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Yeast, the man's best friend
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1. Introduction

In most cultures, bread making depends on a fermentation step. The flour leavening ability was, at first, most probably dependent on spontaneous fermentation. It became a controlled process by the maintenance of fresh innocula from one preparation to the next and this kind of environmental constraints eventually generated a particular type of yeast and bacteria biodiversity, adapted to ferment a certain brand of flour mixture, yielding specific organoleptic characteristics to the dough. Nowadays, although the baking industry generally uses commercially available strains of *Saccharomyces cerevisiae* for bread making, some types of bread are still prepared using dough carried over from previous makings as a starter. This trend decreased worldwide bread diversity and the cultural values associated, simultaneously increasing the dependence of local producers on world-scale yeast producers. Sustainability demands assessing yeast biodiversity, as well as devising simple and cheap methods for maintaining dough and multiply yeast.

1.1 Biodiversity at the bakery

The better example of traditional practises still available is the use of sourdough, an extremely diverse fermented product widely used for the production of bread and sweet leavened baked goods. The production of sourdough bread can be traced back to ancient times (Rothe *et al.*, 1973). The products are characterized by their unique flavour, enhanced shelf life and nutritional value, and favourable technological properties (Hämmes & Gänzle, 1998; Salovaara, 1998). Traditionally, sourdoughs have been used to produce many types of bread with rye, maize or wheat flours. This variety underlies the generalized utilization of the term sourdough as a synonym of leavening bread found in the literature. Although the primary purpose of the sourdough is leavening by the yeasts, a simultaneous souring action

also takes place due to the activity of the lactic acid bacteria (LAB) present, resulting in bread with a good grain, an elastic crumb and, usually, the characteristic sensory quality of sourdough bread (Gobbetti *et al.*, 1994a).

1.1 Sourdoughs: a yeast and bacteria synergistic ecosystem

Three types of sourdough have been defined based on common principles used in artisanal and industrial processes (Bocker *et al.*, 1995). One type is produced with traditional techniques and is characterized by continuous propagation to keep the microorganisms in an active state, as indicated by high leavening ability. Examples of baked goods so obtained are San Francisco sourdough, French bread, the Italian panettone, and three-stage sourdough rye bread. The industrialization of the baking process for rye bread led to the development of another type of sourdoughs, which serve mainly as dough acidifiers. These sourdoughs are fermented for long periods (up to 5 days) at temperatures of 30°C, and high dough yields permit pumping of the dough. The microorganisms are commonly in the late stationary phase and therefore exhibit restricted metabolic activity only. Another type of sourdoughs are dried doughs, which are used as acidifier supplements and aroma carriers. These two last types of dough require the addition of baker's yeast for leavening (Meroth, 2003a, 2003b). Although the fermentation process runs under nonaseptic conditions, microbial associations present may last for years, as shown for certain industrial sourdough processes (De Vuyst & Vancanneyt, 2007).

Hundreds of different types of traditional sourdough breads exist in Europe, in particular in Italy. They differ in the type of flour, other ingredients, and the applied technology and fermentation process. Because of their artisan and region-dependent handling, sourdoughs are an important source of diverse LAB species and strains that are metabolically active or can be reactivated upon addition of flour and water. Some of these strains play a crucial role during the sourdough fermentation process and are or can be used as sourdough starters. Sourdough is a unique food ecosystem in that it (i) selects for LAB strains that are adapted to their environment, and (ii) harbours LAB species specific for sourdough (Dal Bello *et al.*, 2005; De Vuyst & Neysens, 2005; Gobbetti *et al.*, 2005).

Sourdoughs are stable ecosystems obtained using daily propagations of LAB and yeasts. Dough acidification is due mostly to homofermentative and heterofermentative LAB species, mainly belonging to the *Lactobacillus* genus (Hämmes & Vogel, 1995), while yeasts are primarily responsible for the leavening action through the production of carbon dioxide and consequent increase in dough volume.

The microorganisms present in sourdough usually originate from flour, dough ingredients or the environment and the variety and number of species in the dough are influenced by several endogenous and exogenous factors, such as type of flour, temperature and time of fermentation, redox potential, and length of time the ''starter dough'' has been maintained (Hämmes & Gänzle, 1998). In fact, strong effects are exerted by process parameters such as dough yield, amount and composition of the starter, number of propagation steps, and fermentation time. The impact of these parameters during continuous propagation of sourdough causes the selection of a characteristic microflora consisting of LAB and usually yeasts (Gobbetti *et al.*, 1994b).

Extensive research efforts have been directed towards the study of the species diversity and identification of lactic acid bacteria involved in sourdough fermentation processes. Lactobacilli, obligatory homofermentative and facultatively or obligatory heterofermentative, are the typical sourdough LAB. These usually belong to the genus *Lactobacillus*, but occasionally *Leuconostoc* spp., *Weissella* spp., *Pediococcus* spp., and *Enterococcus* spp. have been found. In general, heterofermentative *Lactobacillus* species dominate the sourdough microbiota (De Vuyst & Neysens, 2005). *Lactobacillus sanfranciscensis* (Truper & Clari, 1997), *Lb. plantarum* and *Lb. brevis* are the most frequently isolated lactobacilli.

Recent biodiversity studies of particular sourdough ecosystems throughout Europe resulted in the description of new LAB species. During the last years, several new LAB species have been isolated from traditional sourdoughs that were continuously propagated by back-slopping (repeated cyclic re-inoculation) at ambient temperature: *Lb mindensis, Lb spicheri, Lb rossiae, Lb zymae, Lb acidifarinae, Lb hammesii*, and *Lb nantensis*. Some of these species have been described on one single isolate only. The distribution of the taxa of LAB is highly variable from one sourdough ecosystem to another. Therefore, it is difficult to define correlations between population composition and both the type of sourdough or the geographic location (Gobbetti, 1998).

Adaptations of certain LAB to a sourdough environment include (i) a unique central metabolism and/or transport of specific carbohydrates such as maltose and fructose, maltose being the most abundant fermentable carbohydrate and fructose being an important alternative electron acceptor; (ii) an activated proteolytic activity and/or arginine deiminase pathway; (iii) particular stress responses; and (iv) production of antimicrobial compounds. For instance, dough acidification is a prerequisite for rye baking to inhibit the flour α-amylase (De Vuyst & Vancanneyt, 2007).

Although LAB initially isolated from sourdough are not necessarily unique for sourdough ecosystems, some correlations can be seen between specific LAB species and the type of sourdough, and sometimes the origin of the sourdough. In practice, sourdoughs are either continuously propagated by using a piece of dough from the preceding fermentation process, or produced by using once a week a commercial starter followed by back-slopping for several days. Therefore, large differences can often be seen in species composition within and among sourdough types (De Vuyst &Vancanneyt, 2007).

