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**Enhancement of *Saccharomyces cerevisiae*  
glutathione and micronutrients content for  
nutraceutical applications**

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## *0. Introduction*

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## *0.1 Legislative framework*

Food industry is characterized by a complex legislative framework and the difficulty to move into web of rules and to differentiate between foods for particular nutritional uses, food added with vitamins and minerals and food supplements, at first glance is high.

At national level the *Decreto Ministeriale* 27 January 1992 n. 111, implementing Directive 89/398/EC, for the first time included food supplements and food added with vitamins and minerals in the area of food for particular nutritional uses. Afterwards specific rules for each of them occurred: Directive 2009/39/EC on foods for particular nutritional uses (dietary products and food for infants); Directive 2002/46/EC on food supplements (now replaced by Regulation (EC) 1170/2009) and Regulation (EC) 1925/2006 on food added with vitamins and minerals. The Regulation (EC) 1924/2006 on nutrition and health claims made on foods is placed transversely.

### *Food supplements*

The “Directive 2002/46/EC of the European Parliament and of the Council of 10 June 2002 on the approximation of the laws of Member States relating to food supplements”, implemented in Italy by *Decreto* 21 May 2004, n. 169, establishes harmonized rules for the labeling of food supplements and introduces specific rules on vitamins and minerals in food supplements. The aim is to harmonize legislation and to ensure that these products are safe and appropriately labeled so that consumers can make informed choices.

**Food supplements** are considered as concentrated sources of nutrients or other substances with a nutritional or physiological effect, whose purpose is to supplement the normal diet. They are marketed in dose form as pills, tablets, capsules, liquids in measured doses etc. An adequate and varied diet could, under normal circumstances, provide all necessary nutrients for normal development and maintenance of a healthy life. However, surveys show that this ideal situation is not being achieved for all nutrients and by all groups of the population across the Community.

Inside Annex I, the Directive indicates which vitamins or minerals are allowed to be used in the food supplements, and in Annex II in which form they may be used. Vitamins and mineral substances may be considered for inclusion in the lists following the evaluation by the European Food Safety Agency (EFSA) of an appropriate scientific dossier concerning the safety and bioavailability of the individual substance.

However, in the Directive only the rules applicable to the use of vitamins and minerals in the manufacture of food supplements are laid down. The use of substances other than vitamins or minerals in the manufacture of food supplements therefore continues



to be subject to the rules in force in each national legislation. Directorate General Health and Consumer Protection has commissioned a study on the use of these substances and taking into account all the available information, the Commission, in accordance with the requirement set out in Article 4(8) of Directive 2002/46/EC on food supplements, has prepared a report to the Council and the European Parliament on the use of substances other than vitamins and minerals in food supplements. In this report, the Commission concludes that laying down specific rules applicable to substances other than vitamins and minerals for use in food supplements is not justified ([http://ec.europa.eu/food/food/labellingnutrition/supplements/index\\_en.htm](http://ec.europa.eu/food/food/labellingnutrition/supplements/index_en.htm)).

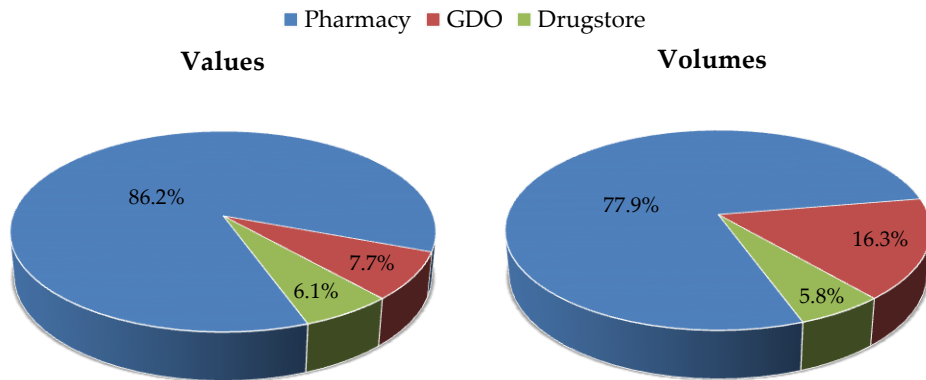
The use of vitamins and mineral supplements together with their sources, and its addition to food, is currently governed by Regulation (EC) 1170/2009 of 30 November 2009 amending Directive 2002/46/EC and Regulation (EC) 1925/2006 "regarding the lists of vitamins and minerals and their forms that can be added to food, including food supplements."

Consequently, for food supplements, Annex I and Annex II of Directive 2002/46/EC are replaced, respectively, in Annex I and Annex II of Regulation (EC) 1170/2009. Food supplements are used from people for improving the support of a normal diet; therefore vitamins, minerals or substances other than vitamins or minerals have to be present in the product in significant amount. At the same time their assumption at excessive levels may cause adverse health effects; that's why minimal and maximal levels present into food supplements have to be fixed: to ensure that the normal use of the product, in the way suggested from the producer, will be safe for costumers. The Community advice is to assume levels within safe limits (upper safe level: UL) as a general reference, taking into account the RDA (recommended dietary allowances) reported in the Annex I of the Directive 2008/100/EC.

### *Market*

The total European market in 2004 was at approximately 15 billion Euros, and the world market 45 billion Euros (revised from France Product Brief French Market for Food Supplements 2005 – Gain Report).

In Italy the food supplements market in 2008 was valued at approximately 1400 million Euros, with an 11.2% increase compared to 2007. Of these 1400 million Euros over 1200 were sold at pharmacies and 108 million at GDO (Grande Distribuzione Organizzata). Referring to the number of sold packages, 116 million of units were sold in 2008, with an 8% increase, compared to 2007. Pharmacies are the leading distributors of food supplements in Italy, followed by GDO and drugstores (Fig. 0.1).



**Figure 0.1.** Distribution of Italian food supplements market in terms of value and volumes.

Among the different channels of sale, there is variability also in the types of product chosen: most of the food supplements sold in pharmacies are probiotics, multivitamins, laxatives and saline supplements, while food supplements sold in the GDO are healthy meals and sport supplements.

The analysis of the types of supplements consumed shows that people use food supplements in order to improve psycho-physical wellness or as an answer to health problems. At the first place in the choices there are vitamins and mineral supplements followed by supplements with probiotics and energetic sport supplements containing vitamins, minerals, amino acids and proteins.

The consumption of food supplements regards 32% of people and this isn't a passing trend: 6.1% of them declare to use them from at least two years.

A FederSalus study shows that two thirds of users are women with a middle-high level of education. Doctor remains the reference point in choosing the most suitable supplements (51.7%) even if 33% people rely upon "self-made". Reported market data do not take into account sales by the e-commerce that should be quite significant: 6 million Italians use web to obtain information about health and wellness (AC/Nielsen for FederSalus, December 2010).

## 0.2 Micronutrients

The improvement of human diet has undergone a transition over the past 50 years. The first half of 20<sup>th</sup> century was focused on the discovery and characterisation of constituents indispensable of an healthy diet such as essential nutrients, vitamins, amino acids and cofactors. In the second half of 20<sup>th</sup> century, environmental factors

became important determinants of human health, and scientists began to explore the relationship between diet and disease. First, many of these new associations were related to nonessential dietary constituents, like fibre or cholesterol, then the question moved from insufficient intake of some nutrients, such as the relation of fibre with colon cancer, to the excess of some of them, like relation of fat intake and cardiovascular disease (CVD) (Caballero, 2003).

Even if clinical deficiency of micronutrients is uncommon in the developed world, suboptimal status of micronutrients such as vitamins C and E and folate, has been proposed to play a role in the development of CVD, cancer at various sites, chronic renal failure and age-related macular degeneration (Fairfield & Fletcher, 2002; Woodside et al., 2005).

Many countries have developed recommendations for intake of micronutrients in the normal diet. Values have been set for the intake of each micronutrient below which a clinical deficiency state is increasingly likely, or above which a toxicity state is likely to develop. Although these are relevant for populations, the difficulty is to determine how adequate is the intake for a particular person (Shenkin, 2006). Moreover there are a number of situations where the intake may be poor or inadequate even in healthy population. This may be due to socio-economic circumstances and so people from a poorer background may well take less fresh fruit and vegetables. It may be also related to social groups like adolescents and teenagers that have an inadequate intake of milk and other sources of calcium, or like elderly people in nursing homes and residential care that are particularly at risk for vitamins B12 and D deficiency, alcohol-dependent individuals are at risk for folate, B6, B12, and thiamin deficiency (McKay et al., 2000; Fairfield & Fletcher, 2002; Shenkin, 2006). Other groups have increased requirements: pregnant women require increased folate; smokers require additional vitamin C and people who are recovering from an acute illness or after surgery probably have multiple requirements.

Nutritional status is profoundly affected by most disease states by reduced intake, such as anorexia (consequence of chronic inflammation, acute infection or neoplastic disease), chronic alcohol misuse or during inadequate parental nutrition; increased requirements or increased losses such as blood loss, diarrhea, fistulas, dialysis (Shenkin, 2006).

Inadequate intake or subtle deficiencies in several vitamins are risk factors for chronic diseases such as cardiovascular disease, cancer, and osteoporosis; according to this information a summary of major vitamin-disease relationships are reported in Tab. 0.1 (Fairfield & Fletcher, 2002).

**Table 0.1.** Summary of cohort studies and randomized trials of major vitamin-disease relationships (Fairfield & Fletcher, 2002).

