Heterologous Expression of Type I Antifreeze Peptide GS-5 in Baker's Yeast Increases Freeze Tolerance and Provides Enhanced Gas Production in Frozen Dough

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The demand of frozen-dough products has increased notably in the baking industry. Nowadays, no appropriate industrial baker's yeast with optimal gassing capacity in frozen dough is however available, and it is unlikely that classical breeding programs could provide significant improvements of this trait. Antifreeze proteins, found in diverse organisms, display the ability to inhibit the growth of ice, allowing them to survive at temperatures below 0ºC. In this study, we expressed a recombinant antifreeze peptide GS-5 from the polar fish grubby sculpin (*Myoxocephalus aenaeus*) in laboratory and industrial baker's yeast strains of *Saccharomyces cerevisiae*. Production of the recombinant protein increased freezing tolerance in both strains tested. Furthermore, expression of GS-5 encoding gene enhanced notably gassing rate and total gas production in frozen and frozen sweet dough. These effects are unlikely due to reduced osmotic damage during freezing/thawing, because recombinant cells showed similar growth behavior than the parental under hypermosmotic stress conditions.

KEYWORDS: Baker's yeast; *Saccharomyces cerevisiae*; Type I antifreeze proteins; GS-5; Frozen dough; Freezing/thawing stress

INTRODUCTION

The ability of yeast to cope with and respond to freezing temperatures is a key point in the bread making process, and improvement of yeast freeze-tolerance has become a major focus of attention during last years (for a review see *1*). This interest has increased by the enormous expansion of the frozen-dough market. Presently, the so-called frozen-dough technology makes up 10% of the sales of baked products in Europe and 25-30% worldwide, and it is expected a growing demand in the next future.

Freezing is often a lethal stress for yeast cells, as it causes denaturalization of macromolecules, ruptures of cell membranes and osmotic shrinkage with loss of water and turgor (*2*). During frozen storage, ice crystal growth further deteriorates the plasma membrane and impairs activity in different cellular systems. Finally, during the thawing process, cells can suffer oxidative stress (*3*). As a consequence, the gassing power of yeast cells is reduced, increasing proofing time and lowering bread volume.

To overcome these problems, it is common for bakers to increase yeast dosages in these products by 4-10% above normal. However, this practice increases process cost and affects negatively the taste and texture. Consequently, the development of yeast strains with better gassing power in frozen dough has a great economic interest. Nowadays, no appropriate industrial baker's yeast with these desired properties is however available, and it is unlikely that classical breeding programs could provide significant improvements of these characteristics. Indeed, our limited understanding of physiological and genetic determinants of freezing tolerance suggests that they are under control of multiple genes and complex regulatory mechanisms (*4*).

In this scenario, the ability to transform baker's yeast by recombinant DNA technology has opened the possibilities to manipulate just a single gene or pathway without altering other key or valuable genes for technological purposes (*5*, *6*). The

transfer of heterologous genes to *S. cerevisiae* has also meant that there is now the possibility of using baker's yeast as a cell factory. This has allowed in the past decade the development of industrial strains with unsuspected properties, giving to a new generation of baker's yeast (*5*).

It has been reported that Antifreeze Proteins (AFPs), play a major role in the freeze avoidance or freeze tolerance response of a wide range of organisms including bacteria, fungi, arthropods, plants and fish (for reviews see *7-9*). The AFPs from polar fish species have been the most extensively characterized and are grouped into four classes on the basis of their amino acid composition. Type I is the smallest and simplest, which structure corresponds with an amphipathic alanine-rich α-helix chain (*10*). Several representatives of this class of protein have been described and all contain between three and five imperfect copies of an amino acid sequence motif composed mainly of alanine residues. These proteins fold with pairs of polar amino acid residues, known as ice-binding motifs (*11*), spaced at regular intervals along the polar face of the helix. These polar residues form hydrogen bonds with ice due to a lattice-matching mechanism (*12*), and the protein inhibits water deposition onto these surfaces of the ice crystal due to steric hindrance.

