer (NanoDrop; Thermo Scientific, Wilmington, DE, USA). Inqaba Biotechnical Industries in Pretoria, South Africa sequenced the purified PCR products. SnapGene sequence editing tool was used for removing ambiguous bases (http://www.snapgene.com). The yeasts were identified by searching databases using BLAST sequence analysis tool (http://www.ncbi.nlm.nih.gov/BLAST/). To confirm the identity, pairwise identification database owned by the Westerdijk Fungal Biodiversity Institute (CBS-NAW) (http://www.westerdijkinstitute.nl/) was also used.

2.5 Investigation of fermentative capacity

To investigate the improvement in fermentative capability of the evolved strains, fermentation was carried out using YPM in 60 mL BD Luer-Lok™ syringes (BD® Syringes) as described in our previous work (Zhou *et al.*, 2017). A single colony (selected based on colony size) from each of the evolved lineages of *W. subpelliculosus* (*Ws_1*, *Ws_2*, and *Ws_3*) was grown overnight in 2 mL of 5 % YPM in 5 mL culture tubes at 26 °C at 200 rpm on a shaker (Infors HT). The yeasts were then harvested, washed and used to inoculate 5 mL YPM in syringes at an initial OD_{600nm} of 1 and incubated under the same conditions as above. The plunger movement, as CO_2 was accumulated, was

recorded after every 2 hours for 20 hours. CO_2 production yields were calculated by dividing the amount accumulated at the end of fermentation by the biomass accumulated. In addition, CO_2 production rate was calculated by determining the slope of the curve using the points at which the accumulation of CO_2 was the fastest. The same procedure was repeated with evolved lineages of *K. gamospora* (*Kg_1*, *Kg_2* and *Kg_3*), both ancestral strains and a control conventional baker's yeast. These experiments were done in triplicates and repeated three times.

2.6 Investigation of leavening ability

The evolved yeast isolates (*Kg_1*, *Kg_2*, *Kg_3*, *Ws_1*, *Ws_2*, and *Ws_3*), the ancestral strains and the control conventional baker's yeast were grown in 2 mL of 5 % YPM in 5 mL tubes and incubated overnight at 26 ºC at 200 rpm on a shaker (Infors HT). The yeasts were then harvested by centrifugation and inoculated into fresh 20 mL YPM and put back on the shaker for another 24 hours to increase cell biomass. 2 mL of cells at an OD_{600nm} of 10 was used to inoculate 10 g of flour dough in Falcon tubes. The Falcon tubes were left to ferment for 1 hour in a 30 ºC incubator. The change in leavening was assessed as dough increased in volume. Respective volume increases after incubation were recorded by photographing. The experiment was done in triplicates and repeated three times.

2.7 Investigation of stress tolerance

The evolved yeast isolates (*Kg_1*, *Kg_2*, *Kg_3*, *Ws_1*, *Ws_2*, and *Ws_3*), the ancestral strains and the control conventional baker's yeast were grown overnight in 5 % liquid YPM in 5 mL tubes at 26 ºC at 200 rpm on a shaker (Infors HT) as above. Cells were then harvested by centrifugation and then washed twice with sterile deionized water. The cells were then adjusted to an initial OD_{600nm} of 0.2. The cells were then serially diluted (2 folds dilution ranges) in sterile phosphate buffer saline and pipetted into 96 well plates. A spot test stamp (replicator) was used to spot cells on solid YPD media plates supplemented with different stressors and incubated at 30 °C for 72 hours. For oxidative stress tolerance, yeasts were grown in YPD agar supplemented with hydrogen peroxide (H_2O_2) at different concentrations (3 mM, 4 mM, 5 mM, 6 mM and 7 mM). For ethanol stress tolerance, yeasts were grown on YPD agar containing different concentrations of ethanol (5 %, 7 %, 9 % and 10 % (v/v)). Similarly, for halotolerance, yeasts were grown in YPD agar supplemented with sodium chloride (NaCl) of different concentrations (0.5 M, 1 M, 1.2 M, 1.5 M, 1.6 M, 1.8 M and 2 M). Osmotolerance tests were carried out by growing cells on YPS agar at different concentrations of

sucrose (50 % and 60 %). For thermotolerance, yeasts cells were grown on YPM agar and incubated at 30 °C, 37 °C, 40 °C, 42 ºC and 44 ºC. Growth of evolved clones was compared to the ancestral strains as well as the control baker's yeasts and scored qualitatively. The experiments were done in triplicates and repeated thrice. The best representative plates were scanned and recorded.

2.8 Baking trials

Leavened dough from the evolved yeast isolates (*Kg_1*, *Kg_2*, *Kg_3*, *Ws_1*, *Ws_2*, and *Ws_3*), the ancestral strains and the control conventional baker's yeast from Section 2.6 were used as starter cultures. 50 g of flour was added to each of the doughs and weighed before and after fermentation to determine the percentage change in weight. After fermentation, the dough was kneaded and moulded into greaseproof muffin moulds. The leavened and moulded dough was baked for 20 minutes at 250 ℃ and 15 minutes in a conventional oven until the bread developed a brownish crust. After baking, the bread was weighed and the percentage change in weight was recorded. The overall texture and pore sizes were photographed and recorded.

2.9 Statistical Analyses

To test whether independently evolved clones and the controls had significantly different fermentative capacity as a function of CO_2 production rate, CO_2 yields and cell-densities, one-way ANOVA was conducted. To test whether the same attributes of independently evolved lines significantly differed from each other, we implemented a post-hoc Tukey's HSD. The significance level was set at $p < 0.05$, $p < 0.01$ and $p < 0.001$. All the analyses were done using STATISTICA, version 13.2 (Statsoft Inc., Tulsa, Oklahoma).

3 Results

3.1 Evolutionary engineering improved the fermentative capacity of 5 out of 6 independent lines

Our previous work (Zhou *et al.*, 2017) reported preliminary observation that potential alternative baking yeasts, *Wickerhamomyces subpelliculosus* and *Kazachstania gamospora* could be adaptively evolved in dough-like conditions. Due to a short incubation time, shorter passaging cycles and absence of characterisation experiments the results were insufficient to ascertain the evolvability and application of the strategy in improving alternative baker's yeasts with poor baking attributes. We were prompted to serially passage the yeasts for longer periods of incubation of 48 h (12 h longer) as