	Evidence	Summary Points
B Vitamins and CHD	Cohort studies	Low serum folate was associated with increased risk of CHD in a retrospective cohort study. <sup>2</sup> In a large prospective cohort, higher dietary intake of folate and vitamin B <sub>6</sub> was associated with decreased risk of CHD. <sup>3</sup>
	Meta-analysis	Folate lowers plasma homocysteine levels by approximately 25%, and addition of B <sub>12</sub> lowers it another 7%, but addition of B <sub>6</sub> resulted in no further reduction. <sup>4</sup>
	Additional new clinical trials of B vitamins	Several large clinical trials of folate, B <sub>6</sub> , and B <sub>12</sub> in relation to CHD are under way. <sup>5,6</sup>
Folate and colorectal cancer	Cohort studies	Separate studies of men and women using multivitamins with folate for >10-15 years observed reductions in colon cancer risk. <sup>7,8</sup> Another prospective study found colon cancer risk reductions associated with higher dietary folate in men but not women. <sup>9</sup>
Folate and breast cancer	Cohort studies	Higher folate consumption was associated with decreased breast cancer risk among women consuming alcohol regularly but not among nondrinkers in 3 cohort studies. <sup>10-12</sup>
Folate and neural tube defect	Cohort study	Women consuming a multivitamin with folate during the first 6 weeks of pregnancy were at decreased risk of having a neonate with neural tube defect. <sup>13</sup>
	Randomized trials	Folate supplementation decreased the risk of first <sup>14</sup> and recurrent <sup>15</sup> neural tube defects.
Vitamin E and CHD	Cohort studies	Vitamin E is an antioxidant and affects smooth muscle proliferation and platelet adhesion. Numerous cohort studies showed reduction in CHD risk with higher vitamin E intake. <sup>16,17</sup>
	Randomized trials	Three of 4 randomized controlled trials showed no benefit of vitamin E supplementation on CHD risk. <sup>18-21</sup> In another study, vitamin E reduced the risk of nonfatal myocardial infarctions but not cardiovascular mortality. <sup>22</sup>
Vitamin E and prostate cancer	Cohort studies	Two prospective studies showed decreased prostate cancer risk with increased vitamin E intake, particularly among smokers. <sup>23,24</sup>
	Randomized trial	In the Alpha-Tocopherol Beta-Carotene trial, $\alpha$ -tocopherol supplementation decreased prostate cancer incidence and mortality. <sup>25</sup>
Carotenoids and CHD	Cohort studies	Prospective studies have failed to show an association between carotenoid intake and CHD. <sup>26,27</sup>
	Randomized trials	Beta carotene did not reduce coronary heart disease risk in 5 primary prevention trials. <sup>28-31</sup> Two studies suggested increased mortality among smokers supplemented with beta carotene. <sup>18,19</sup>
Carotenoids and lung cancer	Cohort studies	Multiple studies showed inverse associations between beta carotene and lung cancer. <sup>32</sup> Three newer cohort studies have shown inverse associations between alpha carotene (but not beta carotene) and lung cancer. <sup>33-34</sup>
	Randomized trials	Two large randomized placebo-controlled trials found increased lung cancer risk among male smokers receiving beta carotene. <sup>25,28</sup> Three other intervention trials reported no increase in risk but included few smokers. <sup>29,30,35</sup>
Carotenoids and prostate cancer	Cohort studies	Beta carotene was not associated with prostate cancer in several prospective studies. <sup>36</sup> Several prospective studies have found decreased risk of prostate cancer associated with increased lycopene intake. <sup>36-39</sup> Serum studies have been mixed. <sup>23,40,41</sup>
	Randomized trials	One intervention study showed increased prostate cancer incidence and mortality in the group receiving beta carotene <sup>25</sup> and 2 showed no association. <sup>28,30</sup>
Vitamin D and bone mass	Cohort studies, randomized trials	Inadequate vitamin D status is a common problem. <sup>42,43</sup> Vitamin D supplementation decreases bone turnover and increases bone mineral density. <sup>42,41</sup> Supplementation with vitamin D and calcium decreases bone loss and fracture rates in elderly people. <sup>45</sup> It remains unclear whether vitamin D alone decreases fracture rates or whether supplemental calcium is required.

\*CHD indicates coronary heart disease.

### *The case of folate*

Folate is a water-soluble B vitamin found in fortified cereal grains, leafy green vegetables, legumes, various seeds, and in liver. There are two forms of folate, the naturally occurring form (folate, also used as the generalized term), and the synthetically produced form (folic acid). Bioavailability of folic acid is higher than folate because folic acid is non-conjugated and more stable than folate (Johnson et al., 2011). The polyglutamate chain of food folate must be cleaved to monoglutamate form before absorption, so bioavailability of food folate is approximately 50%. When folic

acid monoglutamate is consumed without food (as a supplement), it is nearly 100% bioavailable; when it is consumed with food (fortified food) bioavailability decreases to approximately 85%. For this reason data are often reported as Dietary Folate Equivalents (DFE), which would take the higher bioavailability of folic acid compared to food folate into account (1 DFE=1  $\mu\text{g}$  food folate =0.6  $\mu\text{g}$  folic acid added to food= 0.5  $\mu\text{g}$  folic acid taken without food) (Suitor et al., 2000).

Folate plays a critical role in DNA synthesis, methylation and repair, and an imbalance in these three functions may contribute to carcinogenesis. In particular, a deficiency in folate has been implicated in increasing the risk of pancreatic cancer due to hypomethylation of DNA. Although most studies suggest that increased intake of folate may help reducing the risk of pancreatic cancer in both men and women, a recent prospective study conducted by Oaks et al. suggests that only women benefit from higher folate intake (Johnson et al., 2011)

In humans, folate is needed as methyl-group transfer in the conversion of homocysteine to methionine. Inadequate folate intake leads to elevated homocysteine concentrations, which have been associated with an increased risk for cardiovascular diseases. Folate also supplies one-carbon units for the synthesis of deoxyribonucleic acid (DNA). Therefore, folate deficiency can cause single- and double-strand breaks in the DNA, which can contribute to increased cancer risk (Dietrich et al., 2005).

### *The case of copper*

Copper is a transition metal with three oxidation states:  $\text{Cu}^0$ ,  $\text{Cu}^{1+}$  and  $\text{Cu}^{2+}$  and the cupric one is the most common in biological systems. Transition metals are indispensable for life because of their ability to donate and accept electrons. Consequently, copper is a micronutrient that plays a pivotal role in cell physiology, serving as a cofactor for enzymes that modify neuropeptides, generate cellular energy, detoxify oxygen-derived radicals, mobilize iron, coagulate blood, and cross-link connective tissue (Linder et al., 1996; Peña et al., 1999; Lee et al., 2001).

However, these metal ions can be toxic to cells when present in excess. Again, due to the special redox chemistry of this metal ion, Cu participates in reactions that result in the production of highly reactive oxygen species (ROS) (Peña et al., 1999). In addition to the generation of ROS, Cu may manifest its toxicity by displacing other metal cofactors from their natural ligands in key cellular signaling proteins. To maintain levels of metal ions in tight homeostasis, organisms have evolved complex regulatory mechanisms that have been conserved through evolution (De Freitas et al., 2003).

The importance of maintaining this critical balance is highlighted by the existence of two human genetic diseases in Cu transport, Menkes and Wilson's diseases. The

entrapment of Cu in intestinal cells in Menkes disease patients leads to Cu deficiency as ascertained by defects in the activities of Cu containing enzymes. Patients with Wilson disease accumulate Cu in the liver, resulting in liver cirrhosis and neurodegeneration (Peña et al., 1999; Lee et al., 2001).

Cells defend themselves against oxidative damage by tightly controlling the activity of free copper, as well as by detoxifying ROS. Cu, Zn-superoxide dismutase (SOD) is a primary enzyme in the defense against oxidative stress by catalyzing the dismutation of superoxide radicals ( $O_2^-$ ) into hydrogen peroxide (Fridovich, 2001). Peroxidases such as catalase, glutathione peroxidase, and cytochrome-c oxidase help eliminate the excess of hydrogen peroxide ( $H_2O_2$ ).

In mammals, absorption of copper probably occurs primarily in the small intestine, after digestion of food in the stomach and duodenum (Linder et al., 1996; Peña et al., 1999). The efficiency of absorption of the metal ion is high; values for apparent absorption by adult humans average between 55% and 75%. Data from animal studies (mainly rats) as well as from studies of humans indicate that in the range of normal intakes there is some adaptation of absorption relative to need: higher percentages of the available copper are absorbed at lower intakes (over days and weeks) and vice versa (Linder et al., 1996).

Copper is differently distributed in the food: shellfish and organ meats are the richest source of copper, whereas muscle meats have a lower content; seeds have a high abundance of copper while fruit and vegetables tend to have less (Linder et al., 1996).

Copper deficiency is more frequent in preterm infants, especially those with very low birth weights. Copper deficiency has been reported in subjects with malabsorption syndromes, such as celiac disease, tropical and non-tropical sprue, cystic fibrosis and short bowel syndrome. The most common clinical manifestations of this deficiency are anemia, neutropenia and bone abnormalities.

### *The case of iron*

Iron (Fe) exists in two biologically relevant states: the reduced ferrous form ( $Fe^{2+}$ ) and the oxidized ferric one ( $Fe^{3+}$ ). As well as copper, iron is an efficient catalyst for electron transfer and free-radical reactions, meaning also that it is potentially toxic and organism needs to minimize its exposure.

Iron, as a component of hemoglobin in erythrocytes (red blood cells), is required for transporting oxygen around the body and, in the form of myoglobin, for oxygen storage and use in muscles.

Oxygen released in tissues from hemoglobin is used in oxidative metabolism. Hemoglobin binds carbon dioxide in tissues and carries it to lungs, where it is discarded by exhalation. Iron is also present as a component of iron-sulfur complexes, in enzymes that are responsible for electron transport and energy generation in mitochondrial respiration and the citric acid cycle, and for ribonucleotide reductase, which is essential for DNA synthesis. Body iron content is approximately 4.0 g in men and 3.5 g in women. In adults, most body iron is present in hemoglobin (60-70%), in circulating erythrocytes where it is essential for oxygen transport, and in muscle myoglobin (10%). The remaining body iron (20–30%) is found primarily in storage pools located in liver and endothelial-reticulum (macrophage) system as ferritin and hemosiderin. Only about 1% of body iron is incorporated in the range of iron-containing enzymes and less than 0.2% of body iron is in the plasma transport pool where it is bound to transferrin (Geissler & Singh, 2011).

Dietary iron as well as copper is absorbed in the upper small intestine. Dietary non-heme iron is predominantly in the ferric oxidation state and must be reduced for absorption. This may occur by cytochrome B reductase 1 (CYBRD1). After reduction, ferrous iron ( $\text{Fe}^{2+}$ ) is transported into enterocytes by DMT1, coupled to the electrochemical  $\text{H}^+$  gradient from outside to inside cells. In enterocytes, ferrous iron enters in a labile or 'exchangeable' iron pool from which it can enter three different pathways, depending on body requirements: taken into the local mitochondria for heme synthesis; sequestered into ferritin iron depots (and shed into the gut lumen at the end of the enterocyte's lifespan); or transferred to the basal transporter (ferroportin 1) for translocation into the body.

Dietary iron can also be in the heme form, obtained principally from meat sources, but the mechanism of heme iron absorption remains unclear. A recent report described the identification of an apically expressed intestinal heme transporter (heme carrier protein 1, HCP1), but current evidence suggests it functions mainly as an intestinal folate transporter. However, once in enterocytes, the heme molecule is degraded by heme oxygenase to release ferric iron which then enters the enterocytic exchangeable pool (Collins et al., 2010; Geissler & Singh, 2011).