The activity of AFPs has attracted the attention of food biotechnologists interested in engineering freeze hardiness. Antifreeze proteins may inhibit recrystallization during freezing, frozen storage and thawing, thus preserving food texture and minimizing loss of essential nutrients (*13*). AFPs are naturally present in many foods consumed as part of the human diet and there are no evidences of adverse health effects (*14*). In the case of fish AFPs, which intake is estimated as substantial in most northerly regions, is also reasonable to infer a lack of allergenicity (*14*).

AFPs can be introduced into food products either by exogenous addition or by gene transfer. In this late case, the apparent simplicity of structure of the type I AFPs makes them particularly attractive, because it allows a relatively easy genetic manipulation. However, their small size (Mr 3,000-5,000) and lack of globular tertiary structure have apparently rendered this class of protein susceptible to degradation when expressed in heterologous host systems such as *Escherichia coli*, yeast, *Drosophila* and plants (*10*, *15*, *16*).

Here we report how heterologous expression of a type I, alanine-rich AFP (*17*), isolated from the polar fish known as grubby sculpin (*Myoxocephalus aenaeus*) provides a new alternative to face loss of viability of baker's yeast cells upon freezing and frozen storage. GS-5 production through the secretory pathway using the prepro α-mating factor sequence of *S. cerevisiae* also enabled to increase gas production in a frozen liquid dough model system.

MATERIALS AND METHODS

Yeast strains and culture media. The industrial baker's yeast HS13 Ura^- (Lesaffre International, Lille, France) and the laboratory W303-1A (*MATa ade2-1 his3-11,15 leu2-3,112 trp1-1 ura3-1 can1-100 GAL mal SUC2*) wild-type strain (*18*) were used throughout this study. Yeast biomass was prepared by cultivating cells (7.6 units of OD_{600}) on molasses (5.0 g of beet molasses (49% sucrose), 0.5 g of ammonium phosphate, 26.0 g of agar and 20 μg of biotin per liter; adjusted to a final pH 5.0) plates (140-mm diameter) for 20 h at 30ºC. Then yeast cells were recovered by washing the plate surface with 2 x 10 ml of distilled water and the yeast suspension was poured into a tube. After centrifugation, the yeast cake was washed twice with distilled water (4ºC), resuspended in saline solution containing 27 g/l of NaCl, vortexed, and the OD_{600} of the

resulting suspension measured. The final yeast concentration was adjusted to 30 mg (dry weight) per ml (OD_{600} = 1 equals 0.35 mg cells dry weight/ml). Fifteen ml of the yeast mixture was poured into a 250-ml screw cap graduated bottle and placed in a 30ºC water bath. After 15 min, 15 ml of 30ºC liquid dough (LD) was added, which results in a concentration of yeast similar to that used in traditional bread dough (around 2%, flour basis), and the production of CO_2 by the yeast cells measured. The LD model system was prepared as previously reported (*19*). Yeast cells were also grown in SD [0.17% yeast nitrogen base without amino acids (DIFCO) plus 0.5% ammonium sulphate and 2% glucose] supplemented with the appropriate concentrations of essential nutrients (*20*) or YPD (1% yeast extract, 2% peptone, 2% glucose). Cells were grown routinely in 500 ml Erlenmeyer flasks at 30ºC on an orbital shaker (250 rpm).

Phenotype experiments were made by adjusting exponential cultures to OD_{600} = 0.3 and spotting serial dilutions onto SD agar plates containing NaCl (0.7 M) or KCl (1.4 M). Plates were incubated at 30ºC for 2-6 days. For freeze tolerance assays, cells (15 mg dry weight per ml) were inoculated in LD and the suspension was divided in 1 ml portions and transferred directly to -20ºC. After different times, aliquots were thawing at 30ºC for 30 min and the percent of viable cells was measured by counting the number of colonies onto YPD plates.

Escherichia coli DH10B host strain was grown in Luria-Bertani (LB) medium (0.5% yeast extract, 1% peptone, 0.5% NaCl) supplemented with ampicillin (50mg/L). Antibiotics and auxotrophic requirements were filter-sterilized and added to autoclaved medium.