well as increasing passaging cycles, in this case, until the Eppendorf tubes popped before the predetermined incubation time. In addition, in this study we characterised the carbon dioxide production rates and yield using maltose, the dough leavening ability and the improvement in stress tolerance before and after evolution and compared to conventional baker's yeast predominantly used in Southern Africa (*S. cerevisiae,* instant baker´s yeast supplied by Anchor yeast, Co.). Here we report that at 60 passages the tubes started popping before the predetermined incubation time of 48 h suggestive of the improvement in fermentative rates. To confirm the suggestive improvements, we picked a single terminally evolved clone from each of the independently evolved lines and tested their fermentative capacity. Maltose was chosen for testing because it is the most abundant fermentable carbon source in wheat flour (Randez-Gil *et al.*, 2013). Our results show that 5 out of 6 evolved clones accumulated on average 16.43 times more CO_2 (49.28 ± 3.36 mL) at the end of fermentation (after 18 h) as compared to their ancestral strains (3.0 ± 0.94 mL) indicative of a ten-fold improvement in the fermentative capacity using the most abundant carbon source in wheat flour (Figure 1A). In contrast, there was strangely no evident change in one of the six evolved clones from one of the parallel lines analysed (*Kg_2*). Analyses of ITS – ITS4 amplicons suggested that the clone was still *K. gamospora*, which probably lost the ability to ferment maltose efficiently. Overall, our approach was very effective as we observed 4.8 times more CO_2 accumulated when compared to the control conventional baker's yeast (10.3 ± 1.15 mL) within the first 18 hours of incubation. These results suggested that the ancestral strains and the control baker's yeasts are characterised by a longer lag phase during the utilisation of maltose as they later on managed to accumulate more carbon dioxide similar the amounts produced by the evolved clones (results not shown).

In addition to the ability to ferment maltose, we evaluated CO_2 yield of the evolved clones as another important attribute required in leavening the dough. The results showed that there was a significant improvement in CO_2 yield among 5 of the 6 evolved clones (*Kg_1*, *Kg_3*, *Ws_1*, *Ws_2* and *Ws_3*) when compared to the ancestral strains as well as the control baker's yeast (ANOVA, $p < 0.001$) (Figure 1B). The evolved clones exhibited a CO_2 yield that was eight times higher than that of the ancestral strains, which is a significant improvement. Again, *Kg_2* was an outlier. Our approach improved CO_2 yield of 5 out of 6 evolved clones to two times more than that of the control baker's yeast, suggesting that the evolved clones would be a preferable alternative baker's yeasts for the baking industry. Interestingly, there was no significant difference in CO_2 production, production rate and yield among these evolved clones (ANOVA, $p < 0.001$) (see Appendices, Table 4-Table 6) suggesting that the approach is independent of the background of the ancestral strain, which is a

positive attribute to adopt the same strategy to other yeast species of interest. Another important attribute of a model baker's yeast, the gassing power (CO_2 production rate), which reduces the time taken to leaven dough, an important techno-economic factor (Giannone *et al.*, 2010) was tested. We observed a similar trend on the CO_2 production rate, also known as the gassing power, an important attribute determining the speed of dough leavening, among the 5 of the 6 evolved clones in comparison to their ancestral strains and the control baker's yeast (Figure 1C). There was a highly significant improvement in the gassing power of the 5 of the 6 evolved clones when compared to ancestral strains as well as the commercial baker's' yeast (ANOVA, $p < 0.001$). In addition, there was no statistical difference on gassing power attribute tested among the 5 out of 6 evolved clones (Tukey´s HSD, $p < 0.001$) (see Supplementary Materials, Table 8 – Table 10).

3.2 Evolved clones improved the leavening ability

One of the important attributes of baker's yeast is the ability to leaven the dough, an important quality index of a baker's yeast (Ahi *et al.*, 2010). Therefore, the leavening abilities of the evolved clones were evaluated by fermenting the dough and compared them to the dough leavened by the ancestral strains as well as the commercial baker's yeast. Results showed improved leavening ability of the evolved clones *Kg_1*, *Kg_2* and *Kg_3* in comparison to the commercial baker's yeast and their ancestral strain (Figure 2A). Surprisingly, *Kg_2* showed the ability to leaven dough to a volume similar to *Kg_1*. This attribute not observed in the fermentation of maltose, suggesting that *Kg_2* fermented another carbon source found in flour other than maltose is worth investigating in future if at all this strain should be adopted for use in the baking industry. On the other hand, strains *Ws_1*, *Ws_2* and *Ws_3* leavened the dough to double the volume when compared to their ancestor as well as to the control baker's yeast (Figure 2B). These strains evolved from the *W. subpelliculosus* lineage and showed an even higher leavening ability as compared to the *K. gamospora* lineages (*Kg_1*, *Kg_2*, *Kg_3*).

3.3 Evolved clones improved baking associated stress tolerance

Other than fermentative capacity, the ability to withstand baking associated stresses from biomass production to baking is another desirable attribute of a baker's yeast. Baker's yeast may encounter stresses during baking and storage high osmotic pressure, high oxidative stress, high/low temperatures, ethanol stress among others (Attfield, 1999). We firstly investigated the ability to withstand ethanol, a product of dough fermentation, as an important attribute that allows higher

efficiency of leavening ability. The evolved clones *Ws_1*, *Ws_2* and *Ws_3* were resistant to ethanol up to 9 %, which is 2 % higher than the amount tolerable to the conventional baker's yeast (Figure 3). This was a huge improvement in ethanol stress tolerance, as their ancestor did not grow on 5 % ethanol. On the other hand, the evolved lines from the *K. gamospora* background (*Kg_1* and *Kg_3*) tolerated only up to 5 % ethanol. An interesting observation was that *Kg_2* in addition to its poor utilisation of maltose as a carbon source it also did not tolerate ethanol when compared to its ancestor.

Another important attribute of a baker's yeast is the ability to withstand high temperatures. Downstream processing for the preparation of biomass involving drying, storage and rehydration exerts thermal stress as well as oxidative stress to the yeasts (Randez-Gil *et al.*, 2013). Our results suggest that our approach significantly improved thermotolerance of *Ws_1*, *Ws_2* and *Ws_3* evolved clones up to 40 ºC as compared to their ancestor, which did not grow at 37 ºC (Figure 4). A similar improvement in thermotolerance was observed with *Kg_1* and *Kg_3*, except that their ancestor could tolerate 37 ºC. *Kg_2* once again was an outlier in the ability to withstand thermal stress. In addition, the ability to withstand high osmotic stress (as high as 60 %), an attribute of interest for an ideal baker's yeast was investigated. Our findings show that *Kg_2* was more osmotolerant, a trait shared by the parental strain (Anc Kg), than all its evolved counterparts (Figure 5). In addition, a similar trend was also noted for halotolerance (Figure 6). Oxidative stress tolerance was also evaluated as a critical attribute of a baker's yeast because yeasts are exposed to reactive oxygen species generated during dough fermentation. Our results suggest that our approach improved oxidative stress tolerance of all evolved lines (Figure 7).