Dietary reference values (DRVs) for iron are based on estimates of the amount of iron required to replace basal and menstrual iron losses, and for growth. The European estimates are based on an assumed absorption of 15% from the diet.

### *0.3 Antioxidants*

Many physiological and pathological conditions are associated with oxidative stress (Leichert et al., 2007). Reactive oxygen species (ROS), especially hydrogen peroxide, superoxide anions and hydroxyl radicals, are generated in cells through the normal metabolic activity (Benaroudj et al., 2001). These ROS readily react with and damage vital cellular structures. Among them ROS may damage proteins causing modifications of amino acids chains and formation of cross-links. Furthermore ROS can cause lipid peroxidation in cell membrane and modify sugars and bases in DNA (Benaroudj et al., 2001; Leichert et al., 2007).

Nevertheless, lots of factors can cause the body to produce more ROS than are needed. These include smoking, alcohol drinking, an hiperlipidic diet, sun over-exposure, too many pollutants in the air and even too much exercise (Wahlqvist, 1999). The physiological defense systems to counteract free radicals comprises endogenous enzyme systems, such as catalase, glutathione reductase and superoxide dismutase, as well as glutathione, urate and coenzyme Q, or exogenous factors ( $\beta$ -carotene, vitamin C, vitamin E, selenium). All these molecules have an antioxidant effect due to their ability to transform ROS into stable and harmless compounds or by scavenging ROS with a redox-based mechanism (Valko et al., 2006; Brambilla et al., 2008).

One currently recognized characteristic of a healthy diet includes components that counteract oxidative stress and oxidative damage to cell components. Oxidative stress in fact has been linked to the aging process and the etiopathogeny and progression of chronic diseases, including heart disease and cancer (Wahlqvist, 1999; Benaroudj et al., 2001; Herrera et al., 2009).

Many compounds in food have antioxidant properties by interacting with the reactive molecules. Antioxidants from food include not only vitamins, but also some elements such as selenium and copper, respectively part of glutathione peroxidase and superoxide dismutase, and other compounds found in plant foods such as flavonoids and polyphenols. Any factors (excessive dietary fat intake, smoking or alcohol consumption, pollution exposure, intensive exercise), could increase the requirement for antioxidant nutrients (Wahlqvist, 1999).

However, when large amounts of antioxidant nutrients are taken, they can also act as pro-oxidants by inducing oxidative stress and have harmful effects in biologic systems. For some antioxidants there are conflicting data in relation to their adverse effects. For example, favorable effects of vitamin E have been observed in relation to Alzheimer's disease and prostate cancer, but the use of high doses of vitamin E is also associated with increased risk of mortality from some cancers, possibly fatal as opposed to non-

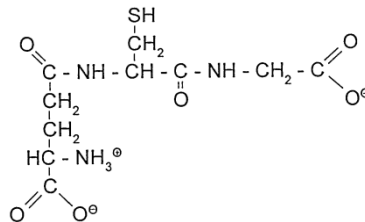


fatal myocardial infarction, and hemorrhagic stroke (Stephens et al., 1996; Brambilla et al., 2008). Another question is how much suppression of oxidation may be compatible with good health, as toxic free radicals are required for defense mechanisms (Wahlqvist, 1999).

Although the role of oxidative stress in aging, neurodegenerative and vascular diseases, cancer, diabetes, and other related diseases is largely accepted, the value of antioxidant strategies is still debatable, above all considering antioxidant supplementation (Herrera et al., 2009).

### *The case of glutathione (GSH)*

Glutathione (GSH) is a biologically active tripeptide consisting of L-glutamate, L-cysteine and glycine (Fig. 0.2). It is the most abundant intracellular thiol compound (0.2-10 mM) widely distributed in living organisms, from prokaryotes to eukaryotes (Meister & Anderson, 1983; Anderson, 1998).



Glutathione (GSH)

**Figure 0.2.** The structure of glutathione (GSH) (Anderson, 1998).

It is synthesized intracellularly in two ATP-dependent steps by the consecutive actions of  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS) (1), feedback inhibited by GSH, and glutathione synthetase (GS) (2). The exceptional peptidic  $\gamma$ -linkage is thought to protect the tripeptide from degradation by aminopeptidases (Anderson, 1998; Sies, 1999).

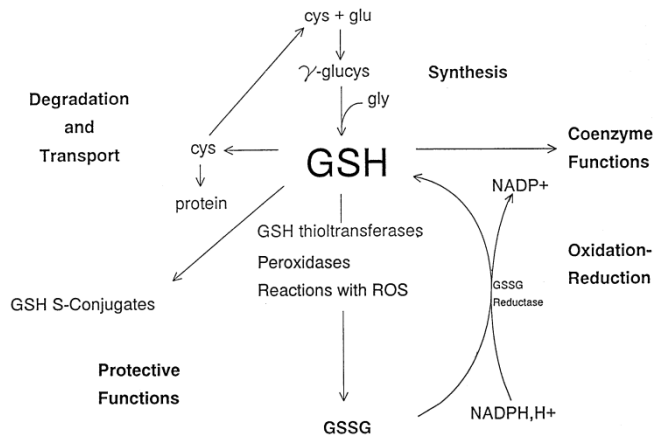


In cells, tissues and plasma, glutathione can exist under the reduced GSH form, the glutathione disulphide GSSG one (oxidized glutathione) and other forms of mixed disulphides GSSR, for example GS-S-CoA and GS-S-Cys (Sies, 1999; Penninckx, 2002) and GSS-protein which is formed via glutathionylation (Li et al., 2004).

Intracellularly, GSH is kept in its thiol form by glutathione disulphide (GSSG) reductase, a NADPH-dependent enzyme (Anderson, 1998). The GSSG/GSH ratio is often used as indicator of cell oxidative stress (Parris, 1997), and values >10 are considered normal physiological conditions (Wu et al., 2004).

Degradation into the constituent amino acids occurs via  $\gamma$ -glutamyltranspeptidase and cysteinyl-glycine dipeptidase. Many important reactions involves GSH on the thiol group, relating to redox reactions, i.e., disulphide formation, and to thioether and thiolester formation. The redox reactions are catalyzed by several GSH peroxidases and GSSG reductases, whereas a major class of enzymes in thioether formation is given by the glutathione transferases (GST). This is a class of enzymes that utilize GSH to generate products (glutathione S-conjugates) that are usually involved in detoxification and elimination (Sies, 1999).

GSH has lots of important cellular functions, summarized in Fig. 0.3 that are related to amino acid transport, protection against oxidative stress, xenobiotic and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism (Anderson, 1998; Penninckx, 2002). GSH has a role in signal transduction, in gene expression, and in apoptosis. There are links between the thiol redox state, glutathione-protein interactions, and cell proliferation, thus, protein glutathionylation may have a role in control of such processes. Human immunodeficiency virus (HIV)-1 protease activity is regulated through cysteine modification, and there are relationships between GSH levels and outcome in HIV patients (Sies, 1999).



**Figure 0.3.** Overview of glutathione metabolism (Anderson, 1998).

GSH has been found to be committed to many physiological processes and thus it plays many important roles, however the major GSH function may be summarized in three main topics: antioxidant, immunity booster and defence molecule (Li et al., 2004). These characteristics make GSH an important biochemical drug for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, gastrointestinal and pancreatic inflammations, as well as neurodegenerative diseases and aging. In fact cellular GSH concentrations are reduced markedly in response to protein malnutrition, oxidative stress, and many pathological conditions (Li et al., 2004; Wu et al., 2004) such as Crohn's disease, arteriosclerosis, diabetes and also neurodegenerative diseases, several tumours and liver diseases. Studies evidenced that GSH may be therapeutically effective when given in high doses to depleted subjects (Perricone et al., 2009).

### ***GSH production***

GSH was discovered in 1888 while its molecular structure was established in 1921. The first approach to GSH recovery consisted in solvent extraction from animal or plant tissue, but this approach gives an expensive end-product. Besides being extracted from some active tissues, GSH may be produced by chemical method, enzymatic reaction and microbial fermentation. Harington and Mead in 1935 demonstrated that GSH could be chemically synthesized but the final product was a racemic mixture that needed an optical resolution for separating the active L-form from its D-isomer (Li et al., 2004). Increased knowledge on GSH and its biosynthetic pathway induced investigations to explore the enzymatic and fermentative GSH production.

The GSH enzymatic production involves: the enzymes involved in GSH synthesis that are  $\gamma$ -GCS and GS, the precursor amino acids (L-glutamic acid, L-cysteine and glycine), ATP, the enzymes cofactors ( $Mg^{2+}$ ) and a proper pH (usually 7.5) (Li et al., 2004).

The ATP requirement in the enzymatic production of GSH makes the entire process too expensive to scale-up. This problem was partially solved employing *Saccharomyces cerevisiae* cells in which ATP regeneration, during glycolysis, compensates ATP consumption as a self-coupling ATP regeneration system. A further evolution dealing with enzymatic GSH production concerned a co-coupled ATP regeneration system in which two or more organisms are employed. Murata et al. (1981) used immobilized cells of *S. cerevisiae* for ATP generation through glycolytic pathway and immobilized *Escherichia coli* and *Brevibacterium ammoniagenes* for GSH synthesis and NADP production. Nevertheless the requirement to enhance the activities of  $\gamma$ -GCS and GS accelerated the application of molecular cloning and genetic engineering approaches in GSH biosynthesis (Li et al., 2004).

GSH fermentative production is currently the most common method employed on industrial scale and *S. cerevisiae* and *Candida utilis* are the most commonly used microorganism employed obtaining a GSH content of 0.1-1% dry cell weight. Fermentative GSH production furnishes yields lower than enzymatic one (up to 9 g/L, Miwa, 1978) but the sugar materials employed as substrates make the fermentative process to be cheaper than enzymatic one. As well as for enzymatic process, higher GSH yields (3-5% dry cell weight) can be obtained using mutants.

To improve GSH volumetric yields means to increase intracellular GSH content and cell density. However, in high-cell-density cultivation, oxygen supply, by-products or other factors may inhibit cell growth. For these reasons biotechnological process optimization is required.