DNA manipulations and plasmids. Plasmid YEpGS5 (*URA3*), which contains the GS-5 expression cassette was constructed as follows: fragments containing sequences of the yeast pheromone α-factor (*MFα1*) prepro region (-13 to +273), *PGK1* terminator,

T_{PGK1} (+1064 to +1544) and *ACT1* promoter, P_{ACT1} (–497 to +11) were obtained by standard PCR using specific synthetic oligonucleotides (Table 1). The amplified P_{ACT1} fragment was digested with *Bam*HI and *Eco*RI and cloned into the pBlueScriptII SK vector (Stratagen, La Jolla, CA), obtaining the plasmid pBS-P_{ACT}. Then, the amplified prepro α-factor sequence was cloned into the pGEM-T Easy Vector (Promega, Madison, WI), treated with *Eco*RI and *Hin*dIII, and inserted into the pBS-P_{ACT} plasmid, resulting in plasmid pBS-P_{ACT}α.

The GS-5 coding DNA fragment was synthesized by PCR using first two specific 60-bp oligonucleotides, GSL-1 and GSL-2, designed to overlap in 10 bp (Table 1). After a first cycle of primer extension, amplification of GS-5 DNA was continued using primers GSC-1 and GSC-2 (Table 1). The amplified fragment was subcloned into the pGEM-T Easy Vector (Promega), obtaining the plasmid pGEM-GS5. This was digested with *Hin*dIII and *Cla*I, and the released GS-5 fragment was inserted into the pBS-P_{ACT}α plasmid, obtaining the plasmid pBS-P_{ACT}αGS5. This was treated with *Cla*I and *Sal*I, and used to accommodate the T_{PGK1} fragment previously digested with the same set of enzymes, obtaining the plasmid pBS-P_{ACT}αGS5T_{PGK}. Finally, the GS-5 expression cassette was accommodated into the vector YEplac195 (*21*) by digestion with *Bam*HI and *Sal*I.

Yeast cells were transformed according to (*22*) and transformants were selected by auxotrophic complementation in SD plates. *E. coli* was transformed by electroporation following the manufacturer's instructions (Eppendorf).

Gas production measurements. Yeast biomass was inoculated in LD, incubated at 30ºC with low shaking (80 rpm), and the amount of CO_2 evolved recorded in a home-made fermentometer (Chittick apparatus) by measuring the displacement of a manometric solution placed in a graduated buret (*23*). The manometric solution

contained 10% $CaCl_2$ and 0.5% $CuCl_2$ at pH < 5. CO_2 production was recorded at 20 min intervals for 180 min. Values are expressed as ml of CO_2 per mg of yeast cells, and were normalized to the initial dry weight of the yeast sample tested. We assume that yeast growth over the course of the experiment is almost identical for all the strains tested.

RESULTS AND DISCUSSION

Construction of a yeast expression vector containing the GS-5 coding sequence. The mature form of grubby sculpin GS-5 type I AFP contains only 33 amino acids, making practical to synthesize the gene *de novo*, as a 99 nucleotide DNA fragment. The strategy relied on commercial synthesis of two 10 bp-overlapping oligonucleotides, which contain the GS-5 sequence adjusted to the *S. cerevisiae* preferred codon usage (*24*). In addition, the oligonucleotides were designed to remove the ATG of the wild-type GS-5 encoding sequence by the change of A to T and to add a stop codon. This change allows the in-frame expression of the GS-5 sequence and introduces a single replacement at residue 1 from methionine to leucine. Nucleotides at the 5'-ends for the restriction sites *Hin*dIII and *Cla*I, were also included to allow its later manipulation. After an extension phase of the assembled oligonucleotides, the complete fragment was amplified by PCR, ligated into an appropriate vector and the nucleotide sequence of the insert was determined to confirm its identity. A schematic representation of the strategy of synthesis and cloning of the AFP is shown in Figure 1.

To obtain the expression of the recombinant peptide, the GS-5 sequence was placed under the control of the *S. cerevisiae ACT1* promoter, a strong constitutive promoter widely used for the production of heterologous proteins in baker's yeast (*25*, *26*). The expression cassette also comprised the terminator of the *PGK1* gene and the signal

sequence and leader region of the yeast prepro α-mating factor (*MFα1* gene), which was fused in-frame upstream of the GS-5 sequence (Figure 1). The prepro α-factor sequence is an efficient secretion vehicle for heterologous proteins, as it is processed through the secretory pathway and digested by specific proteases, releasing thus the biologically active protein (*27*). The final construct (Figure 1) generated in the shuttle vector YEplac195 (*21*) was used to transform the laboratory W303-1A and the industrial HS13 strain. Transformants were selected by auxotrophic complementation and the functionality of the AFP-producing strains analyzed.