3.4 Evolved clones show improved baking attributes

Baking trials were conducted to evaluate the relevant attributes and impression of the baked bread, which are crucial to fulfil consumer demands (Rouillé *et al.*, 2010). Sliced portions of the bread baked with different yeasts were evaluated. Bread baked with evolved clones had higher loaf volume when compared to bread baked with their ancestral strain as well as the control conventional baker's yeast (ANOVA, $p < 0.001$) except bread baked with *K.g_2*. This is in agreement to the higher amounts of CO_2 produced in maltose fermentation as the loaf volume is proportional to the amount of CO_2 trapped in the gluten matrix of the dough (Struyf *et al.*, 2017). Although the dough leavened using *Kg_2* had risen to a height just like other evolved strains, the height of the bread was lower (4.9 cm) than breads baked with the other evolved strains (Figure 8) (ANOVA, $p < 0.001$). Our results

296 suggest that the highest loaf was attainable using *W. subpelicullosus* derived strains (*Ws_1*, *Ws_2* and
297 *Ws_3*) (7.3 ± 0.36 cm) (Figure 8).

298 Another important factor in final quality of the bread is the pore sizes, which influences the texture of
299 bread. Bread baked with evolved clones had much more bigger and uniform pore sizes when
300 compared to bread baked with the ancestral strains as well as the control conventional baker's yeast
301 (Figure 8). Our evolutionary engineering approach improved the baking traits as we observed an
302 improvement in loaf volume and overall appearance of the bread baked with 5 out of 6 evolved
303 clones when compared to both their ancestors and the baker's yeast. Bread baked with *Kg_2* was in
304 agreement to poor attributes of other traits investigated above.

305 To further reveal the change in baking attributes, we investigated the change in weight of dough
306 before and after fermentation as well as that of bread after baking. Change in weight is considered a
307 desirable quality attribute for the best outcome of bread (Sanchez-Garcia *et al.*, 2015). The results of
308 change in weight of dough after fermentation and weight of dough after baking are shown in Figure
309 9. It should be noted that the best producer of CO_2 should be the best to leaven dough and, hence, the
310 best in producing the best quality of bread in terms of texture and size. Results show that leavening
311 of the dough and bread baked using the evolved clones lost more weight after baking as compared to
312 their ancestors and the control baker's yeast.

313

314 **4 Discussion**

315 *Saccharomyces cerevisiae*, the industrial workhorse of all times, remains the baker's yeast of choice
316 despite its limitations. The development of alternative baker's yeasts is attractive due to modern
317 baking associated with a huge change in preference for healthy lifestyles, improved foods, improved
318 resource utilisation efficiency, and diversity of food products in our generation's consumer driven
319 markets. This study examined the hypothesis that adaptive evolution of previously reported potential
320 baking yeasts strains in flour dough conditions improves their baking traits. Indeed, not only were the
321 strains evolved to efficiently ferment the most abundant carbon source in flour leading to an elevated
322 speed of leavening, but the strains also improved the ability to withstand baking-associated stress, as
323 well as improving the outcome of the bread. This work demonstrated a direct relationship between
324 evolutionary engineering and improvement in baking traits. To our knowledge, this work is the first
325 work to report on the benefits of evolutionary engineering of non-conventional yeasts for

development of alternative baker's yeasts. Use of evolutionary engineering to improve specific fermentation capabilities is well described (Kim *et al.*, 2013), although not in the case of non-conventional yeasts. This work is in agreement with reports that possible selection over several decades, as a long term evolutionary engineering in fermentations, could be the main reason why the conventional baker's yeast has the best baking attributes (Randez-Gil *et al.*, 2013).

Our observations are consistent with our previous preliminary observations (Zhou *et al.*, 2017) which suggested that baking attributes can be improved by passaging potential baking yeasts using dough conditions. Increased maltose fermentative capacity associated with higher leavening ability is supported by our experimental results. Maltose is the most abundant sugar in wheat flour and therefore a baker's yeast is desirable if it has an efficient maltose fermentation capacity (Struyf *et al.*, 2017). Relatively short fermentation time and leavening ability within a short space of time is of commercial interest. This work did not test the effects of our evolutionary engineering approach on sweet dough, which contains sucrose and fructan. However, it should be noted that sucrose and fructan are degraded within the first hour of fermentation, leaving only maltose to sustain fermentation (Struyf *et al.*, 2017). The ability to leaven dough relies on carbohydrate composition of which efficient utilisation of these carbon sources is very important for an alternative baker's yeast (Randez-Gil *et al.*, 2003). Although the rate of fermentation of flour dough by *S. cerevisiae* is known to be the best in the baking industry, the results presented here suggest that it is possible to develop alternative baking strains with comparable or better baking traits than the conventional baker's yeast currently used in the baking industry.

Survival and performance under baking associated stresses is another important attribute of a baker's yeast. Thermotolerance is one of the most relevant traits because yeasts are subjected to thermal stress during preparation of biomass, transportation and during fermentation of dough (Panadero *et al.*, 2007). Here, we report that evolutionary engineering improved resistance to higher temperatures. Another stress of importance is oxidative stress, which has a well-known effect on dough rheology during bread making (Bonet *et al.*, 2006). Since most yeast biological systems generate reactive oxygen during growth (Sies, 2014), an alternative yeast should develop resistance to this stress. This work suggests that the evolutionary approach exploited led to an improved resistance in oxidative stress. In addition, yeasts fermentation of sugars in the flour dough produces ethanol which can reduce rates of growth, fermentative capacity and cell viability (Nagodawithana *et al.*, 1976). Ethanol production also contributes to the increased rate of H_2O_2 diffusion into the cells and thereby

357 increasing oxidative stress during dough fermentation (Banat *et al.*, 1998). In this sense, it is worth
358 mentioning that we observed an improved resistance to ethanol, extreme temperature and oxidative
359 stresses. In this way, the alternative baker's yeast reported in this work showed the improvement of
360 critical desirable phenotypes.

361 Overall, our study demonstrated the potential in developing alternative baking yeasts with improved
362 phenotypes. It also highlights how this approach leads to improved bread appearance as an important
363 characteristic on which the acceptability of bread depends on. The volume and texture of the bread
364 are the major attributes of desirable bread. Evolutionary engineering improved bread attributes as
365 noted from the results. The loss of ability to ferment and leaven the dough exhibited by one strain in
366 this work is not surprising because the loss of phenotype or trade-offs during evolution is well known
367 (Zeyl, 2006, Kumar & Gayen, 2011, Charlesworth *et al.*, 2017, Van den Bergh *et al.*, 2018). Further
368 studies to reveal the observed phenotypes would be worth exploring.

369 Although there are multiple strategies to develop yeast strains towards specific industrial
370 characteristics as extensively reviewed by Steensels et al. (2014), evolutionary engineering is one of
371 the simplest, yet very powerful approach to develop non-recombinant strains for the baking industry
372 (Deckers *et al.*, 2020). The search for baking yeasts from natural biodiversity, genetic and other non-
373 genetic improvements are associated with many drawbacks. Searching for baking yeasts from nature
374 is attractive, but the traits of such yeasts meant for survival and reproduction in their natural
375 environments are not directly transferrable to highly stressful man-made environments, such as
376 baking, brewing or other industrial processes (Steensels *et al.*, 2014). Before such strains are used,
377 artificial strain development strategies are necessary (Nevoigt, 2008). Genetic improvements of food
378 grade yeasts are controversial for consumer acceptance (Da Silva & Srikrishnan, 2012). Non-genetic
379 modification techniques, as evolutionary engineering, used to improve strains are therefore more
380 attractive.