Lots of papers report different process optimization strategies taking into account culture conditions, i.e. selection of nutrient and their concentration. For example Santos et al. (2007) used experimental designs to find the best conditions of temperature, agitation rate, initial pH, inoculum concentration and glucose concentration for GSH production by *S. cerevisiae*. Different strategies to enhance GSH production were applied and generally fed-batch culture is one of the most efficient method for achieving high-cell-density (Li et al., 2004; Shang et al., 2008). Wei et al. (2003) reported to enhance GSH production with *Candida utilis* (till 2.5% w/w) employing a two-stage temperature control strategy: a higher temperature for cell growth (30°C) and a lower one (26°C) for increasing GSH production. Liang et al. (2009) enhanced GSH production inducing H<sub>2</sub>O<sub>2</sub> multiple oxidative stress in *Candida utilis* and obtained up to a 2-fold increase respect to the control.

Although sugars were the principal substrate in the fermentative production of GSH, the addition of precursor amino acids required for GSH, was found to increase its production (Wen et al., 2005; Nisamedtinov et al., 2010). In particular cysteine was confirmed to be a key amino acid for increasing the specific GSH production rate, but it showed a growth inhibition effect (Li et al., 2004). In this context several papers have dealt with optimisation of cysteine addition to increase GSH production without causing growth inhibition. Alfafara et al. (1992) found that single-shot additions of L-cysteine furnished better yields than a continuous feeding. Cysteine is not the only amino acid influencing GSH accumulation: Wen et al. (2005) indicated glycine as the most important amino acid after cysteine in GSH synthesis (for more details see Chapters 1 and 5).

#### **0.4 *Saccharomyces cerevisiae***

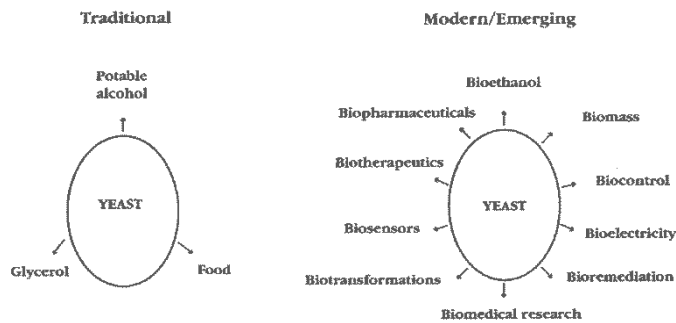
The yeast *Saccharomyces cerevisiae* is one of the most studied microorganisms and it is considered a model for eukaryotes especially for molecular genetic research. In fact its basic mechanisms of replication, recombination, cell division and metabolism are very similar to those of higher eukaryotes, including mammals (Waites et al., 2001). A model organism should have several important traits. Among these are size, generation time, manipulation, genetics, and economic benefit. *S. cerevisiae* has developed as a model organism having several appreciable characteristics, as follows:

1. It is a unicellular eukaryotic organism with a relatively uncomplicated and short life cycle (Gershon & Gershon, 2000): asexual cell division involves budding of a daughter cell from a mother cell. *S. cerevisiae* cells are generally ellipsoidal in shape with a large diameter ranging from 5-10  $\mu\text{m}$  and a small one 1-3 to 1-7  $\mu\text{m}$  (Walker, 1998) having a doubling time of 1.25–2 hours at 30 °C (Goffeau et al., 1996).
2. It has a small genome comprising about 6000 genes, which has been completely sequenced (Goffeau et al., 1996) and extensively mapped (Gershon & Gershon, 2000).
3. Special characteristics of this organism have enabled the development of essential molecular genetic tools that contribute significantly to the understanding of some of the major processes in cell biology as signal transduction, control of cell cycle progression, the basis of the switch from mitosis to meiosis, genetic recombination, intracellular trafficking of proteins, response to stress, and protein degradation (Gershon & Gershon, 2000).
4. Many yeast genes have been shown to have orthologs in the human genome, including some disease-causing genes. Several human proteins can functionally substitute for their yeast analogs following transfection of human genes into yeast (Gershon & Gershon, 2000).
5. It is relatively cheap to grow in large quantities on simple medium (Gershon & Gershon, 2000) and so easy to study.

#### ***Technological applications***

The yeast *S. cerevisiae* is used from ancient times, it is one of the oldest food microbial starters employed in the production of food and alcoholic beverages. Each of these traditional process converts substrates into ethanol, carbon dioxide and biomass Demirci et al., 1999) differently exploited. Nowadays it is also used in many other processes, exploiting the same metabolic characteristics, such as the production of fermentation products, particularly fuel ethanol, single cell protein, enzyme and heterologous proteins, e.g. human insulin (Waites et al., 2001); inactivated yeast and yeast derivatives have been used as nutritive complements and as food ingredients for

the formulation of a variety of industrial food products (Yamada & Sgarbieri, 2005). In Fig. 0.4 a summary of traditional and modern/emerging yeast technologies is reported.



**Figure 0.4:** Summary of yeast technologies (Walker, 1998).

Among traditional technologies, alcoholic beverages are produced throughout the world from locally available fermentable sugar materials derived from fruit juices, plant sap and honey, or from hydrolysed grains and root starch. The fermentation products are ethanol, a range of desirable organoleptic (flavour and aroma) compound and CO<sub>2</sub> (Waite et al., 2001). The alcoholic beverage obtained can be drunk fresh, or aged to modify its flavour or distilled to increase alcoholic strength. Although bacteria may be involved in some processes, yeasts are primarily used and mostly strains of *S. cerevisiae*.

Among modern technologies, increasing interest has been addressed in the production of bioethanol as a source of renewable energy and as alternative to petroleum. Generally the fermentable sources employed for bioethanol production comes from plant biomasses and lignocellulosic hydrolysates. Numerous genetic and physiological approaches have been applied aimed at improving yeast fermentation performance that in the scale of bioethanol industry would be economically significant (Walker, 1998).

Besides ethanol, *S. cerevisiae* metabolism naturally leads to biomass production. Baker's yeast, represent the largest bulk production of any single-celled microorganism throughout the world (Walker, 1998).

Baker's yeast production involves a multi stage propagation of selected *S. cerevisiae* strains on sugar cane and sugar beet molasses supplemented with additional sources of nitrogen (ammonium salts or urea), phosphorus and essential mineral ions such as

magnesium. The objective is to obtain a high biomass yield characterized by high fermentative activity and good storage properties. Thus, to preserve yeast fermentative ability, only the last stages are performed in high aerobically conditions and molasses medium is delivered incrementally to the growing cells in a feed-batch manner to avoid the Crabtree effect and maximize respiratory growth.

Yeast cells, originating from freeze-dried sample or agar-medium culture, are initially transferred to small liquid culture flasks, then to larger intermediate vessels before being finally used to inoculate the large production fermenters of 50-350 m<sup>3</sup> capacity. The process is conducted at 28-30°C and pH adjusted to 4.0-4.5. The obtained culture (containing normally 60 g biomass/L) is centrifuged and harvested cells are washed and differently dried according to the required yeast commercial forms: liquid (having about 18-20% dcw) compressed (about 30% dcw) and dried form ( 90% dcw).

Beside the direct food use as baking agent, *S. cerevisiae* may be employed as whole cell because of its proteins and micronutrients source for animal and food nutrition. Moreover, yeast extracts are used in food industry for their flavour or in the preparation of microbiological growth media. Yeast cell walls, end-product of yeast extract production, may be used as biosorbents for removal of heavy metals from industrial wastewaters. Recently, great interest has been aroused by this last application, biosorption, which exploits microbial cell envelopes to remove metals from water solutions (Volesky, 2001; De Philippis et al, 2007).

### **GSH and *S. cerevisiae***

GSH is the major (95%) nonprotein thiol compound in *S. cerevisiae* where it plays several important roles in response to nutritional and oxidative stress (Penninckx, 2000).

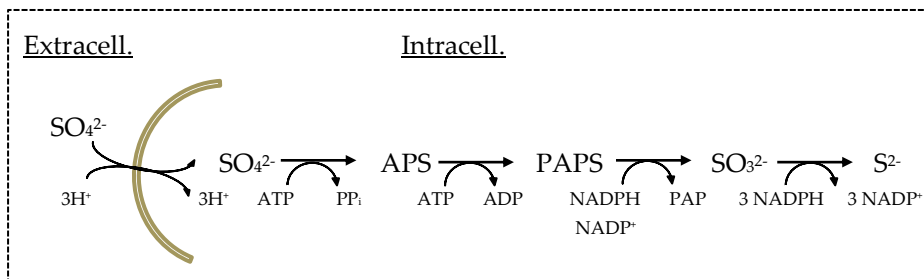
**Nutritional stresses** - The work of Penninckx (2000) demonstrated that most of the excess sulphur is incorporated into GSH, while it may serve as an endogenous sulphur source when cells were starved for sulphate. Moreover nitrogen starvation provokes the shift of more than 90% GSH toward the vacuolar compartment and a strong decrease in cellular GSH with a release of the constituent amino acids of GSH in the cytoplasm by vacuolar  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT) and cytoplasmic cysteinylglycine dipeptidase. In strains deficient in GSH biosynthesis (mutant strains), no accumulation of GSH in the central vacuole was observed, thus the GSH in these strains may serve as an endogenous source of amino acids for growth and maintenance.

**Environmental stresses** – This mechanism deals with the presence of xenobiotics and heavy metal ions in the culture medium. Glutathione S-transferase catalyses (GST) the formation of GSH-conjugates then sequestered in the vacuole.

**Oxidative stresses** – GSH, thanks to its sulphhydryl group, acts as a radical scavenger with oxidants to produce oxidised glutathione (GSSG). GSH is an electron donor for glutathione peroxidase (GPx) reaction, and GSSG is reduced to GSH by glutathione reductase (GR) in the presence of NADPH (Izawa et al., 1995). Penninckx demonstrated that most of the excess sulphur is incorporated into GSH, and it may serve as an endogenous sulphur source when cells were starved for sulphate. Moreover, nitrogen starvation provokes the shift of more than 90% GSH toward the vacuolar compartment and a strong decrease in cellular GSH with a release of the constituent amino acids of GSH in the cytoplasm by vacuolar  $\gamma$ -glutamyltranspeptidase ( $\gamma$ -GT) and cytoplasmic cysteinylglycine dipeptidase (Fig. 0.5). In strains deficient in GSH biosynthesis (mutant strains), no accumulation of GSH in the central vacuole was observed, thus GSH in these strains may serve as an endogenous source of amino acids for growth and maintenance.

***S. cerevisiae sulphate assimilation pathway***

The biosynthesis of organic sulphur compounds first requires sulphate to be taken up and reduced (Fig 0.5 and 0.6 reaction 13).