This is well illustrated in a more recent study of spontaneously fermented wheat sourdoughs from two regions of Greece, where a total of 136 lactic acid bacteria strains were isolated. *Lactobacillus sanfranciscensis* were dominant in the sourdoughs from Thessaly and *Lb. plantarum* sub spp. *Plantarum* in the sourdoughs from Peloponnesus. The latter was accompanied by *Pediococcus pentosaceus* as secondary microbiota. In this case, none of the lactic acid bacteria strains isolated produced antimicrobial compounds (Paramithiotis *et al.*, 2010).

1.2 The particular roles of yeasts in sourdoughs

Several of the most important functions in bread making are fulfilled by yeast. They contribute to leavening and produce metabolites such as alcohols, esters, and carbonyl compounds which are involved in the development of the characteristic bread flavour (Corsetti *et al.*, 1998; Damiani *et al.*, 1996; Hansen & Hansen, 1994; Martinez-Anaya *et al.*, 1990a, 1990b). Furthermore, the enzymatic activities of yeasts by enzymes such as proteases, lecithinases, lipases α-glucosidase, β-fructosidase, and invertase have an influence on the dough stickiness and rheology as well as on the flavour, crust colour, crumb texture, and firmness of the bread (Antuna & Martinez-Anaya, 1993; Collar *et al.*, 1998; Meroth *et al.*, 2002).

S. cerevisiae is the species most frequently found in sourdoughs but several other yeast species may be present in these ecosystems. In early studies the amount of *S. cerevisiae* may have been overestimated due to the lack of reliable systems for identifying and classifying yeasts from this habitat (Vogel, 1997).To study the sourdough yeast microbiota traditional cultivation methods in combination with phenotypic (physiological and biochemical) and/or genotypic (randomly amplified polymorphic DNA [RAPD]-PCR and restriction fragment length polymorphism [RFLP] analysis) identification methods have commonly been used (Corsetti *et al.*, 2001; Galli *et al.*, 1998; Mäntynen *et al.*, 1999; Paramithiotis *et al.*, 2000; Rocha & Malcata, 1999). These studies focused mainly on the characterization of ripe doughs and revealed the presence of 23 yeast species belonging especially to the genera *Saccharomyces* and *Candida* (Brandt, 2001; Ottogalli, 1996; Rossi, 1996). In particular *S. exiguus* (imperfect state *Torulopsisholmii* or *Candida holmii*, physiologically similar to *C. milleri*), and *C. krusei*, *Pichia norvegensis* and *P. anomala* are yeasts associated with LAB in sourdoughs. The LAB/yeast ratio in sourdoughs is generally 100:1 (Gobbetti *et al.*, 1994a). Like other fermented foods produced by mixed microflora, the organoleptic, health and nutritional properties of baked sourdough goods depend on the cooperative activity of LAB and yeasts (Gobbetti, 1998)**.** No data are available on the competitiveness of yeasts; thus, the effects of ecological factors and process conditions on the development of yeast biota during sourdough fermentation processes are virtually unknown **(**Meroth *et al.*, 2003a**).** In a simulation of the complex natural sourdough ecosystem, the competition for substrates was studied in model co-cultures showing that yeasts only partially compete with the LAB for the nitrogen sources present and synthesize and excrete essential and stimulatory amino acids which enhance the cell yield of the LAB (Gobetti *et al.*, 1994a). These findings contribute to the interpretation of some of the complex interactions which occur during sourdough leavening and which are difficult to understand because of the extensive proteolytic activity that occurs in sourdough (Spicher & Nierle, 1984).

Several recent studies have given emphasis to the yeast microbiota associated with spontaneous sourdough fermentations (Paramithiotis *et al.*, 2010; Valmorri *et al.*, 2010; Vrancken *et al.*, 2010). A total of 167 yeast and 136 lactic acid bacteria strains were isolated from spontaneously fermented wheat sourdoughs from two regions of Greece, namely Thessaly and Peloponnesus. Identification of the isolates exhibited dominance of *Torulaspora delbrueckii* with sporadic presence of *S .cerevisiae* (Paramithiotis *et al.*, 2010)**.** In another study, conducted in 20 sourdoughs collected from central Italy, PCR-RFLP analysis identified 85% of the isolates as *S. cerevisiae*, with the other dominant species being *C. milleri* (11%), *C. krusei* (2.5%), and *T. delbrueckii* (1%). RAPD-PCR analysis performed with primers M13 and LA1, highlighted intraspecific polymorphism among the *S. cerevisiae* strains. The diversity of the sourdoughs from the Abruzzo region is reflected in the chemical composition, yeast species,

and strain polymorphism (Valmorri *et al.*, 2010). In contrast with the Greek study, the high presence of *S. cerevisiae* had already been reported in Italian sourdoughs by other authors (Corsetti *et al.*, 2001; Gobbetti *et al.*, 1994b; Iacumin *et al.*, 2009; Pulvirenti *et al.*, 2004; Succi *et al.*, 2003). The current general opinion is that cross contamination from bakery equipment and working environment by baker's yeast is commonly associated with the presence of *S. cerevisiae* in sourdoughs. The other species detected (*Candida milleri, C. krusei* and *Torulaspora delbrueckii*) are typically associated with sourdoughs (Corsetti *et al.*, 2001; Garofalo *et al.*, 2008; Gobbetti *et al.*, 1994b; Halm *et al.*, 1993; Iacumin *et al.*, 2009; Obiri-Danso, 1994; Ottogalli *et al.*, 1996; Pulvirenti *et al.*, 2004; Rossi, 1996; Succi *et al.*, 2003; Sugihara *et al.*, 1971; Vernocchi *et al.*, 2004a, 2004b).