381 In conclusion, our work highlighted that evolutionary engineering is an attractive tool to improve the
382 baking performance of non-conventional yeasts, which has been a major limitation for entrance into
383 the market monopolised by *Saccharomyces cerevisiae*. However, further studies are required to
384 reveal the molecular mechanisms behind the observed improvements. Other studies to investigate
385 more attributes such as the ability to withstand other baking associated stresses, changes in aroma
386 complexity after evolution as well as the ability to utilise other carbon sources are required to
387 develop more efficient alternative baker's yeasts.

5 Conflict of Interest

Authors declare no conflict of interest.

6 Author Contributions

T.S performed the experiments. N.Z conceived the experiments and partly designed the experiments. T.S, T.B, A.G and Senior Author N.Z wrote the paper.

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9 List of Figure Legends

Figure 1. Fermentative capability of evolved clones in comparison to their ancestors.

A. CO_2 production profile **B**. CO_2 yield **C**. CO_2 production rate. Anc Kg is an ancestor for *Kg_1* *Kg_2* and *Kg_3* strains, Anc Ws is an ancestor for *Ws_1*, *Ws_2*, *Ws_3* strains and the baker's yeast as a control. The evolved clones show elevated fermentative capacity within 18 h of fermentation (profile of fermentation after 18 h was excluded for brevity). This experiment was performed in triplicates and repeated twice. Error bars represent the standard deviation from the mean (see Supplementary Materials, Table 1-Table 3).

Figure 2. Qualitative leavening abilities of strains after being adaptively evolved in flour dough.

A. Left to right: control with no yeast (NC), control baker's yeast (control), ancestral *K. gamospora* and evolved clones (*Kg_1*, *Kg_2*, *Kg_3*) **B**. Left to right: control with no yeast (NC), control baker´s yeast, ancestral *W. subpelliculosus* and evolved strains. The evolved clones showed improved leavening ability. Images were taken after an hour of incubation at room temperature.

Figure 3. Ethanol stress tolerance of the evolved clones.

Parental strains Anc *Kg* and Anc *Ws*, evolved clones and commercial baker's yeast were spotted (OD_{600nm} 0.2, 0.1 and 0.05) for growth on YPM media supplemented with different ethanol concentrations (5 %, 7 %, 9 %, and 10 %). The evolved clones show improved ethanol tolerance as compared to the ancestral strains and conventional baker's yeast.

Figure 4. Thermotolerance of the evolved clones.

Parental strains Anc Kg and Anc Ws, evolved clones and commercial baker's yeast were spotted (OD_{600nm} 0.2, 0.1 and 0.05) for growth on YPM incubated at different temperatures (30 ºC, 37 ºC, 40 ºC, 42 ºC and 44 ºC). 5 out of 6 evolved clones show improved thermotolerance in contrast with the ancestral strains and the conventional baker's yeast. *Kg_2* lost its ability to withstand thermal stress as compared to its ancestor.

Figure 5. Osmotolerance of the evolved clones.

511 Parental strains Anc Kg and Anc Ws, evolved clones and commercial baker's yeast were spotted
512 (OD_{600nm} 0.2, 0.1 and 0.05) for growth on YPS media supplemented with different concentrations of
513 sucrose (50 % and 60 %). *Ws_1*, *Ws_2*, *Ws_3* and *Kg_2* evolved clones retained a similar
514 osmotolerance capability compared to parental strains. *Kg_1* and *Kg_3* lost the osmotolerance trait.

515 **Figure 6. Halotolerance of the evolved clones.**

516 Parental strains Anc Kg and Anc Ws, evolved clones and commercial baker's yeast were spotted
517 (OD_{600nm} 0.2, 0.1 and 0.05) for growth on YPM media supplemented with different NaCl
518 concentrations (0.5 M, 1 M, 1.2 M, 1.5 M, 1.6 M, 1.8 M and 2 M). *Kg_1* and *Kg_3* lost the
519 halotolerance ability whereas *Kg_2* retained the attribute. *Ws_1*, *Ws_2* and *Ws_3* strains maintained
520 their poor halotolerance as their ancestral strain (Anc Ws).

521 **Figure 7. Oxidative stress tolerance of the evolved clones.**

522 Parental strains Anc Kg and Anc Ws, evolved clones and commercial baker's yeast were spotted
523 (OD_{600nm} 0.2, 0.1 and 0.05) for growth on YPM media supplemented with different H_2O_2
524 concentrations (3 mM, 4 mM, 5 mM, 6 mM and 7 mM). All the evolved clones showed improved
525 oxidative stress tolerance as compared to the ancestral strains and the conventional baker's yeast.

526 **Figure 8. Images of cross section of breads baked with different yeast clones.**