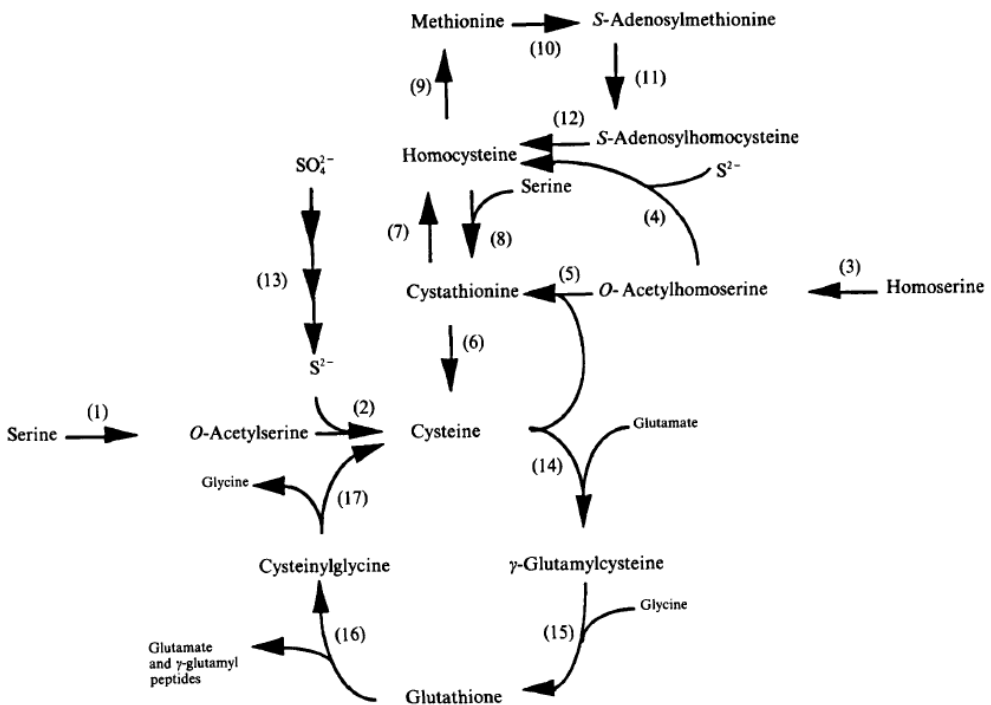


**Figure 0.5.** Yeast sulphate assimilation pathway.

Sulphate is co-transported into the cells with 3 H $^+$  by specific plasma membrane permeases and it is activated to adenylyl sulphate (APS) by ATP sulphurylase (ATPS). Then APS is phosphorylated by an APS kinase using ATP to produce phosphoadenylyl sulphate (PAPS) (Mendoza-Cózatl et al., 2005). PAPS is then reduced to sulphite by PAPS-reductase and then further reduced to sulphide by sulphide reductase. At the end of this process, the reduced sulphur atom can be incorporated into carbon chains (Thomas & Surdin-Kerjan, 1997).



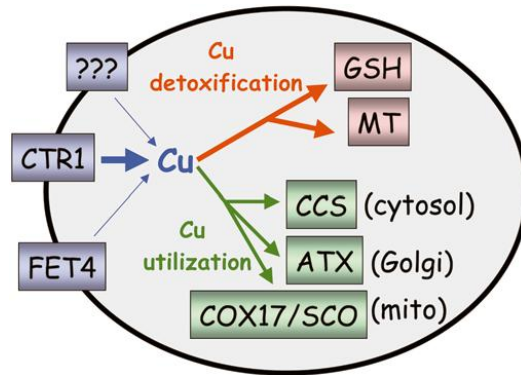
*S. cerevisiae* can grow both on inorganic sulphur sources and on organic ones (Thomas & Surdin-Kerjan, 1997) as methionine, homocysteine, cysteine or GSH as a sole sulphur source because of its metabolic systems, in which these compounds can readily exchange their sulphur atom (Fig. 0.6) (Miyake et al., 1999). These systems are the trans-sulphuration pathways that allow the interconversion of homocysteine and cysteine via the intermediary formation of cystathionine (Thomas & Surdin-Kerjan, 1997; Ono et al., 1999).



**Figure 0.6.** A model for the main fluxes of sulphur in *S. cerevisiae*: (1) Serine acetyltransferase; (2) cysteine synthase; (3) homoserine acetyltransferase; (4) homocysteine synthase; (5)  $\gamma$ -cystathionine synthase; (6)  $\gamma$ -cystathionase; (7) P-cystathionase; (8) P-cystathionine synthase; (9) homocysteine methyltransferase; (10) S-adenosylmethionine synthetase; (11) S-adenosylmethionine demethylase; (12) adenosylhomocysteinase; (13) sulphate-reducing pathway; (14)  $\gamma$ -glutamylcysteine synthetase; (15) GSH synthetase; (16)  $\gamma$ -GT; (17) cysteinylglycine dipeptidase (Elskens et al., 1991).

### *Metals transport in S. cerevisiae: copper and iron*

Some heavy metals such as  $\text{Cu}^{2+}$ ,  $\text{Co}^{2+}$ ,  $\text{Fe}^{2+}$ ,  $\text{Mn}^{2+}$  and  $\text{Zn}^{2+}$  are essential in trace amount for cell metabolism, acting both as enzyme cofactors, mediating redox reactions, and interacting with nucleic acids and proteins. Others metals instead enter into the cell through the same transport systems used by essential heavy metals, but altering cell functions. GSH may bind to a variety of metals in the cytosol and metal-(GSH)<sub>2</sub> complexes are actively transported into the vacuole (Mendoza-Cózatl et al., 2005).



**Figure 0.7.** Trafficking pathways for copper. The cell surface transporters (blue boxes), copper chaperone-like molecules (green boxes) and detoxification factors (pink boxes) for copper are shown, based on pathways established for baker's yeast. CTR1, being a major high-affinity transporter, is the predominant source of environmental copper under physiological conditions (depicted by heavy arrow). When environmental copper becomes more available, low-affinity transporters such as FET4 and other unknown molecules ("???"), can also contribute to the pool of intracellular copper. Once the metal enters the cell, a substantial fraction is subjected to detoxification (red arrows) by factors such as metal-binding metallothioneins (MT) or glutathione (GSH). Another pool of copper is reserved by copper chaperone molecules for copper utilization pathways (green arrows), i.e. delivery of the metal to copper-requiring targets in the cytosol, mitochondria (mito) or Golgi (from Luk et al., 2003).

Copper largely enters the cell (Fig. 0.7) through the action of one or more high-affinity cell surface transporters (CTRs); it can also enter via one or more low-affinity transporters (generally not specific for copper) when the medium is supplemented with elevated, but non-toxic, copper concentration (Luk et al., 2003). Under low copper conditions, transcription of high affinity transporters is enhanced, facilitating copper uptake. Conversely, copper overloaded cells cause high affinity transporters to be down-regulated to decrease copper uptake (De Freitas et al., 2003).

Iron can be transported into yeast cells bound to low molecular weight compounds or as ions. The high affinity system is specific for iron. As well as copper, reduced iron is

also transported through a less specific, lower affinity plasma membrane transporter (De Freitas et al., 2003). Once copper enter the cells it may be detoxified, and in this case sequestered by metal-binding factors such as metallothioneins or GSH, alternatively used from copper enzymes in different cellular districts, delivered by chaperons (De Freitas et al., 2003; Luk et al., 2003). How iron is safely carried within the cytosol to subcellular compartments is not fully understood (De Freitas et al., 2003).

### 0.5 References

**AA. VV.** Council Directive 89/398/EC on the approximation of the laws of the Member States related to foodstuff intended for particular nutritional use. Off J Eur Union 1989;L 186:27–32.

**AA. VV.** Council Directive 2002/46/EC on the approximation of the laws of the Member States relating to food supplements. Off J Eur Union 2002;L 183:51–57.

**AA. VV.** Council Regulation (EC) 1924/2006 on nutrition and health claims made on foods. Off J Eur Union 2006;L 404:9–25.

**AA. VV.** Council Regulation (EC) 1925/2006 on the addition of vitamins and minerals and of certain other substances to foods. Off J Eur Union 2006;L 404:26–38.

**AA. VV.** Council Directive 2008/100/EC amending Council Directive 90/496/EEC on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions. Off J Eur Union 2008;L 285:9–12.

**AA. VV.** Council Directive 2009/39/EC on foodstuffs intended for particular nutritional uses. Off J Eur Union 2009;L 124:21–29.

**AA. VV.** Council Regulation (EC) 1170/2009 amending Directive 2002/46/EC of the European Parliament and of Council and Regulation (EC) No 1925/2006 of the European Parliament and of the Council as regards the lists of vitamin and minerals and their forms that can be added to foods, including food supplements. Off J Eur Union 2009;L 314:36–42.

**AA. VV.** Tendenze di consumo. Integratori. AC/Nielsen Federsalus, 2009, 23-33.

**AA. VV.** *Nutrient and Energy Intakes for the European Community*. Report of the Scientific Committee for food (thirty-first series) Report- Food Supplements 2005 – Gain Scientific Committee on Food (SCF).

**Alfara C, Miura K, Shimizu H, Shioya S, Suga K.** 1992. Cysteine addition strategy for maximum glutathione production in fed-batch culture of *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 37:141-146.

**Anderson ME.** 1998. Glutathione: an overview of biosynthesis and modulation. Chem Biol Interact 111:1–14.

**Benaroudj N, Lee DH, Goldberg AL.** 2001. Trehalose accumulation during cellular stress protects cells and cellular proteins from damage by oxygen radicals. *J Biol Chem* 276:24261-24267.

**Brambilla D, Mancuso C, Scuderi MR, Bosco P, Cantarella G, Lempereur L, Di Benedetto G, Pezzino S, Bernardini R.** 2008. The role of antioxidant supplement in immune system, neoplastic, and neurodegenerative disorders: a point of view for an assessment of the risk/benefit profile. *Nutr J* 7:29-37.

**Caballero B.** 2003. Fortification, supplementation, and nutrient balance. *Eur J Clin Nutr* 57: S76-S78.

**Collins JF, Prohaska JR, Knutson MD.** 2010. Metabolic crossroads of iron and copper. *Nutr Rev* Vol. 68:133-147.

**Decreto Ministeriale** 27 January 1992, n. 111 Attuazione della direttiva n. 89/398/CEE concernente i prodotti alimentari destinati ad una alimentazione particolare. G.U. Serie Generale n. 39, 17 February 1992.