Production of the recombinant AFP GS-5 increases freeze tolerance in yeast. Transformants of each strain under assay were selected for testing the ability of recombinant GS-5 to enhance freeze tolerance in yeast. Cells were inoculated in a flour-free liquid dough model system (LD), shifted to -20ºC, and at different times, samples were thawed and the number of viable cells was determined by their colony-forming ability. We choose the LD system because it mimics the main nutrient composition of bread dough and permit the easy collection of isolated yeast cells and testing of functional properties (*19*). In all cases, yeast cells transformed with the empty plasmid YEplac195 were used as control. As it can be seen (Figure 2), the two strains analyzed differed in their susceptibility to freezing and frozen storage. Cells of the industrial HS13 strain showed a rapid and strong loss of viability, even after only 1 day at -20ºC. On the contrary, at least 50% of the laboratory wild-type strain cells survived 13 days at this temperature. The degree of freeze tolerance acquired by overexpression of GS-5 was also dependent on the strain tested. Although production of the AFP in the laboratory strain resulted in a certain protection against freeze damage, this effect was significantly larger in transformants of the HS13 strain (Figure 2). As an example, the number of viable cells in YEpGS5 transformants of the HS13 strain was 32-fold higher

than in the wild-type after 13 days at -20ºC. Thus the expression of the AFP GS-5 increases freeze tolerance in yeast, but the magnitude of this response appears to correlate with the basal sensitivity of the yeast strain assayed.

We also tested if the increase of freeze tolerance observed by expression of the AFP GS-5 in the industrial strain, determines an enhanced CO_2 production in frozen dough. Again, cells were inoculated in the LD model system and their gassing power tested after freezing at -20ºC and frozen storage (Figure 3). CO_2 production by HS13 cells was reduced markedly after freezing for 3 h and thawing (see point marked as 0' in Figure 3). This loss was similar in either of the transformants tested, around 30% with respect to unfrozen samples, suggesting that the recombinant AFP does not impart osmoprotection. Effects on leavening activity of yeast cells upon freezing and thawing has been mainly attributed to desiccation and electrolyte release giving to hyperosmotic stress (*28*). On the contrary, expression of the antifreeze peptide GS-5 enhanced gas production capacity during frozen storage. Indeed, after 40 days at -20ºC the production of CO_2 in samples inoculated with cells of the YEpGS5 transformant was 30% higher than that found in control (YEplac195) samples (Figure 3). Nevertheless, these differences were less pronounced than those seen in the viable cells assay (Figure 2). Thus, it seems that a number of cells unable to form colonies on YPD plates are still metabolically active, at least in terms of CO_2 production.

The production of the AFP GS-5 does not confer osmotic stress resistance in yeast. Studies by Meijer et al. (*29*) reported that expression of fish AFPs in *E. coli* has an osmoprotective function. This property has been attributed to an interaction between antifreeze peptides and membrane lipids, which would reduce membrane permeability and inhibit leakage from cells (*30*). However, the CO_2 production data reported in this study (Figure 3) did not appear to support a functional role of the AFP GS-5 in

osmoprotection. In order to clarify this point, we tested if expression of the recombinant antifreeze peptide could also confer improved osmotolerance in baker's yeast cells. The ability of yeast to withstand increasing osmotic pressures is a key point in the bread making process, and improvement of yeast osmotolerance has become a main issue during years (*1*). As it is shown in Figure 4, production of the AFP by yeast host cells of the W303-1A strain produced no significant growth improvement on media containing high concentrations of NaCl or KCl. No effects were also found on growth of transformants of the industrial HS13 strain.