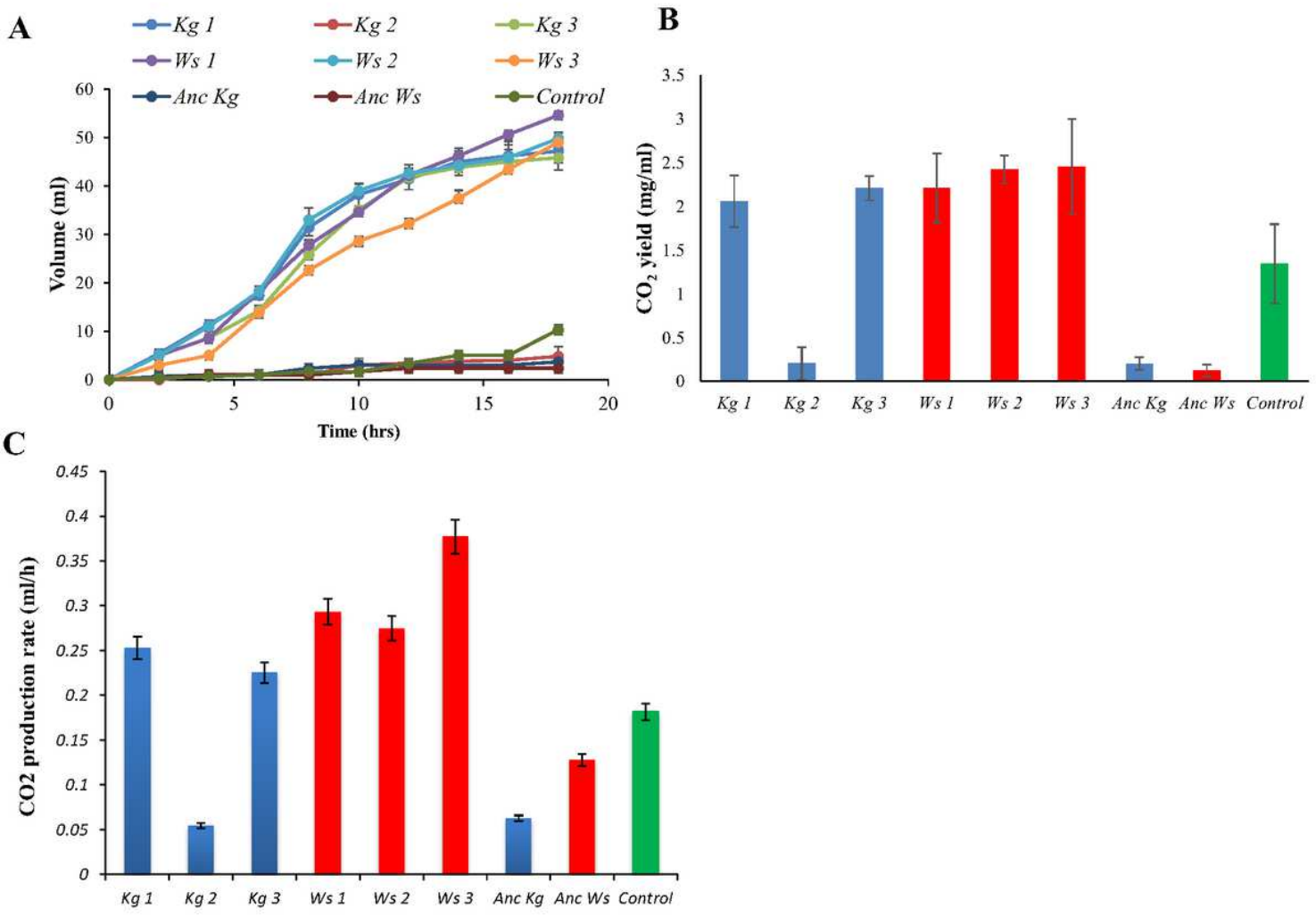
527 Left to right: Unleavened bread NC (without yeast), control baker's yeast (control), ancestral strains
528 (Anc *Kg* and Anc *Ws*) followed by their respective evolved strains. The height of each loaf was
529 recorded and used for comparing the loaf volumes after baking with a respective yeast strain.

530 **Figure 9. Percentage change in weight of the dough after fermentation and after baking.**

531 Evolved clones show more weight change as compared to their ancestral strains and the control
532 baker's yeast. (See also Supplementary Materials, Table 4).

533

Figures



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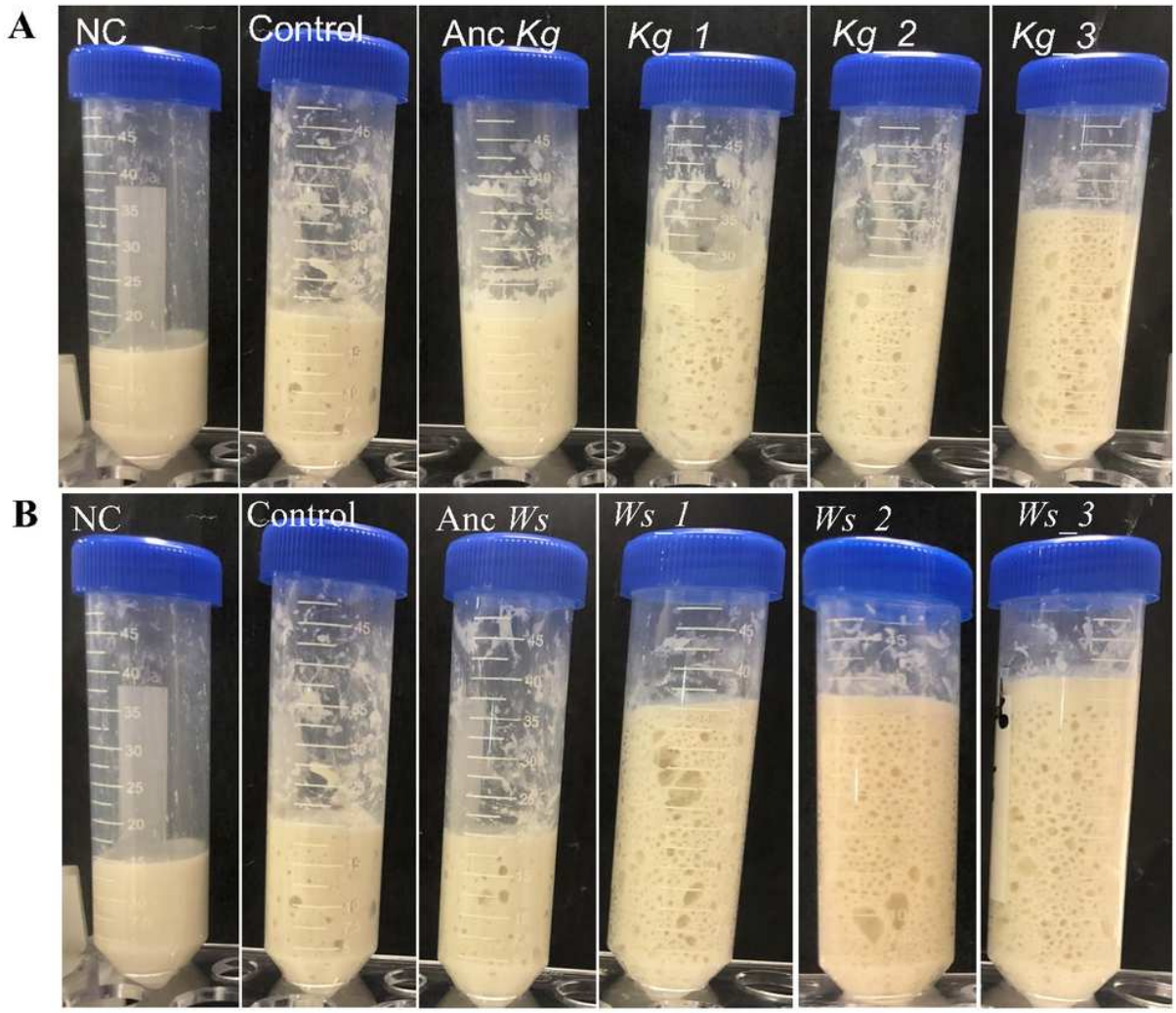