**Decreto Ministeriale** 21 May 2004, n. 169 Attuazione della direttiva 2002/46/CE relativa agli integratori alimentari. G. U. n. 164, 15 July 2004.

**De Freitas J, Wintz H, Kim JH, Poynton H, Fox T, Vulpe C.** 2003. Yeast, a model organism for iron and copper metabolism studies. *BioMetals* 16:185-197.

**De Philippis R, Paperi R, Sili C.** 2007. Heavy metal sorption by released polysaccharides and whole cultures of two exopolysaccharide-producing cyanobacteria. *Biodegradation* 18:181-187.

**Demirci A, Pometto AL, Cox DJ.** 1999. Enhanced organically bound selenium yeast production by fed-batch fermentation. *J Agric Food Chem* 47:2496-2500.

**Dietrich M, Brown CJP, Block G.** 2005. The effect of folate fortification of cereal-grain products on blood folate status, dietary folate intake, and dietary folate sources among adult non-supplement users in the united states. *J Am Coll Nutr* 24:266-274.

**Elskens MT, Jasper CJ, Penninckx MJ.** 1991. Glutathione as an endogenous source of sulfur in the yeast *Saccharomyces cerevisiae*. *J Gen Microbiol* 137:637-644.

**Fairfield KM, Fletcher RH.** 2002. Vitamins for chronic disease prevention in adults. *J Am Med Associat* 287:3116-3126.

**Fridovich I.** 2001. Reflections of a fortunate biochemist. *J Biol Chem* 276:28629-28636.

**Geissler C, Singh M.** 2011. Iron, meat and health. *Nutrients* 3:283-316.

**Gershon H, Gershon D.** 2000. The budding yeast, *Saccharomyces cerevisiae*, as a model for aging research: a critical review. *Mech Ag Develop* 120:1-22.

**Goffeau A, Barrell BG, Bussey H, Davis RW, Dujon B, Feldmann H, Galibert F, Hoheisel JD, Jacq C, Johnston M, Louis EJ, Mewes HW, Murakami Y, Philippsen P, Tettelin H, Oliver SG.** 1996. Life with 6000 genes. *Science* 563-567.

**Harington CR, Mead TH.** 1935. Synthesis of glutathione. *Biochem XXIX*:1602-1611.

**Herrera E, Jiménez R, Aruoma OI, Hercberg S, Sánchez-García I, Fraga C.** 2009. Aspects of antioxidant foods and supplements in health and disease. *Nutr Rev* 67:S140-S144.

**Izawa S, Inoue Y, Kimura A.** 1995. Oxidative stress response in yeast: effect of glutathione on adaptation to hydrogen peroxide stress in *Saccharomyces cerevisiae*. *FEBS Lett* 368:73-76.

**Johnson J, Gonzalez de Mejia E.** 2011. Dietary factors and pancreatic cancer: the role of food bioactive compounds. *Mol Nutr Food Res* 55:58-73.

**Lee J, Prohaska JR, Thiele DJ.** 2001. Essential role for mammalian copper transporter Ctr1 in copper homeostasis and embryonic development. *Proc Natl Acad Sci USA*. 98:6842-6847.

**Leichert LI, Gehrke F, Gudiseva HV, Blackwell T, Ilbert M, Walker AK, Strahler JR, Andrews PC, Jakob U.** 2007. Quantifying changes in the thiol redox proteome upon oxidative stress in vivo. *PNAS* 105:8197-8202.

**Li Y, Wei G, Chen J.** 2004. Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* 66:233-242.

**Liang G, Liao X, Du G, Chen J.** 2009. A new strategy to enhance glutathione production by multiple H<sub>2</sub>O<sub>2</sub>-induced oxidative stresses in *Candida utilis*. *Biores Technol* 100:350-355.

**Linder MC and Hazegh-Azam M.** 1996. Copper biochemistry and molecular biology. *Am J Clin Nutr* 63:797S-811S.

**Luk E, Jensen LT, Culotta VC.** 2003. The many highways for intracellular traffic of metals. *J Biol Inorg Chem* 8:803-809.

**McKay DL, Perrone G, Rasmussen H, Dallal G, Hartman W, Cao G, Prior RL, Roubenoff R, Blumberg JB.** 2000. The effects of a multivitamin/mineral supplement on micronutrient status, antioxidant capacity and cytokine production in healthy older adults consuming a fortified diet. *J Am Coll Nutr* 19:613-621.

**Meister A, Anderson ME.** 1983. Glutathione. *Annu Rev Biochem* 52:711-760.

**Mendoza-Cózatl D, Loza-Tavera H, Hernández-Navarro A, Moreno-Sánchez R.** 2005. Sulfur assimilation and glutathione metabolism under cadmium stress in yeast, protists and plants. *FEMS Microbiol Rev* 29:653-671.

**Miwa N.** 1978. Production of glutathione by fermentation. JP patent 53,094,090.

**Miyake T, Sammoto H, Kanayama M, Tomochika KI, Shinoda S, Ono BI.** 1999. Role of the sulphate assimilation pathway in utilization of glutathione as a sulphur source by *Saccharomyces cerevisiae*. *Yeast* 15:1449-1457.

- Murata K, Tani K, Kato J, Chibata I.** 1981. Glutathione production by immobilized *Saccharomyces cerevisiae* cells containing an ATP regeneration system. *Eur J Appl Microbiol Biotechnol* 11:72–77.
- Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T.** 2010. Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding. *Appl Microbiol Biotechnol* 87:175-183.
- Ono Bi, Hazu T, Yoshida S, Kawato T, Shinoda S, Brzvwczy J, Paszewski A.** 1999. Cysteine biosynthesis in *Saccharomyces cerevisiae*: a new outlook on pathway and regulation. *Yeast* 15:1365–1375.
- Parris MK.** 1997. Glutathione: systemic protectant against oxidative and free radical damage. *Alt Med Rev* 2:155-176.
- Peña MMO, Lee J, Thiele DJ.** 1999. A delicate balance: homeostatic control of copper uptake and distribution. *J Nutr* 129:1251-1260.
- Penninckx M.** 2000. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. *Enzyme Microb Technol* 26:737–742.
- Penninckx M.** 2002. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res* 2:295–305.
- Perricone C, De Carolis C, Perricone R.** 2009. Glutathione: a key player in autoimmunity. *Autoimmun Rev* 8:697–701.
- Santos LO, Gonzales TA, Úbeda BT, Monte Alegre R.** 2007. Influence of culture conditions on glutathione production by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 77:763-769.
- Shang F, Wang Z, Tan T.** 2008. High-cell-density cultivation for co-production of ergosterol and reduced glutathione by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 77:1233-1240.
- Shenkin A.** 2006. Micronutrients in health and disease. *Postgrad Med J* 82:559–567.
- Sies H.** 1999. Glutathione and its role in cellular functions. *Free Radic Biol Med* 27:916-921.
- Stephens NG, Parsons A, Schofield PM, Kelly F, Cheeseman K, Mitchinson MJ.** 1996. Randomised controlled trial of vitamin E in patients with coronary disease: Cambridge Heart Antioxidant Study (CHAOS). *Lancet* 347:781-786.
- Suitor CW, Bailey LB.** 2000. Dietary folate equivalents: interpretation and application. *J Am Diet Assoc* 100:88-94.
- Thomas D, Surdin-Kerjan Y.** 1997. Metabolism of sulphur amino acids in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* 61:503-532.
- Valko M, Rhodes CJ, Moncol J, Izakovic M, Mazur M.** 2006. Free radicals, metals and antioxidants in oxidative stress-induced cancer. *Chem Biol Interact* 160:1-40.

**Volesky B.** 2001. Detoxification of metal-bearing effluents: biosorption for the next century. *Hydrometallurgy* 59:203–216.

**Wahlqvist ML, Wattanapenpaiboon N.** 1999. Antioxidant nutrients. *Aus Prescr* 22:142-144.

**Waits MJ, Morgan NL, Rockey JS, Higton G.** *Industrial Microbiology: an introduction.* Blackwell Science, Oxford, 2001.

**Walker GM.** *Yeast Physiology and Biotechnology.* John Wiley & Sons, Chichester, 1998.

**Wei G, Li Y, Du G, Chen J.** 2003. Application of a two-stage temperature control strategy for enhanced glutathione production in the batch fermentation by *Candida utilis*. *Biotechnol Lett* 25:887-890.

**Wen S, Zhang T, Tan T.** 2005. Optimization of the amino acid composition in glutathione fermentation. *Process Biochem* 40:3474-3479.

**Woodside JV, McCall D, McGartland C, Young IS.** 2005. Micronutrients: dietary intake v. supplement use. *Proc Nutr Soc* 64:543–553.

**Wu G, Fang YZ, Lupton JR, Turner ND.** 2004. Glutathione metabolism and its implications for health. *J Nutr* 134:489–492.

**Yamada EA, Sgarbieri VC.** 2005. Yeast (*Saccharomyces cerevisiae*) protein concentrate: preparation, chemical composition, and nutritional and functional properties. *J Agric Food Chem* 53:3931-3936.

## 0.6 Outlines of the thesis

This research concerns nutritional and microbiological aspects. The final aim of this project is to obtain new food supplements formulations containing *Saccharomyces cerevisiae*, enriched with micronutrients and antioxidant molecules, by biotechnological processes.

This idea is supported by the fact that even if clinical deficiency of micronutrients is uncommon in the developed world, a suboptimal intake of certain micronutrients has been linked with an increased risk of chronic diseases such as CVD (cardiovascular disease), cancer and osteoporosis. Moreover external factors, such as smoke, UV radiations and pollution, contribute to oxidative stress and to the formation of free radicals that are considered to contribute to the risk of cancer.

The yeast *S. cerevisiae* is one of the most studied microorganisms and it is considered a model for eukaryotes. It is used both in industrial productions and in human diet. As well as leavening agent for baking products and fermenting agent for alcoholic beverages such as wine and beer, *S. cerevisiae* is used in the industrial production of ethanol, enzymes and dried yeast both for animal-feed and food supplement.

From an overlook of the information reported in the State of the Art section, it can be concluded that both antioxidant and micronutrients play very important roles for human health. *S. cerevisiae* may represent an efficient delivery system for these compounds, suitable for human nutrition and therapeutic treatments. In particular subsequently to the identification of some potential molecules, with which the yeast might be enriched, the research focused mainly on cell enrichment with reduced glutathione (GSH) and Copper-conjugated glutathione, with particular regard to the set-up of biotechnological processes in order to increase product yields. The research also investigated biological activity of the obtained enriched biomass, in particular the fate of GSH when the biomass is swallowed, by determining GSH stability during gastro-intestinal digestion and any possible protective role of the yeast cell; GSH transport/absorbtion by intestinal cell lines and any possible toxicity has been also analysed. The final goal of the research was to obtain GSH and copper-enriched cells of *S. cerevisiae* furthering the range of application of yeast cell cultures.