In conclusion, the expression of AFPs in baker's yeast appears to be a promising strategy to increase cell viability in frozen dough. However, it seems unlikely that this approach could be successful to provide osmoprotection. At present stage, production of a native form of the antifreeze peptide GS-5 from the polar fish grubby sculpin allowed a modest but significant improvement in fermentative performance. Further increases in freeze tolerance would be however required to ensure satisfactory leavening of frozen dough at the conventional yeast doses employed in unfrozen products. Directed evolution by random mutation based on the GS-5 AFP could be thus an attractive technique to provide additional freeze protection. Experiments in this line are currently in progress.

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Figure Captions

Figure 1. Partial DNA sequence of the insert contained in plasmid YEpGS5 and construction of the GS-5 expression cassette. The DNA sequence of the yeast prepro α-factor fused in-frame upstream of the GS-5 sequence, and adjacent nucleotides is shown. The deduced amino acid sequence of the recombinant protein and mature GS-5 (bold) is shown below. Unique restriction sites are shown in bold and underlined. The start and stop codon are marked by *. Strategy to construct plasmid YEpGS5 is detailed in Material and methods. Oligonucleotides used for PCR amplifications are shown in Table 1. P_{ACT1}, *ACT1* promoter. prepro α-factor, signal sequence and leader region of the yeast prepro α-mating factor (*MFα1* gene). T_{PGK1}, *PGK1* terminator. ori, replication origin in *E. coli*. MCS, multicloning site. ori 2 μm, replication origin in *S. cerevisiae*. *lacZ*, a reporter gene. *URA3* and *ampR* are selectable markers, used in *S. cerevisiae* and *E. coli*, respectively. Plasmid and fragment sizes are not to scale.

Figure 2. Viability during freezing and frozen storage of transformants of *S. cerevisiae* strains expressing the AFP GS-5. Yeast strains HS13 and W303-1A were transformed with plasmids YEpGS5 (●) or YEplac195 (○) and analyzed for freeze tolerance. The graph represents the log of colony forming units (CFU) of *S. cerevisiae* per ml after different periods of frozen storage. Unfrozen samples were used as control (0 days). Cells were grown, shifted to -20ºC, thawing and platted onto YPD solid medium as described in the Materials and methods section. Values represent the means of at least three independent experiments. The error associated with the points was calculated by using the formula: $(1.96 \times SD) / \sqrt{n}$, where n is the number of measurements.

Figure 3. Effect of freezing and frozen storage on CO_2 production by HS13 baker's yeast cells producing the AFP GS-5. Liquid dough (LD) samples were inoculated with cells of the YEplac195 (○) or YEpGS5 (●) transformants, shifted to -20ºC and stored at the same temperature for 3 h (0') or up to 40 days. After each storage stage the CO_2 production level was tested in a home-made fermentometer. Unfrozen samples were used as control (0 days). Values are given as means of three independent experiments. The error associated with the points was < 5% of the value of the point. dw, dry weight.

Figure 4. The heterologous expression of the AFP GS-5 does not alter growth of yeast cells under hyperosmotic conditions. Cells of the W303-1A and HS13 strains were transformed with plasmids YEpGS5 (GS-5) or YEplac195 (wt) and assayed for growth in the presence of 0.7 M NaCl or 1.4 M KCl. Cultures were grown in SD at 30ºC until early-exponential phase and adjusted to OD_{600} = 0.3. Serial dilutions ($1\text{-}10^{-3}$) of the adjusted cultures were spotted (3 μl) onto SD plates containing the indicated solutes and incubated at 30ºC for 2 to 6 days, respectively.