In rural areas in the north of Portugal a corn and rye bread is still prepared using a piece of dough usually kept in cool places, covered with a layer of salt. Prior to bread making this piece of dough is mixed with fresh flour and water and, when fully developed, serves as the inoculum for the bread dough. This starter dough is a natural biological system characterized by the presence of yeast and lactic acid bacteria living in complex associations in a system somewhat similar to that existing in sourdough. In a survey carried in 33 dough samples from farms mainly located in the north of Portugal, 73 yeast isolates were obtained belonging to eight different species. The predominant species was *S. cerevisiae* but other yeasts also occurred frequently, among which *Issachenkia orientalis, Pichia membranaefaciens* and *Torulaspora delbrueckii* were the most abundant, being present in about 40% of the doughs examined. Only six of the doughs contained a single yeast species. Associations of two species were found in 48% of the bread doughs, 30% presented three different species and the remainder consisted of a mixture of four yeast species. Associations of *S. cerevisiae* and *T. delbrueckii, I. orientalis* and/ or *P. membranaefaciens* were the most frequent. All mixed populations included at least one fermentative species with the exception of the association between *P. anomala*, *P. membranaefaciens*and *I. orientalis,* which was found in one of the doughs (Almeida & Pais, 1996). Apparently this dough is somehow similar to the San Francisco sour dough in which maltose-negative *S. exiguus* is predominantly found and the fermentation may be carried out by lactic acid bacteria (Sugihara *et al.*, 1971). In another Portuguese study in which, besides sourdough, maize and rye flour were examined the most frequently isolated yeasts were *S. cerevisiae* and *C. pelliculosa* (Rocha *et al.*, 1999).

In conclusion, yeasts and lactic acid bacteria (LAB) are often encountered together in the fermentation of wheat and rye sourdough breads. To optimize control of the fermentation, there has been an increased interest in understanding the interactions that occur between the LAB and yeasts in the complex biological ecosystem of sourdough.

2. Sustainability: *the old made new*

Sustainability aims is common sense operating at a global scale: using simple ideas, mixing with old procedures and new materials, adding inventive solutions, generating innovation. In the baking market, in particular in the baking industry, there is considerable space for improvement. The present procedure of bread making in developed countries consists of using block or granular baker's yeast identically produced all around the world. Although the flour types, geographical origin and mixtures introduce organoleptic diversity in bread, the leavening is a crucial step in order to achieve the traditional specific flavours and textures of each population and region. The old procedure above mentioned, of using the

old leaven to the next leavening step, was lost as baking became progressively an industrialized process. Producing and conserving large amounts of yeast though requires energy wasting biotechnological plants and expensive technical support, favouring the standardization and centralization of production. Additionally, the conservation processes involving freezing temperatures seriously compromise *S. cerevisiae* viability but also its desirable leavening ability and organoleptic properties.

2.1 Frozen yeast and frozen dough

2.1.1 Yeast response to cold

All living organisms, from prokaryotes to plants and higher eukaryotes are exposed to environmental changes. Cellular organisms require specific conditions for optimal growth and function. Growth is considered optimal when it allows fast multiplication of the cells, and the preservation of a favourable cell/organism internal composition, *i.e*., homeostasis. Therefore, any circumstance that provokes unbalance in a previous homeostatic condition may generally be considered stressful, as is the case of sudden changes in the external environment. These generally cause disturbances in the metabolism/regulation of the cells, tissues or organs, eventually disrupting their functions and preventing growth. Cellular organisms have to face this constant challenge and, therefore, rapidly adapt to the surroundings, adjusting their internal milieu to operate under the new situation. For this purpose, uncountable strategies have been developed to sustain the homeostasis. Whereas, multicellular organisms can make use of specialized organs and tissues to provide a relatively stable and homogenous internal environment, unicellular organisms have built up independent mechanisms in order to adjust to drastic environmental changes. Several approaches have been described for the most diverse microbes, from bacteria to fungi, involving responses at the level of gene expression as well as metabolism adaptation by faster processes like protein processing, targeting and inactivation, or iRNA interference (Hansen *et al*., 2005), just to mention the more general processes.

Yeasts in particular, in their natural habitat can be found living in numerous, miscellaneous and changeable environments, since they can live as saprophytes on, either plants, or animals. As examples we can name fruits and flowers, humans, animals, etc. Likewise, in their substrates, yeasts are also exposed to highly variable milieus. On such diverse ambiences, it can be expected that yeasts regularly withstand fluctuations in the types and quantities of available nutrients, acidity and osmolarity, as well as temperature of their environment. In fact, the most limiting factors cells have to cope are the low water activity (a_w), *i.e.* availability of water, and temperature. Being yeast unicellular organisms, cell wall and plasma membrane are the first barriers to defeat environment and its alterations. Both changes on the water content and temperature lead to physical and functional modifications on plasma membrane, altering its permeability that are on the basis of cell lyses and ultimately cell death (reviewed by D'Amico *et al*., 2006, Simonin *et al*., 2007).

Actually, the variations on the permeability of plasma membrane, attributed to transitions of the phospholipid phase in the membranes (Laroche and Gervais, 2003; Leslie *et al*., 1994), are associated to loss of viability during dehydration/rehydration stress (Laroche and Gervais, 2003; Simonin *et al*., 2007). In a physical perspective, membrane phospholipid bilayers, under an optimum temperature level and favourable availability of water, are supposedly in

a fluid lamellar liquid-crystalline phase. When temperature levels drop or under any other cause of dehydration, such organization suffers alterations as the hydrophilic polar head groups of phospholipids compulsorily gather. This phenomenon leads to the loss liquid-crystalline regular phase and conversion into a gel phase and consequent reduction of membrane fluidity (D'Amico *et al.*, 2006, Simonin *et al.*, 2007; Aguilera *et al.*, 2007).

Still, a decline in temperature has other effects besides the reduction in membrane fluidity. Aside with the alterations on plasma membrane permeability (primarily but on the other physiological membranes as well) and hence changes on the transport of nutrients and waste products, occurs the formation of intracellular ice crystals, which damage all cellular organelles and importantly reduces the a_w, under near-freeze temperatures. Furthermore, it has been evoked that temperature downshifts can cause profound alterations on protein biosynthesis, alterations in molecular topology or modifications in enzyme kinetics (Aguilera *et al.*, 2007). Other crucial biological activities involving nucleic acids, such as DNA replication, transcription and translation can also suffer from exposure to low temperatures. This happens through the formation and stabilization of RNA and RNA secondary or super-coiled structures (D'Amico *et al.*, 2006, Simonin *et al.*, 2007; Aguilera *et al.*, 2007). In turn, the stabilization of secondary structures of RNAs takes place for instance at the level of the inhibition of the expression of several genes, that would be unfavourable for cell growth at low temperatures (Phadtare and Severinov, 2010). The latter occurs since the transcription of the mentioned genes is impaired, as well as the RNA degradation becomes ineffective (Phadtare and Severinov, 2010).