## *1. GSH production during cell growth*

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## 1.1 State of the art

Several studies have been performed in bacteria and yeast related to glutathione production. Many of them reported different strategies to increase GSH production in a high-cell-density cultivation of *Saccharomyces cerevisiae* varying culture media, incubation time, addition of amino acids and/or precursors at different times and concentrations. Here follows some examples.

Alfara et al. (1992a) reported that cysteine addition increased specific GSH production rate of about two-fold compared with cultures grown without cysteine; a single shot addition of cysteine proved also to be better than a continuous feeding. Wang et al. (2007) reported that cysteine addition would cause growth inhibition, while the simultaneous introduction of glutamic acid and glycine, that are cheaper than cysteine, can weaken the inhibition effect. Authors obtained the best results in flasks by adding 4 mM of the three precursor amino acids, with an intracellular GSH content of about 1.12% dry cell weight (dcw). When only cysteine was added, intracellular GSH content was about 0.94% dcw, so the increase in GSH yield was respectively 87 and 56% (0.60% dcw obtained without any amino acids addition, control). In process carried out in fermenter, when amino acids were added after 32 h cultivation, GSH productivity was maximized: intracellular GSH was found 1.53% dcw with an increase of about 32% respect to the control (1.16% dcw), without amino acids supplementation.

Results obtained from Alfara et al. (1992b) showed that cysteine was the only amino acid that could enhance GSH production. Wen et al. (2005) and Wang et al. (2011) reported that the addition of glutamic acid was not necessary to improve GSH production. Wei et al. (2008) investigated the effects of cysteine in combination with a temperature-shift strategy on GSH production in *Candida utilis*; applying these strategies in a fed-batch culture, they enhanced GSH production content up to 3.75%, which was 90% higher than in control trials.

In this preliminary phase of the research, the effect of cysteine addition on GSH production was investigated. Trials were initially performed in flask and only after scale-up studies were performed in fermenter. The aim of this part of the study was to produce GSH enriched yeast cells by optimize culture conditions during cell growth.

## 1.2 Materials and Methods

### Strains

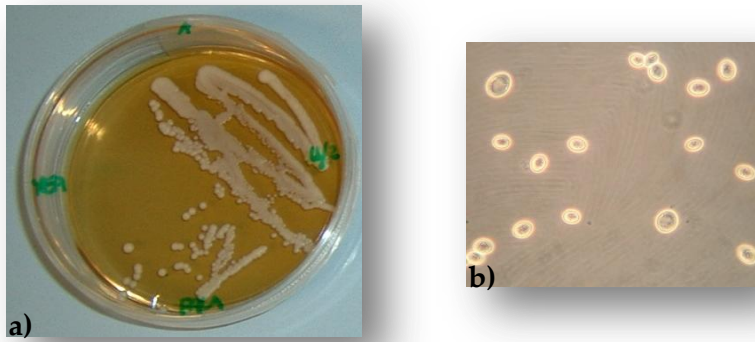
In the first part of the study different commercial baker's yeast were considered with the aim to individuate the most suitable strain to be employed. *S. cerevisiae* is a strain that naturally produces intracellular GSH as defense mechanism that cells use when in stress conditions, i.e. in fermentation process during leavening or alcoholic beverage production. Generally strains employed for large scale baker's yeast production resulted from a selection and isolation procedure among *S. cerevisiae* strains able to accumulate levels of intracellular GSH of about 1% on cell dry weight. Commercial baker's yeast is sold in different forms: compressed, dried and recently liquid, anyway in Italy it is mainly purchased in compressed form (30-35% dcw) (Fig. 1.1).



**Figure 1.1.** Commercial baker's yeast in compressed form.

Baker's yeast is obtained in large scale multistep processes, in which each phase is conducted in adequate aeration condition. In particular the first stages run in semi anaerobic condition (limited air flux) to retain yeast-fermentative ability. Only the last two stages (generally about 150 and 400 m<sup>3</sup>) are managed in total aerobic conditions. Moreover the inoculum of these stages is high (up to 20-30% v/v) and the fermentation time is not more than 15 hours. With these strategies, the obtained biomass has high fermentative capacity. In the last stage cells accumulate metabolites such as glycogen, trehalose and GSH, molecules related to both yeast performance and resistance to technological stress.

In the preliminary steps of the research, the characteristics of different *S. cerevisiae* strains were evaluated. In particular GSH content, both in reduced (GSH) and oxidized form (GSSG) was determined, in strains belonging to official collections and in isolated ones. Among the tested *S. cerevisiae*, the isolated from commercial compressed *Fala* baker's yeast (Lesaffre Italia, Trecasali Parma, Italy) presented the highest attitude to produce glutathione, in particular in reduced form (GSH). Due to its interesting GSH intracellular content, this type of yeast was employed for the research (Fig. 1.2).



**Figure 1.2.** Culture isolated from *Fala* commercial baker's yeast: a) Petri dish, b) microscope image (1000x).

### ***Culture conditions***

Different media were employed according to the experiment plan; the composition of each media is reported below (g/L).

- **Ti:** bacto peptone 20, yeast extract (Costantino, Turin, Italy) 10, glucose 20, pH 6.0;
- **Tr:**  $(\text{NH}_4)_2\text{SO}_4$  8,  $\text{MgSO}_4$  0.25, yeast extract (Costantino) 1, glucose 30, pH 6.0;
- **TN:**  $(\text{NH}_4)_2\text{SO}_4$  5,  $\text{K}_2\text{HPO}_4$  1,  $\text{MgSO}_4$  0.2, yeast extract (Costantino) 1, glucose 10, pH 5.8;
- **MEB:** malt extract (Costantino) 20, soybean peptone (Costantino) 1, glucose 20, pH 5.8.

Media were sterilized at 118 °C for 20 min. Trials were performed in 1 L Erlenmeyer flasks containing 100-200 mL of the selected medium, inoculated (10% v/v) with an overnight-old liquid pre-culture obtained by inoculating 5 mL cell suspension from a 24 h-old solid culture (MEA, Malt Extract Agar). Cultures were incubated on an alternative shaker (60 spm, 4 cm run) at different temperatures and time. Trials were subsequently carried out in a 14 L fermenter (OMNITEC, Sedriano - MI) with a 10 L working volume equipped with two Rushton impellers (4 blades, 8 cm diameter, 0.32 impeller/fermenter internal diameter). The following conditions were employed: 10% (v/v) inoculum from overnight-old culture, 30°C temperature, 1 vvm aeration rate and 300 rpm agitation speed.

### ***Analytical procedures***

Intracellular GSH was determined according to Rollini & Manzoni (2006). Briefly, samples were centrifuged (7,000 rpm, 7 min) and collected cells, washed with distilled water, were suspended in a solution of 0.5 g ascorbic acid/L HPLC-grade H<sub>2</sub>O. The suspension was then thermally treated at 100°C for 10 min. After cooling in ice bath, samples were centrifuged (12,000 rpm, 12 min) to eliminate cell residues and, on obtained supernatant fractions, intracellular GSH was evaluated.

GSH identification and quantification were carried out by HPLC, equipped with a UV detector (210 nm), at 30°C using a (250 × 4) mm Purospher® RP-18 endcapped column (Merck), eluted with 25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 2.8, at 0.3 mL/min. Standard of GSH in reduced form was purchased by Sigma and HPLC-grade H<sub>2</sub>O was obtained by a Milli-Q A10 Gradient System (Millipore Corporation).

Results were expressed in terms of percent intracellular GSH content with respect to dry cell weight:

$$\text{Intracellular GSH content (\%dcw)} = \frac{\text{GSH concentration } \left(\frac{\text{mg}}{\text{L}}\right)}{\text{DCW} \left(\frac{\text{g}}{\text{L}}\right) \times 10} \times 100\%$$

Determination of dry cell weight was performed by drying cells at 105°C (CEAL, Milano mod. SC4) to constant weight (thermobalance Gibertini mod. TB2).

### **1.3 Results**

#### ***Cysteine addition and temperature shift***

In this first part of the research, trials were performed taking into account the promising results obtained by Wei et al. (2008) by adding cysteine and applying a temperature shift procedure for GSH production in *Candida utilis*. *Fala* yeast strain pre-culture grown on T<sub>i</sub> medium, was inoculated (10% v/v) in 1 L flasks containing the T<sub>i</sub> medium. All cultures were incubated overnight (16 h) at 30 °C in an alternative shaker and then added or not (control culture) with 8 mM cysteine (CYS). Cultures were subsequently incubated at two different temperature, 25 or 30°C, for further 8 h, for a total of 4 combinations of experiments:

- CYS-25°C
- CYS-30°C
- NO CYS-25°C
- NO CYS-30°C

**Table 1.1.** Intracellular GSH content (% dcw) and DCW (g/L) of *Fala* yeast.

	25°C		30°C	
	GSH (% dcw)	DCW (g/L)	GSH (% dcw)	DCW (g/L)
CYS	0.93 ± 0.11	3.1	0.83 ± 0.09	3.4
NO CYS	0.70 ± 0.04	4.1	0.64 ± 0.04	3.7

Obtained results (Tab. 1) showed that with cysteine addition a slight growth inhibition was evidenced (3.1-3.4 g/L) with respect to no-cysteine added cultures (3.7-4.1 g/L). On the contrary, higher GSH levels (0.8-0.9% dcw) were evidenced in samples obtained with cysteine addition, with respect to control cultures (0.6-0.7% dcw). As regards temperature no significant difference were found.

This behavior made it possible to highlight that cysteine addition produced a little increase in GSH accumulation, even if a partial inhibition of biomass production was seen.

Subsequent experiments were carried out employing  $T_i$  medium in an automatized fermenter (Fig. 1.3), in the conditions described in the previous section. Culture was inoculated with 10% (v/v) of a 16 h-old culture obtained in flasks with  $T_i$  medium.



**Figure 1.3.** Yeast culture for GSH production in automatized fermenter.

After overnight growth (16 h) cysteine was added to the culture to reach a final 8 mM concentration. Intracellular GSH and biomass yield (g/L dcw) were evaluated at different incubation times (16, 20 and 24 h).