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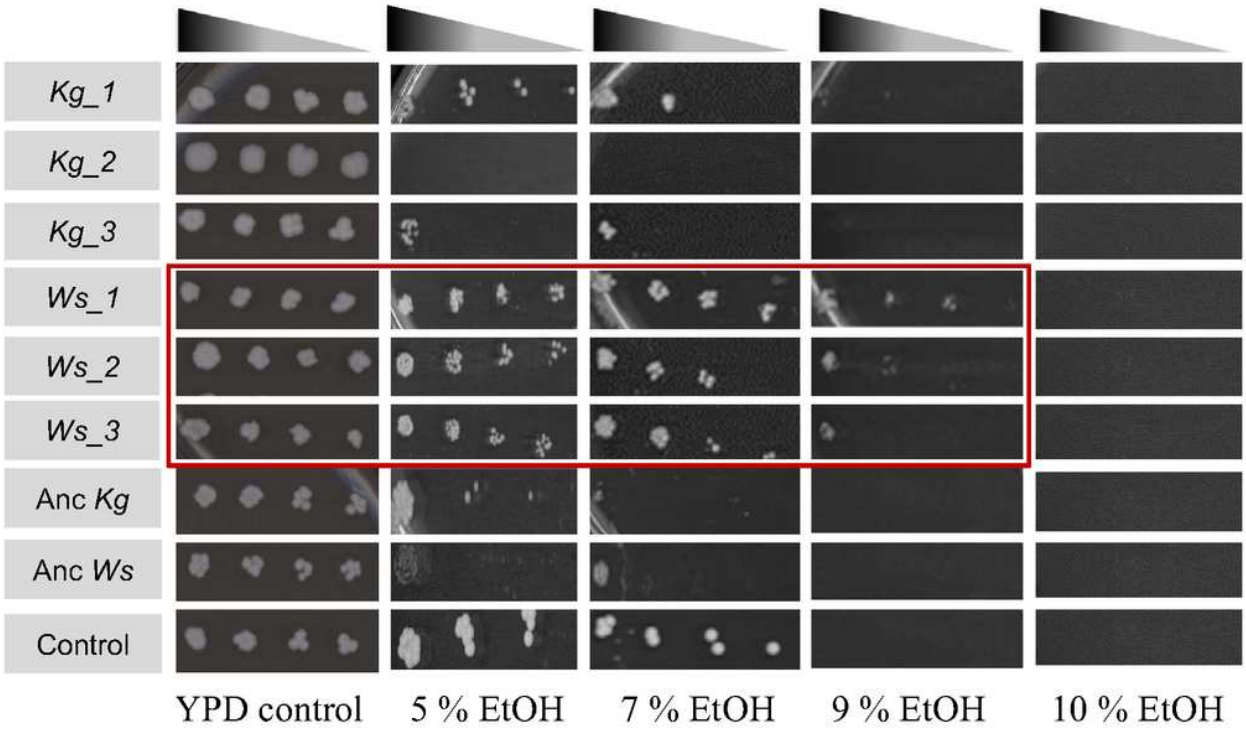

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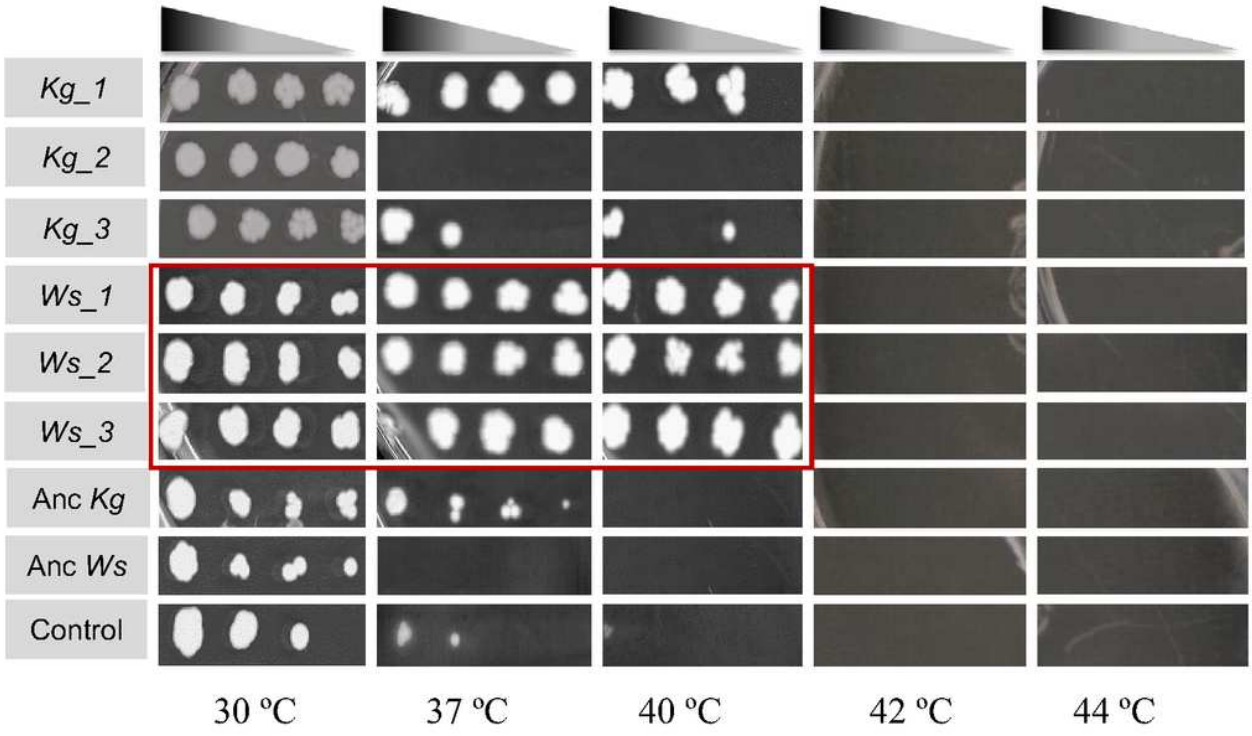

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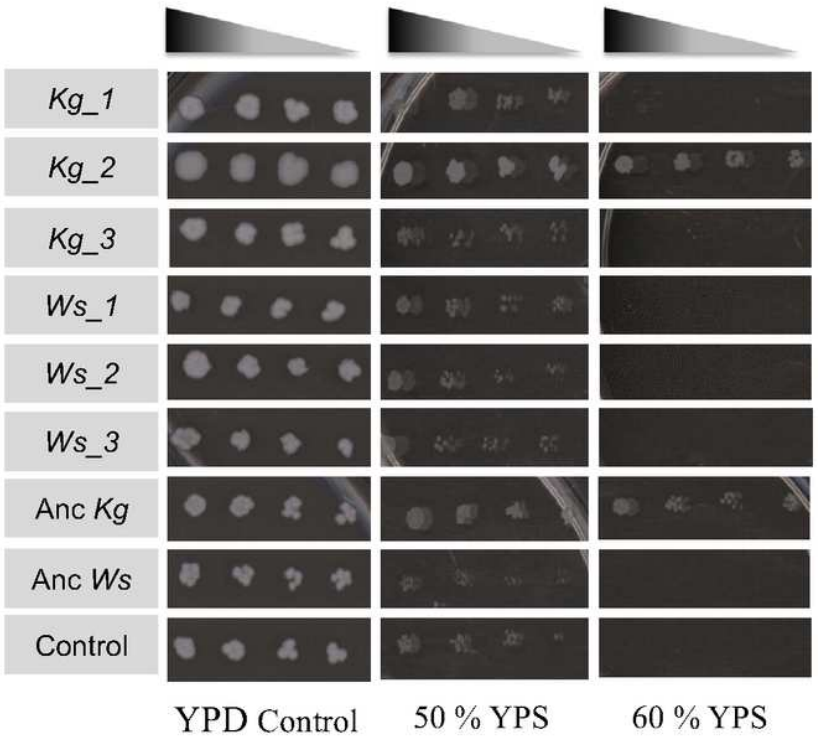