The adaptive response to temperature downshifts, commonly referred to as the cold-shock response, in yeast as in most organisms comprises orchestrated adjustments on the lipid composition of membranes, and on the transcriptional and translational machinery, including protein folding. These adjustments are mostly elicited by a drastic variation in the gene expression program (reviewed by Aguilera *et al.*, 2007; Simonin *et al.*, 2007). Still, some authors name cold-shock response to temperature falls in the region of 18- 10°C and near-freezing response to downshifts below 10°C. In fact, yeast cells appear to initiate quite different responses to one or another situation, which can be rationalized since yeast can actively grow at 10–18 °C, but growth tends to stop at lower temperatures (reviewed by Al-Fageeh and Smales, 2006) To withdraw misinterpretations we will focus mainly in low/near-freezing temperatures, which is also a cold response. Some works on genome-wide expression analysis have explored the genetic response of *S. cerevisiae* to temperature downshifts (Zhang *et al.*, 2001; Rodrigues-Vargas *et al.*, 2002; Sahara et al 2002; Murata *et al.*, 2006). In *S. cerevisiae* exposed to low temperature, 4°C, together with the enhanced expression of the general stress response genes, other groups of genes were induced as well. These include genes involved in trehalose and glycogen synthesis (*TPS1, GDB1, GAC1*, etc.), which may suggest that biosynthesis and accumulation of those reserve carbohydrates are necessary for cold tolerance and energy preservation. Genes implicated on phospholipids biosynthesis (*INO1, OPI3,* etc.), seripauperin proteins (*PAU1, PAU2, PAU4, PAU5, PAU6* and *PAU7*), and cold shock proteins (*TIP1, TIR1,* etc.) displayed as well increased expression, which is consistent with membrane maintenance and increased permeability of the cell wall. Conversely, the observed induction of Heat Shock genes (*HSP12, HSP104, SSA4* etc,) can possibly be linked with the demand of enzyme activity revitalization, and the induction of glutathione related genes (*TTR1, GTT1, GPX1,* etc.) required for the detoxification of active oxygen species. On the other hand, it is also described the down-

regulation of some genes, like the ones associated with protein synthesis (*RPL3, RPS3*, etc.), reflecting the reduction of cell growth, which in turn may be a sign of a preparation for the following adjustment to the novel conditions (Fig. 1). A rationalization of all the data from genome-wide expression analysis and also from the numerous works on yeast cold response developed on the last years, led to the idea that there are two separated responses to temperature downshifts (reviewed by Aguilera *et al.*, 2007; Al-Fageeh and Smales, 2006). One is a general response, which involves certain clusters of genes. These include members of the *DAN/TIR* family encoding putative cell-wall mannoproteins, temperature shock inducible genes (*TIR1/SRP1, TIR2* and *TIR4*) and seripauperins family, which have some phospholipids interacting activity. The other is a time dependent separated response, meaning that the transcriptional profile changes are divided in a time succession (reviewed by Aguilera *et al.*, 2007; Al-Fageeh and Smales, 2006). For instance, within the first two hours would be observed an over-expression of genes involved in phospholipid synthesis, (like *INO1*, *OPI3*, etc) in fatty-acid desaturation (*OLE1*), genes related to transcription, including (9pt)

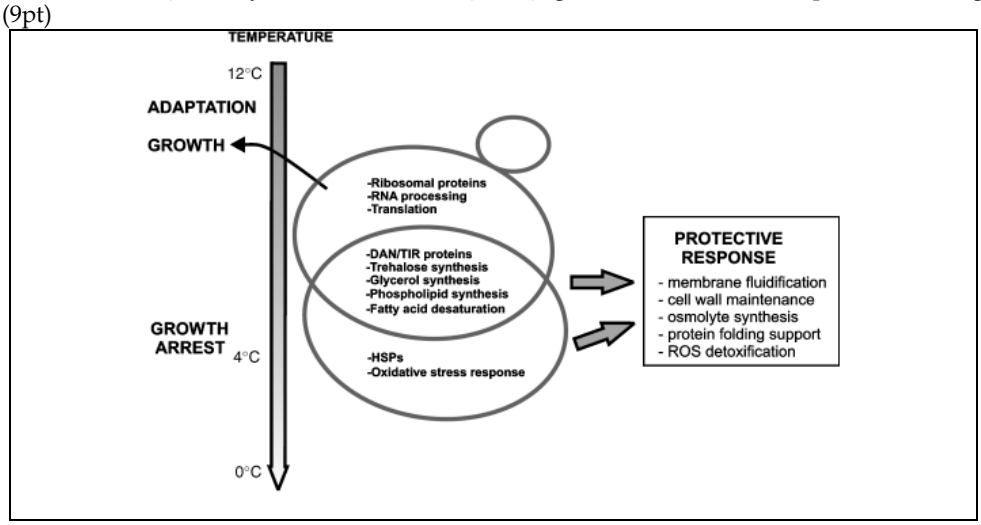

Fig. 1. *S. cerevisiae* major response to a temperature downshift (Adapted from Aguilera et al, 2007)

RNA helicases, polymerase subunits and processing proteins, and also some ribosomal protein genes. Whereas, in a second stage the latter genes (transcription related ones) are silenced and is promoted the induction of another set of genes, such as some of the heat shock protein (*HSP*) genes, also of genes associated with the accumulation of glycogen (*GLG1, GSY1, GLC3, GAC1, GPH1* and *GDB1*) and trehalose (*TPS1, TPS2* and *TSL1*), of genes in charge of the detoxification of reactive oxygen species (ROS) and defence against oxidative stress (including catalase, *CTT1*; glutaredoxin, *TTR1*, thioredoxin, *PRX1*, and glutathione transferase, *GTT2*).

2.2.2 Improving baker's yeast frozen dough performance

Preservation by low temperatures is widely accepted as a suitable method for long-term storage of various types of cells. Specially, freezing has become an important mean of preservation and storage of strains used for many types of industrial and food processing, such as those used in the production of wine, cheese and bread. Bread, in particular, is a central dietary product in most countries of the world, and presently frozen dough technology is extensively used in the baking industry. Yet, the loss of leavening ability, of organoleptic properties, but mainly the lost of viability of the yeasts after thawing the frozen dough, is a problem that persists nowadays.