Table 1. Oligonucleotides used in this study

Primer	Sequence 5´ to 3´	Comments
Alpha-1	AACGAaTtcAAGAATGAGATTTCC	PCR, forward *MFα1* (*Eco*RI site underlined)
Alpha-2	ATGCCAAGCTTCAGCCTC	PCR, reverse *MFα1* (*Hin*dIII site underlined)
PGK-1	ACGAAGTTGTCAAGAGCTCTGCTGC	PCR, forward *tPGK1*
PGK-2	ATCCTTgTCGAcAGCTTTAACGAAC GC	PCR, reverse *tPGK1* (*Sal*I site underlined)
ACT-1	CCTACATTCTTCCTTATCGGATCC	PCR, forward *pACT1* (*Bam*HI site underlined)
ACT-2	CCAGAATCCATTGTgAATTCAG	PCR, reverse *pACT1* (*Eco*RI site underlined)
GSC-1	CTGAAGCTTTGGACGCA	PCR, forward GS-5 (*Hin*dIII site underlined)
GSC-2	ACATCGATTATGGCTTC	PCR, reverse GS-5 (*Cla*I site underlined)
GSL-1	CTGAAGCTTTGGACGCACCTGCTAT AGCCGCAGCAAAAACAGCCGCAG ATGCATTAGCTGCTGCCAA	PCR, forward GS-5 Synthesis. First extension (*Hin*dIII site underlined)
GSL-2	ACATCGATTATGGCTTCGCCGCGGC TGCAGCAGCATCAGCAGCAGTCTT TTTGGCAGCAG	PCR, reverse GS-5 Synthesis. First extension (*Cla*I site underlined)

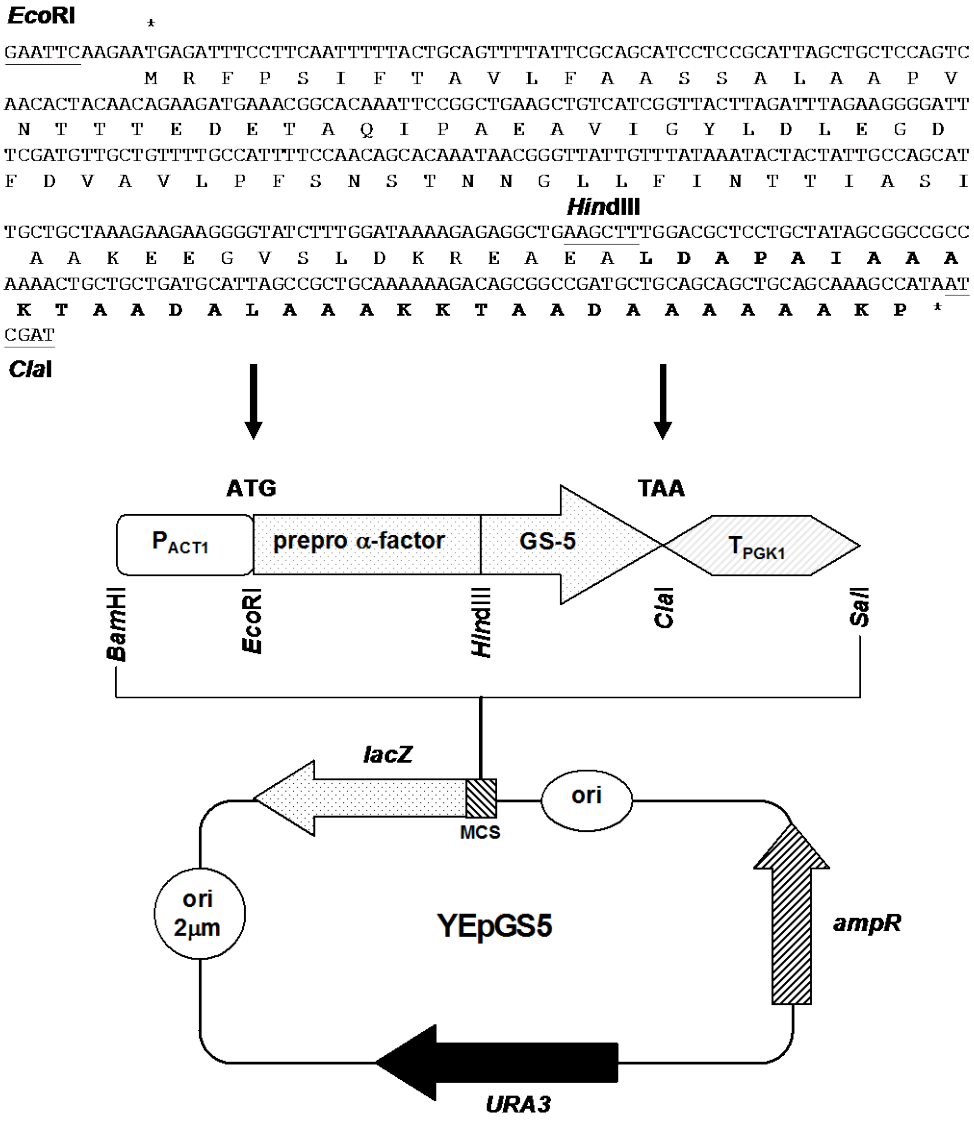
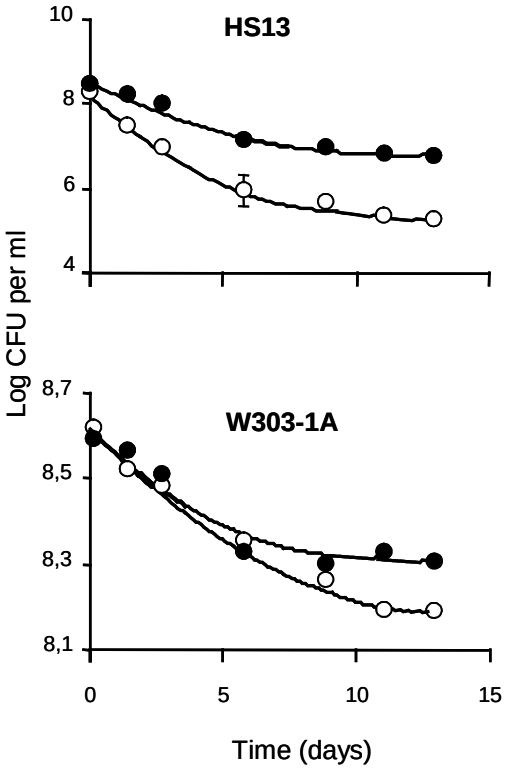