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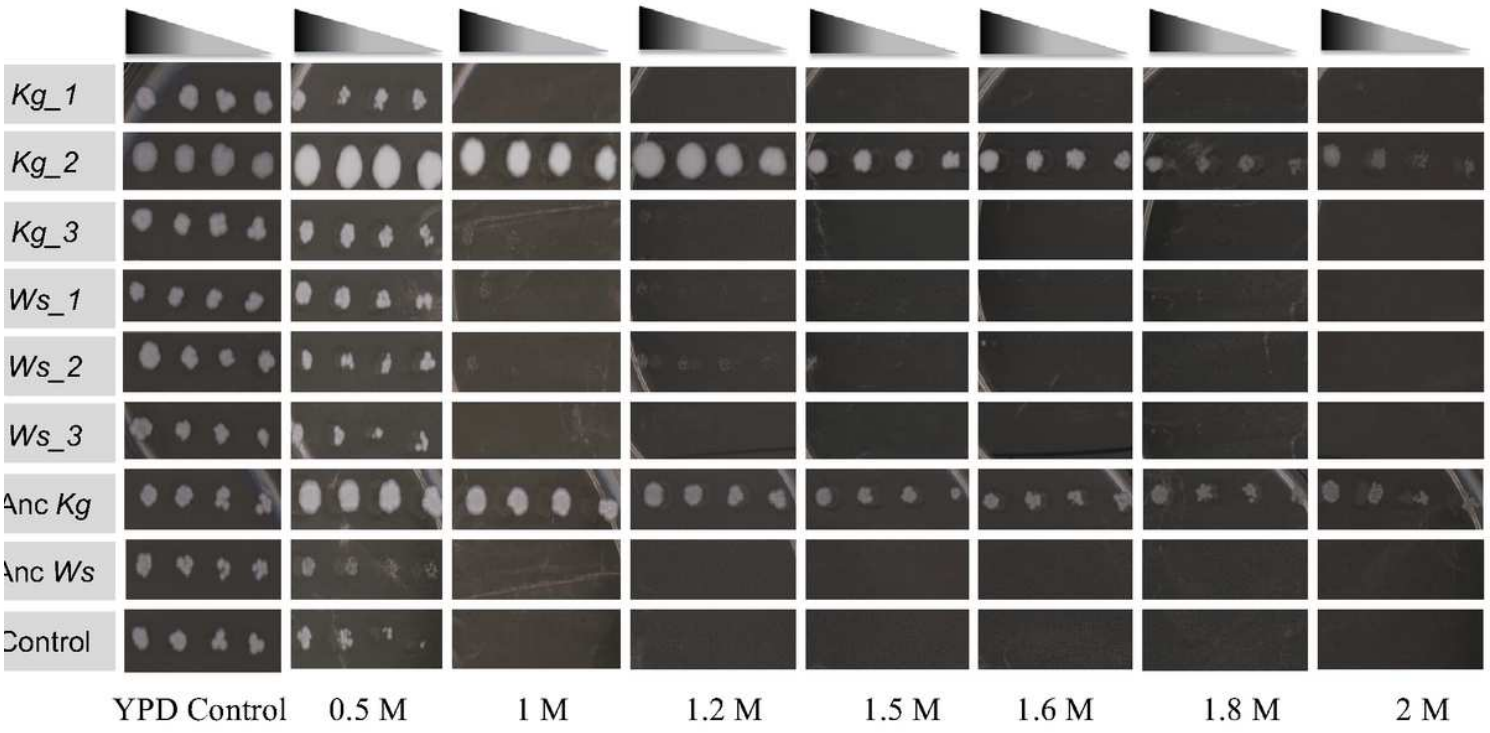

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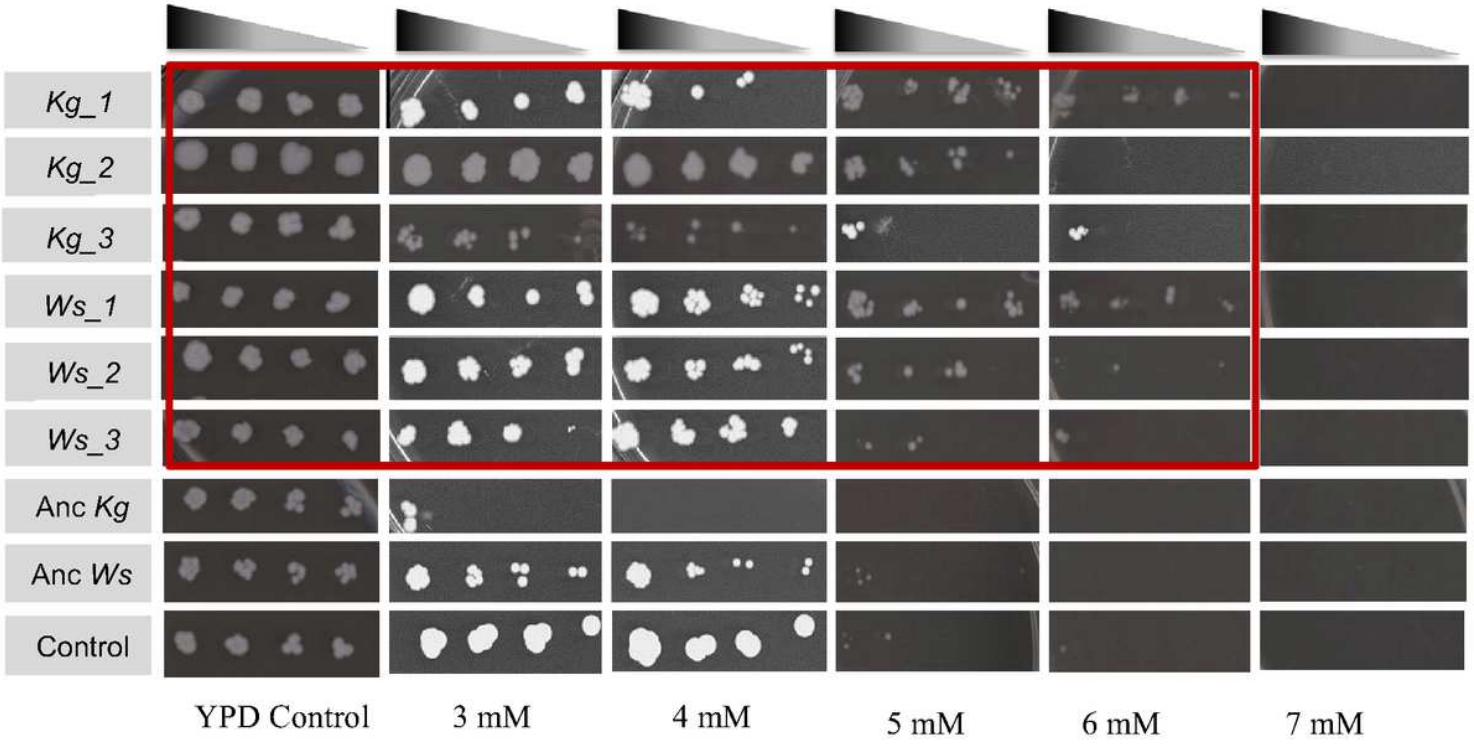