In-depth knowledge concerning yeast genetics, physiology, and biochemistry as well as engineering and fermentation technologies has accumulated over the time, and naturally, there have been several attempts to improve freeze-thaw stress tolerance in *S. cerevisiae*. A recent work, described that genes associated with the homeostasis of metal ions were upregulated after freezing/thawing process and that mutants in some of these genes, as *MAC1* and *CTR1* (involved in copper homeostasis), exhibited freeze-thaw sensitivity (Takahashi *et al.*, 2009). Furthermore, the researchers showed that cell viability after freezing/thawing process was considerably improved by supplementing the broth with copper ions. Those results suggest that insufficiency of copper ion homeostasis may be one of the causes of freeze-thaw injury; yet, these ions toxicity does not allow their easy incorporation in food products. A very promising study reported an improved freeze-resistant industrial strain, in which the aquaporin was overexpressed (Tanghe *et al.*, 2002). Nonetheless, this enhancement was not attained in larger dough preparations (under industrial conditions), wherein freezing rate is not that rapid (Tanghe *et al.*, 2004). Another recent approach addressed the impact of unsaturated fatty acids on freeze-thaw tolerance by assaying the overexpression of two different desaturases (FAD2-1 and FAD2-3) from sunflower in *S. cerevisiae*. This resulted into increased membrane fluidity, and freezing tolerance (Rodriguez-Vargas *et al.*, 2007). Also the heterologous expression of antifreeze proteins (antifreeze peptide GS-5 from the polar fish grubby sculpin (*Myxocephalusaenaeus*) was tested in an industrial yeast strain, leading to both improved viability and enhanced gas production in the frozen dough (Panadero *et al.*, 2005). A very current study confirmed the role of hydrophilins in yeast dehydration stress tolerance yeast cells, since overexpression of *YJL144W* and *YMR175W* (*SIP18*) become yeast more desiccation and freezing tolerant (Dang and Hincha, 2011). An alternative work, showed improved freezing resistance by expressing of *AZI1* (Azelaic acid induced 1) from *Arabidopsis thaliana* in *S. cerevisiae* (Xu *et al.*, 2011). Other approaches devoid of genetic engineering were also taken. Cells were cultured in diverse conditions, including media with high concentration of trehalose or glycerol (Hirasawa *et al.*, 2001; Izawa *et al.*, 2004a); with poly-γ-glutamate (Yokoigawa *et al.* 2006), and with soy peptides (Izawa *et al.*, 2007) acquiring improved tolerance to freeze–thaw stress and also retaining high leavening ability.

The benefits of cryoprotectants, substances that promote the excretion of water, decreasing that way the formation of ice crystals that happens during the freezing process, were also addressed. These include Me_2SO, (Momose *et al.*, 2010); proline (Terao *et al.*, 2003; Kaino *et al.*, 2008) and charged aminoacids as arginine and glutamate (Shima *et al.*, 2003); trehalose (Hino *et al.*, 1990; Kandor *et al.*, 2004) as well as glycerol (Izawa 2004a,b; Tulha *et al.*, 2010). A comparative analysis of yeast transcriptional responses to Me_2SO and trehalose, revealed that exposure to cryoprotectants prior to freezing not only reduce the freeze-thaw damage but also provide various process to the recovery from freeze-thaw injury (Momose *et al.*,

2010). Yet, the use of Me_2SO in food preparation is not possible due to its toxicity. Intracellular proline accumulation was found to enhance freeze-thaw tolerance, thus several engineering strains emerged, overexpressing glutamyl metabolic related enzymes *PRO1* and *PRO2* or specific alleles (Terao *et al.*, 2003), and self-cloned strains in which *PRO1* specific alleles combined with disruption of prolineoxidase *PUT1* (Kaino *et al.*, 2008). Moreover, it was shown that an arginase mutant (disrupted on *CAR1* gene) accumulates high levels of arginine and/or glutamate (depending on the cultivation conditions), with increased viability and leavening ability during the freeze-thaw process (Shima *et al.*, 2003).

Trehalose and glycerol are not only cryoprotectants but also confer resistance to osmotic stress. A correlation between the intracellular trehalose content and freeze–thaw stress tolerance in *S cerevisiae* was described (Hino *et al.*, 1990; Kandor *et al.*, 2004). The same correlation has been described for glycerol (Izawa *et al.*, 2004a,b; Panadero *et al.*, 2006; Tulha *et al.*, 2010). Furthermore, it has been reported that, beyond the cryoprotection, an increased level of intracellular glycerol has several benefits for the shelf life of wet yeast products and for the leavening activity (Myers *et al.* 1998; Hirasawa and Yokoigawa 2001; Izawa *et al.*, 2004a) and no effect on final bread quality in terms of flavor, color, and texture (Myers *et al.* 1998).

2.2.3. Role of glycerol for the baker's yeast frozen dough

S. cerevisiae accumulates intracellular glycerol as an osmolyte through the high osmolarity glycerol signaling pathway (HOG pathway) under osmotic stress but also under temperature (high and low) stress (Siderius *et al.* , 2000; Hayashi and Maeda, 2006; Panadero *et al.*, 2006, Ferreira & Lucas, 2007; Tulha *et al.*, 2010). Moreover it was reported that a pre-treatment of yeast cells with osmotic stress was an effective way to acquire freeze tolerance probably due to the intracellular glycerol accumulation attained. Some engineering approaches were performed in order to increase the intracellular glycerol accumulation in baker's yeast. For instance, Izawa and co-authors showed that the quadruple mutant on the glycerol dehydrogenase genes (*ara1Δgcy1Δgre3Δypr1Δ*), responsible for the alternative pathway of glycerol dissimilation (Fig. 1) has an increased level of intracellular glycerol with concomitant to freeze-thaw stress resistance (Izawa *et al.* 2004a). Similarly, the overexpression of the isogenes *GPD1* and *GPD2* that encode for glycerol-3-phosphate dehydrogenase (Fig. 2) (Ansell *et al.*, 1997), also lead to an increase in intracellular glycerol levels (Michnick *et al.* 1997; Remize *et al.* 1999), and probably improved freeze-thaw tolerance. One of the most promising genetic modifications was the deletion of *FPS1* encoding the yeast glycerol channel. Fps1p channel opens/closes, regulating extrusion and retention of massive amounts of glycerol in response to osmotic hyper- or hipo-osmotic shock (Luyten *et al.*, 1995; Tamás *et al.*, 1999). The engineered cells deleted on *FPS1*, showed an increased intracellular glycerol accumulation accompanied by higher survival after 7 days at -20°C (Izawa *et al.*, 2004b). Yet the dynamics of the channel under this type of stress remains unexplored. The mentioned study, was considered quite innovative, it was even suggested the possibility that the *fps1Δ* mutant strain could be applicable to frozen dough technology. This because the *fps1Δ* mutant strain displayed the higher intracellular glycerol content attained so far, and (similarly to the previous engineered strains) avoided the exogenous supply of glycerol into the culture medium, which was at the time too expensive for using at an industrial scale. Our group has recently described a simple recipe with high biotechnological potential (Tulha *et al.*, 2010), which also avoids the use of transgenic strains.