Figure 1 (Panadero et al., 2005)



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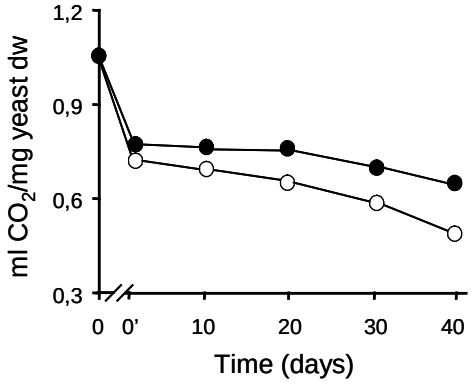

Figure 3 (Panadero et al., 2005)

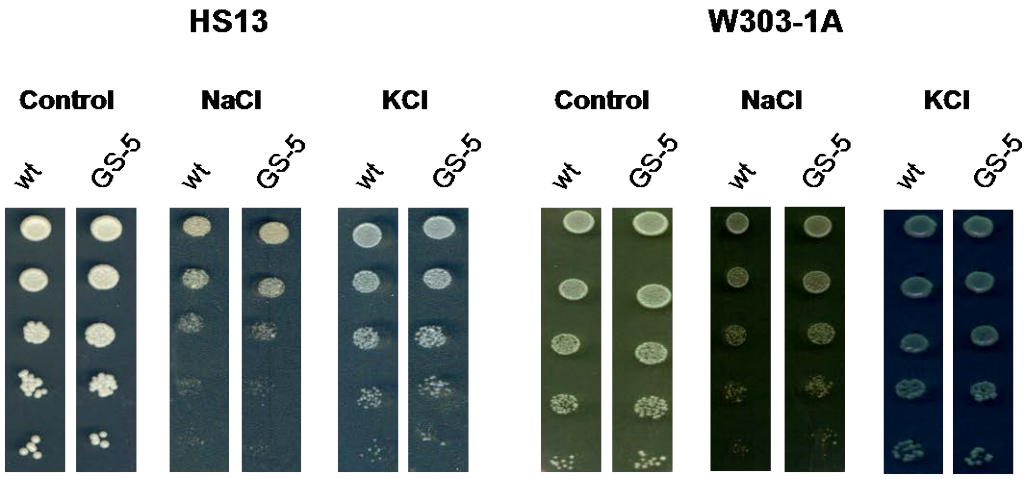

Figure 4 (Panadero et al., 2005)