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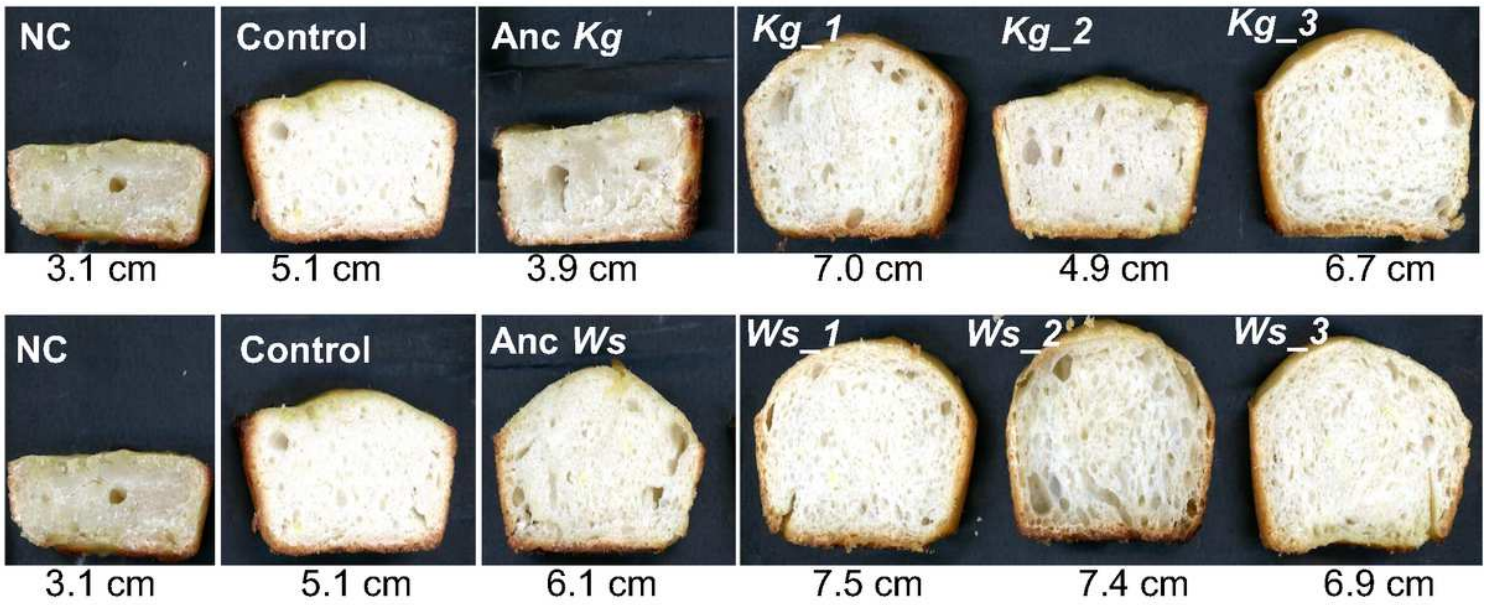

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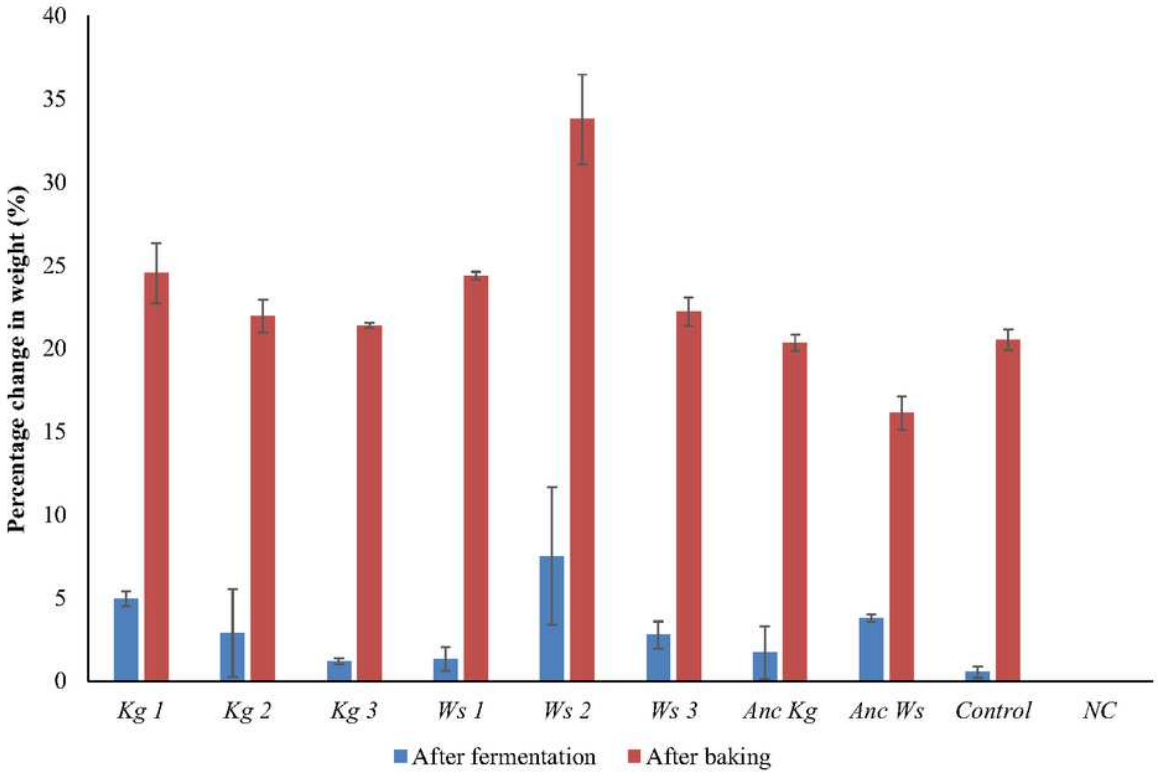

Figure 9

Percentage change in weight of the dough after fermentation and after baking. Evolved clones show more weight change as compared to their ancestral strains and the control 531 baker's yeast. (See also Supplementary Materials, Table 4).

Supplementary Files

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