We found that yeast cells grown on glycerol based medium and subjected to freeze-thaw stress, displayed an extremely high expression of theglycerol/H+ symporter, Stl1p (Ferreira *et al.*, 2005), also visible at activity level. This permease plays an important role on the fast accumulation of glycerol; under those conditions, the strains accumulated more than 400 mM glycerol (whereas the mutant *stl1Δ* presented less than 1 mM) and survive 25-50% more. Therefore, any *S. cerevisiae* strain already in use can become more resistant to cold/freeze-thaw stress just by simply adding glycerol (presently a cheap substrate) to the broth. Moreover, glycerol also improves the leavening activity and has no effect on final bread quality in terms of flavor, color, and texture (Izawa 2004a, Myers *et al.* 1998).

3. Low-cost yeasts, a new possibility

The industrial production of baker's yeast is carried out in large fermentors with working volumes up to 200.000 l, using cane or sugar beet molasses as carbon source. These are rich but expensive substrates. Quite the opposite, glycerol, once a high value product, is fast becoming a waste product due to worldwide large surplus from biofuels industry, with disposal costs associated (Yazdani & Gonzales, 2007). It represents approximately 10% of the fatty acid/biodiesel conversion yield. Due to its chemical versatility, glycerol has countless applications. Yet, new applications have to be found to cope with the amounts presently produced. This underlies the global interest for glycerol, which became an attractive cheap substrate for microbial fermentation processes (Chatzifragkou *et al.*, 2011).

3.1 Metabolism of glycerol in yeasts

A significant number of bacteria are able to grow anaerobically on glycerol (for a review see da Silva *et al.*, 2009). In the case of yeasts, most of the known species can grow on glycerol (Barnett *et al.*, 2000), but this is achieved under aerobic conditions. *S. cerevisiae* is a poor glycerol consumer, presenting only residual growth on mineral medium with glycerol as sole carbon and energy source. In order to obtain significant growth on glycerol synthetic medium a starter of 0.2% (w/v) glucose is needed (Sutherland *et al.*, 1997). Yet glycerol is a very important metabolite in yeasts, including *S. cerevisiae*, namely because its pathway is central for bulk cell redox balance, because it couples the cytosolic potential with mitochondria's, and because it is the only osmolite known to yeasts, in which accumulation cells depend for survival under high sugar, high salt (Hohmann, 2009), high and low temperature (Siderius *et al.*, 2000), anaerobiosis and oxidative stress (Påhlman *et al.*, 2001).

Recently, it was suggested that *S. cerevisiae* glycerol poor consumption yields could be due to a limited availability of energy for gluconeogenesis, and biomass synthesis (Zhang *et al.*, 2009). Nevertheless, the weak growth performances have long been attributed to a redox unbalance caused by the intersection of glycerol pathway with glycolysis at the level of glycerol-P shuttle (Fig. 2) (Larsson *et al.*, 1998). Fermenting cultures of *S. cerevisiae* produce glycerol to reoxidize the excess NADH generated during biosynthesis of aminoacids and organic acids, since mitochondrial activity is limited by oxygen availability and ethanol production is a redox neutral process (van Dijken & Scheffers, 1986) (Fig. 2). This is the reason why glycerol is a major by-product in ethanol and wine production processes. Consistently, the mutant defective in the above mentioned isogenes encoding the glycerol 3P dehydrogenases (Δ*gpd1*Δ*gpd2)* are not able to grow anaerobically (Ansell *et al.*, 1997;

Påhlman *et al.*, 2001). This ability was restored supplementing the medium with acetic acid as electron acceptor (Guadalupe Medina *et al.*, 2010).
(9pt)

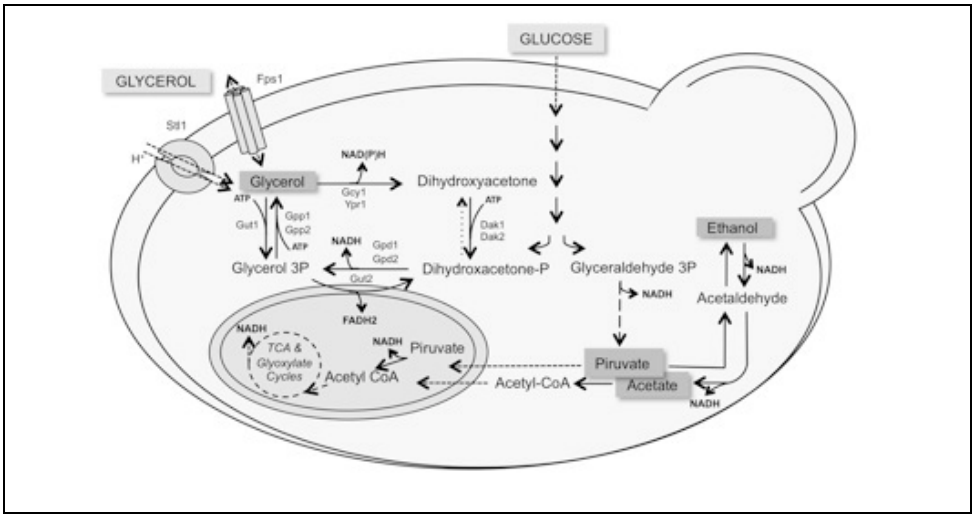

Fig. 2. *Preencher pf Dra Candida*

S. cerevisiae takes up glycerol through the two transport systems above mentioned, the Fps1 channel and the Stl1 glycerol/H^+ symporter (Ferreira *et al.*, 2005). Fps1 is expressed constitutively (Luyten *et al.*, 1995; Tamás *et al.*, 1999), while *STL1* is complexly regulated by a number of conditions (Ferreira *et al.*, 2005; Rep *et al.*, 2000). It is derepressed by starvation, and inducible by transition from fermentative to respiratory metabolism, as happens during diauxic shift at the end of exponential growth on rich carbon sources. Additionally, it is also the most expressed gene under hyper-osmotic stress (Rep *et al.*, 2000), and highly expressed at high temperature, overcoming glucose repression (Ferreira & Lucas, 2007). In yeasts glycerol can be consumed through two alternative pathways (Fig. 2), the most important of which involving the glycerol 3-P shuttle above mentioned, directing glycerol to dihydroxyacetone-P through respiration and mitochondria. According to very disperse literature, other yeasts, better glycerol consumers than *S. cerevisiae*, appear to have equivalent pathways, though they should differ substantially in the underlying regulation to justify the better performance. At the level of transcription, significant ability to consume glycerol depends on the constitutive expression of active transport (Lages et al, 1999). Possibly, unlike in *S. cerevisiae* where *GUT1* is under glucose repression (Rønnow & Kielland-Brandt, 1993; Grauslund *et al.*, 1999), glycerol consumption enzymes could be identically expressed. This should be in accordance with the yeasts respiratory/fermentative ability. Related or not, *S. cerevisiae* respiratory chain differs from a series of other yeasts classified as respiratory, which are resistant to cyanide (CRR - Cyanide resistant respiration) (Veiga *et al.*, 2003). Cyanide acts at the level of Cytochrome Oxidase complexes. CRR owes its resistance to an alternative oxidase (AOX) that short-circuits the main respiratory chain, driving electrons directly from ubiquinone to oxygen, bypassing complex III and IV.

Although exhaustive data are not available, CRR appears to occur quite frequently in yeasts that are Crabtree[1] negative or simply incapable of aerobic fermentation (Veiga *et al.* 2003), all of which are good glycerol consumers (Lages et al, 1999; Barnett *et al.*, 2000). Interestingly, CRR may not be constant, occurring only under specific physiological conditions like diauxic shift, in *P. membranifaciens* and *Y. lipolytica*, or early exponential phase, in *D. hanseni* (Veiga et al. 2003). In *S. cerevisiae*, both conditions highly and transiently induce the glycerol transporter *STL1* expression (Ferreira *et al.*, 2005; Rep *et al.*, 2000).

Baker's yeast is a Crabtree[1] positive yeast. Fermentation begins instantly when a glucose pulse is added to glucose-limited, aerobically grown cells. Crabtree has been seldom addressed in the last two decades, although it is still a recognized important variable in industrial processes (Ochoa-Estopier *et al.*, 2011). The molecular regulation and main players of this process remain obscure. A relation of Crabtree effect with respiration was discarded (van Urk *et al.*, 1990). Instead, the piruvate decarboxilase levels were found to be 6 times higher in the Crabtree positive yeasts *S. cerevisiae, T. glabrata* (today *C. glabrata*) and *S. pombe*. This presented an increased glucose consumption rate that the authors attributed to glucose uptake (van Urk *et al.*, 1990) that did not correspond to equivalent growth improvement, but instead to ethanol production through fermentation. Concurrently, growth on glycerol is supposedly entirely oxidative (Gancedo *et al.*, 1968; Flores *et al.*, 2000), which underlies the good and bad performance of respectively Crabtree negative and positive yeasts. In order to turn glycerol broths commercially attractive for *S. cerevisiae*-based biotechnology, in particular baker's yeast cultivation, several approaches were assayed.

One of the most straightforward strategies is metabolic engineering, obtained through genetic manipulation (Randez-Gil *et al.*, 1999). However, this need precise knowledge on the strain/species genome, available molecular tools (mutants and vectors to the least), and deep knowledge of the metabolic process involved, which are not always available. Additionally, cellular processes are hardly under the control of a single gene and simply regulated. Because of this, available molecular and informatics tools are combined for engineering industrial strains of interest (Patnaik, 2008). In the particular case of baker's yeast, the industrial strains are mostly aneuploids and homothallic, impairing easy genetic improvement (Randez-Gil *et al.*, 1999). In view of the complexity above, and these genetic characteristics, the improvement of glycerol consumption by baker's yeast is hardly possible to obtain by genetic engineering. All this, and the general skepticism of consumers towards the use of genetically modified organisms in the food industry, led to the search of alternative strategies for the baking industry.

3.2. Improbable hybrids

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[1] Crabtree effect is the phenomenon whereby *S. cerevisiae* produces ethanol aerobically in the presence of high external glucose concentrations. Crabtree negative yeasts instead produce biomass via the tricarboxylic acid cycle. In *S. cerevisiae*, high concentrations of glucose accelerate glycolysis, producing appreciable amounts of ATP through substrate-level phosphorylation. This reduces the need of oxidative phosphorylation done by the TCA cycle via the electron transport chain, inhibits respiration and ATP synthesis, and therefore decreases oxygen consumption.

The traditional way of producing new strains is through the generation of hybrids through mating. This approach allows the indirect *in vivo* genetic recombination and the propagation of phenotypes of interest. It can be achieved through intra- or inter-specific hybridization. The most resourceful way is the intra-specific recombination of strains with desirable phenotypes. To achieve this, it is necessary to induce sporulation of the target diploid strains, usually through nitrogen starvation. The haploid ascospores are then isolated and their mating type determined, followed by the mating of ascospores from opposite mating type, and the formation of a new heterozygous diploid. Several wine and baker's yeast strains available commercially are the result of such hybridization (Higgins *et al.*, 2001; Pretorius & Bauer, 2002; Marullo *et al.*, 2006).

All of these strategies demand for a deep knowledge of the phenotypes and the underlying metabolic and molecular processes. As an example, Higgins and collaborators (2001) generated a *S. cerevisiae* strain able to combine efficient maltose metabolism, indispensable for fermentative ability of unsugared dough's, with hyperosmotic resistance for optimization of growth on sugared dough's. Loading *S. cerevisiae* with glycerol has been shown to improve the fermentation of sweet doughs (Myers *et al.*, 1998), therefore the selection for osmotolerant phenotype. On the other hand, unlagged growth on maltose is due to the constitutive derepression of maltase and maltose permease (Higgins *et al.*, 1999), as well as of invertase (Myers *et al.*, 1997), but this was previously reported to negatively influence the leavening of sweet doughs (Oda *et al.*, 1990). This difficulty was overcome by the use of massive random mating upon sporulation enrichment, yielding approximately 10% of interesting isolates for further detailed screening (Higgins *et al.*, 2001).

In the particular case of baker's yeast, the sporulation ability of industrial strains is extremely reduced, and most strains are homothallic yielding random-mating spores. This is due to their frequent aneuploidy and the consequent heterogenous coupling of their chromosomes during meiosis. This raises the need of using *asexual* approaches, as spheroplast fusion or cell-spore mating (Sauer, 2001), as well as other mass mating strategies that may circumvent the inability to mate isolated spores (Higghins *et al.*, 2001). In spheroplast fusion, after appropriate cell wall digestion, it is possible to force the fusion of cells with different levels of ploidy. These are though in many cases phenotypically and reproductively unstable and non-resilient multinuclear cells unfit for industry.

3.3 Evolutionary engineering

The alternative solution to extensive and expensive genetic manipulation is evolutionary engineering (Chatterjee & Yuan, 2006, Fong, 2010). This strategy allows the improvement of complex phenotypes of interest, for example stress resistance combined with carbon source utilization. The methodology is based in the combination of confined environmental selection and natural variability. It was first used in a work of Butler and co-workers, who selected different genetic strains of *Streptomyces griseus* under defined and selective conditions (Butler *et al.*, 1996). Evolutionary engineering aims the creation of an improved strain based in selection of behavioral differences between individual cells within a population. For this reason, the generation of genetic variability is vital to this approach, although the adaptive confined evolution based on spontaneous mutations may prove to be useful, using extremely prolonged cultivation under selective conditions (Aguillera *et al.*, 2010, Oliveira F., Ferreira C. &Lucas C. unpublished results).

One of the simplest ways of generating variability within a population is the introduction of random genetic mutations. Within a population, there is naturally occurring mutagenesis, either through local changes in the genome or larger modifications like DNA rearrangements and horizontal transfers (Sauer, 2001). Nevertheless, spontaneous mutations occur at very low rate, mainly due to the DNA proof-reading mechanism of the organisms and high fidelity of the DNA polymerases. However, it is known that under adverse conditions the mutation rate is enhanced. This feature is crucial to increase the genetic variability within the population to a level propitious to adaptation to challenging environmental constraints. This selection through survival is the basic principle behind the evolutionary engineering.

Several methodologies are available for the generation of variability, namely physical or chemical mutagenesis, sporulation followed by mating, spheroplast fusion, whole genome shuffling (Petri & Schmidt-Dannert, 2004), and so on (Fong, 2010). Mutagenesis is the most common practice, being technically simple and applicable to most organisms (Fong, 2010). The most common mutagens are either chemicals, like ethyl methane sulfonate (EMS), ethidium bromide (EB), or radiation, namely ultraviolet (UV). These mutagens are rather unspecific, and for this reason are widely used (Sauer, 2001). The main drawback of such approaches is low rate of useful mutations, and the high rate of lethal and neutral mutations. Most chemicals, like EB, introduce preferentially alterations to the DNA like nucleotide exchanges or frame shifts, but other like EMS can induce deletions (Nair & Zhao, 2010). These are responsible for important DNA rearrangements and severe phenotypic alterations. Yet, some chemicals have affinity for certain genome sub-regions, and its utilization in sequential rounds of mutation/selection can be rather reductive. Physical mutagens, namely UV radiation and X-rays, are more prone to chromosomal structural changes and nucleotide frame shifts.

The simplest method to ameliorate a baker's yeast strain relies, as mentioned above, on spontaneous mutations and prolonged cultivations. This strategy has the advantage of doing without the manipulation of dangerous chemicals or radiation, and the disadvantage of the long time needed to obtain results. These batch or fed-batch cultivations have to cover more than 100 generations (Aguilera *et al.*, 2010, Merico *et al.*, 2010, Ochoa-Estopier *et al.*, 2011; Oliveira F., Ferreira C. & Lucas C. unpublished results) and can last for several months depending essentially on the severity of the environmental constraints. This procedure was applied with success to transform *S. cerevisiae* into a good lactose consumer (Guimarães *et al.*, 2008), a good improving its freeze and salt tolerance (Aguilera *et al.*, 2010), and turning baker's yeast into an efficient consumer of glycerol as sole carbon and energy source to industrially acceptable biomass yields (Oliveira F., Ferreira C. & Lucas C. unpublished results). This was obtained through the use of a simple evolution strategy consisting of sequential aerobic batch cultivations on synthetic glycerol-based media for 150 generations, followed by several cycles of cultivation on rich media for ensuring phenotypic/mutation stability. The resulting strains were able to grow up to 4 g biomass dry weight in 2% (w/v) raw biodiesel centrifuged glycerol[2] which corresponds to a biomass yield of 0,4 g/g^{-1}

2 Raw glycerol was diluted with water (1:3) and the pH adjusted to 4.5 with HCl. This was centrifuged at 5000 rpm for 15 min at 4°C. Fat separates from glycerol forming a upper layer

glycerol. Although yields were not significantly different from cultures from reagent grade glycerol, specific growth rates were 3 times higher in raw glycerol (μ_g 0.12 h^{-1}) and lag phase was reduced to a minimum of 2h. Merico and collaborators (2010) describe the selection and characterization of an identically evolved *S. cerevisiae* strain which, additionally, also exhibited a high resistance to freeze and thaw stress after prolonged storage at -20°C. Screening capacity can nowadays be expanded by high throughput techniques (Sauer, 2004), but more importantly, as exemplified above, there has to be prior extensive knowledge to be able to design clever phenotype selection platforms.

Alternatively, yeast species other than *S. cerevisiae*, displaying good characteristics for the baking industry can be used. For example, *Torulaspora delbrueckii* strains isolated from corn and rye traditional bread doughs display dough-raising capacities and yield production similar to the ones found in commercial baker's yeasts (Almeida & Pais, 1996 a) and maintain approximately the same leavening ability during storage of frozen doughs for 30 days, showing a very high tolerance to freezing (Almeida & Pais, 1996 b). Furthermore, in one of these strains no loss of cell viability was observed after 120 days of freezing at -20ºC, whereas a loss of 80% was observed in a commercial baker´s yeast after 15 days (Alves-Araújo et al., 2004). These characteristics make them candidates of great potential value to the baking industry, mainly to be used in frozen dough products.

4. Conclusion

Nowadays, the manmade baker's yeast strains, as well as their associated technological process particularities are fast disappearing due to globalization of yeast and dough industry. Nevertheless, sustainability demands ask for solutions empowering local populations with tools that allow their own survival. Yeasts, as always, have a role in this desired change of paradigm.

Going after the regional tastes for unique types of bread and other bakery products could improve the revenue for the local economical players. This can be achieved through the reintroduction of lost biodiversity in the leavening processes. The industrial production of such yeasts and bacteria can be done using unconventional substrates like biodiesel-derived glycerol, since most bacteria and yeast can consume this substrate naturally, in opposition to the traditional baker's yeast strains. Yet still, baker's yeast can be improved for producing interesting biomass yields at the expense of glycerol by clever and simple accelerated evolution strategies. Finally, the glycerol-based broths can by themselves improve the shelf-life span of doughs and leavens.

Biotechnology needs improvement in order to meet sustainability objectives. For this there is not a simple unique solution. Instead, sustainability can be achieved by increasing diversity of processes, tools and products, for which clever simplicity-generating solutions are needed.

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which is sucked with a vacuum pump to liberate the clean glycerol fraction. The pH adjustment increases the separation efficiency.

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