Obtained results from these experiments evidences in this scale-up phase a 20% increase of GSH accumulation was produced by overnight cysteine addition (from 0.65 to 0.85% dcw at 20 h), with a biomass growth of about 4.7 g/L. The subsequent monitoring of GSH level evidenced that it was maintained by further incubation for up to 24 h.

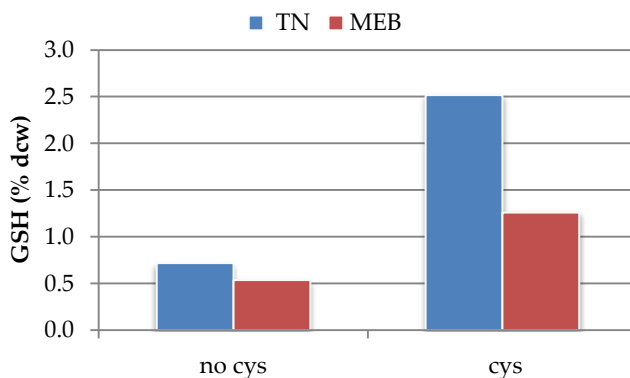
### Culture medium formulation and cysteine addition

As before, in this part of the research experiments were preliminary performed in flasks (1 L with 200 mL medium), employing TN and MEB culture media (see *Materials and Methods* section), for comparative purpose, both supplemented with 4 mM cysteine. Again intracellular GSH and biomass yield (g/L dcw) were evaluated.

Results reported in Tab. 1.2 show that biomass production was higher employing MEB medium than TN. Cysteine addition caused a decrease in biomass yield in both the media, more evident in TN medium. However, as reported in Fig. 1.4 when cysteine was added, intracellular GSH increased and this was much more evident in TN media. From these results it was possible to conclude that biomass yield and GSH production appear to be inversely correlated: the higher the biomass, the lower the GSH content.

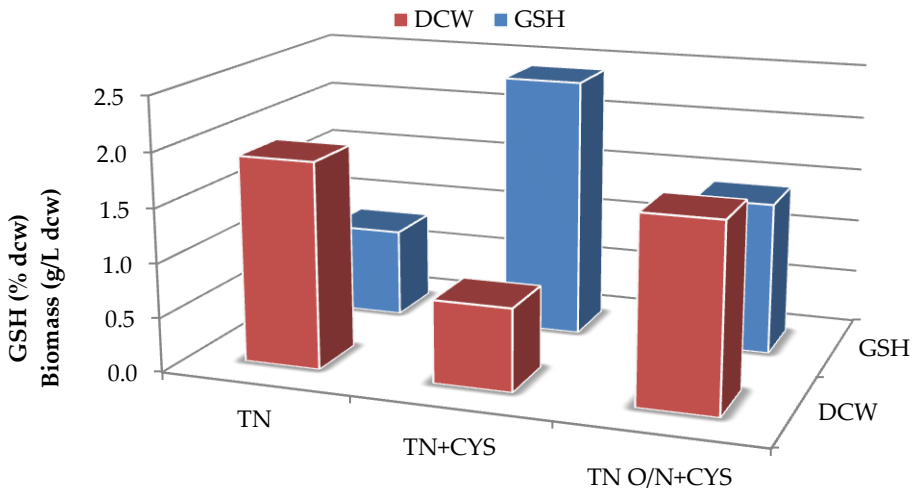
**Table 1.2.** Biomass yield (g dcw/L) in TN and MEB media with or without cysteine addition.

Medium	Biomass (g/L dcw)	
	NO CYS	CYS
TN	2.4±0.2	0.7±0.1
MEB	4.2±0.3	2.7±0.2



**Figure 1.4.** Intracellular GSH accumulation (% dcw) in TN and MEB media with or without cysteine addition (data are means of three replicates, Coefficient of Variation, CV, 3-5% in “no cys” trials and 7-10% in “cys” trials).

On the basis of the fact that the highest GSH yields were obtained with TN, optimization of cultural condition was carried out employing this medium. Trials were performed in 1 L flasks, and cysteine comparatively added (4 mM) directly to the medium (t<sub>0</sub>) or overnight (16 h) (Fig. 1.5).



**Figure 1.5.** Biomass yield (g/L dcw) and intracellular GSH accumulation (% dcw) employing TN medium in different condition: no cysteine (TN), with cysteine at t<sub>0</sub> (TN+CYS) and cysteine added at 16 h (TN O/N + CYS). Data are means of three replicates, CV between 5 and 10%.

As evidenced in previous experiments, results confirm that cell growth was partially inhibited when cysteine was added directly to the medium (sample TN+CYS), with a cell yield of 0.76 g/L with respect to 1.90 g/L of the control (TN). This effect was practically not present when the addition was done overnight, after 16 h (1.70 g/L, TN O/N+CYS) in fact at that incubation time cell growth was almost completed.

Again GSH accumulation was in inverse relation with growth levels, in fact the highest GSH accumulation (2.40% dcw) was evidenced in the sample obtained with cysteine directly added to the medium (TN+CYS), while a lower level was obtained in sample with cysteine added overnight (TN O/N+CYS). The lowest GSH production (0.82% dcw) was observed in control (TN) sample.



In conclusion, cysteine addition at overnight growth seemed a good compromise to reach a satisfactory cell growth associated to GSH accumulation. This condition allowed to obtain 1.70 g dcw/L of biomass with a content of GSH 1.40% dcw. Trials were then repeated in fermenter. 5 L TN culture medium was inoculated with 10% (v/v) of a 16 h-old culture obtained in flasks with the same medium. After overnight growth, cysteine was added (4 mM). Intracellular GSH and biomass yield were evaluated at overnight, 20 and 24 h of incubation. Results are reported in Tab. 1.3.

**Table 1.3.** Intracellular GSH (% dcw) and biomass yield (g/L) obtained in fermenter at different incubation time (O/N, 20 h and 24 h).

Incubation time (h)	Biomass (g/L dcw)	GSH (% dcw)
16→ cysteine addition	1.9±0.4	0.75±0.09
20	2.0±0.5	1.02±0.07
24	2.1±0.3	1.17±0.06

An increase of GSH accumulation with respect to previous experiments carried out in flasks was evidenced. A nearly 30% GSH increase was found already after 4 h cysteine addition (20 h sample), and reached +40% after 8 h (24 h sample). GSH levels increased from 0.75% before cysteine addition (16 h) to 1.02% at 20 h, to reach 1.17% at 24 h.

#### 1.4 Conclusions

From an overall view of the obtained results it was possible to evidence cysteine role to enhance GSH intracellular accumulation, anyway taking also in account its inhibition effect on cell growth. Results are in agreement with those reported by other authors (Alfara et al., 1992a-b; Wen et al., 2005 and Wei et al., 2008), which describe cysteine growth inhibition, effect associated with an increased GSH production.

To overcome this limitation a biotechnological strategy of adding cysteine after a well-defined growth time may represent the solution. From the obtained results cysteine addition after overnight incubation (16-18 h) represents a good compromise to maximize yeast growth and GSH accumulation.

## 1.5 References

**Alfajara CG, Kanda A, Shioi T, Shimizu H, Shioya S, Suga K.** 1992a. Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 36:538–540.

**Alfajara CG, Miura K, Shimizu H, Shioya S, Suga K.** 1992b. Cysteine addition strategy for maximum glutathione production in fed-batch culture of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 37:141–146.

**Rollini M, Manzoni M.** 2006. Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. *Process Biochem* 41:1501–1505.

**Wang Z, Tan T, Song J.** 2007. Effect of amino acids and feedback control strategies on the high-cell-density cultivation of *Saccharomyces cerevisiae* for glutathione production. *Process Biochem* 42:108-111.

**Wang M, Sun J, Xue F, Shang F, Wang Z, Tan T.** 2011. The effect of intracellular amino acids on GSH production by high-cell-density cultivation of *Saccharomyces cerevisiae*. *Appl Biochem Biotechnol* DOI: 10.1007/s12010-011-9435-4.

**Wei GY, Whang DH, Chen G.** 2008. Overproduction of glutathione by L-cysteine addition and a temperature-shift strategy. *Biotech Bioproc Eng* 13:347-353.

**Wen S, Zhang T, Tan T.** 2005. Optimization of the amino acid composition in glutathione fermentation. *Process Biochem* 40:3474-3479.

## *2. Post-growth intracellular GSH production*

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## 2.1 Post-growing procedure: activation

*Activation* is a term generally employed on industrial scale to identify a post growing procedure in which microbial cells are allowed to increase/accumulate a metabolite as a function of the incubation conditions. When applying this strategy for GSH production, yeast cells were suspended in a solution containing substances directly or indirectly involved in GSH synthesis with the aim to increase its physiological intracellular content.

For this purpose, different molecules and minerals, involved in GSH biosynthesis with different roles, were considered:

- glutamic acid (GLU), cysteine (CYS) and glycine (GLY), GSH precursors;
- glucose, for ATP generation in the glycolytic pathway;
- minerals, essential in several steps of the metabolic pathways. In particular, magnesium takes part in ATP-dependent reactions and is the cofactor of  $\gamma$ -glutamylcysteine-synthetase ( $\gamma$ -GCS); magnesium also stabilizes yeast phospholipid membrane.

## 2.2 Materials and Methods

In this part of the study, trials were carried out employing the following commercial baker's yeast, both in compressed and dried forms.

- **Compressed form**



*Zeus*  
(ZEUS IBA, Firenze)



*Fala*  
(Lesaffre, Trecasali, Parma)

- **Dried form**



*Fermipan Brown*



*Fermipan Red*

(GB Ingredients, Casteggio, Pavia)

This approach was justified by the fact that, as previously reported (par. 1.2), commercial baker's yeasts are obtained employing selected *S. cerevisiae* strains in which GSH physiological level is higher than that present inside conventional strains obtainable from official collections. This aspect might be related to a specific attitude to synthesize GSH.

Yeast cells were suspended (5% dcw) in an activation mixture, generally set-up in tap-water, containing glucose, sodium citrate, ammonium sulphate,  $\text{KH}_2\text{PO}_4$ , magnesium sulphate, cysteine (CYS), glycine (GLY), glutamic acid (GLU) and when present, other ingredients, differently combined according to the detailed arrangement of each experiment. The appropriate composition (g/L) of the activation mixture is reported in each section.

Trials were carried out in 100 mL Erlenmeyer flasks, each containing 10 mL reaction mixture, incubated at 28°C on an alternative shaker (60 spm, 4 cm run). Samples were collected at appropriate intervals, and intracellular GSH determined as previously reported (1.2). Results were expressed in terms of GSH percent related to dry cell weight (% dcw).

### **2.3 Preliminary trials**

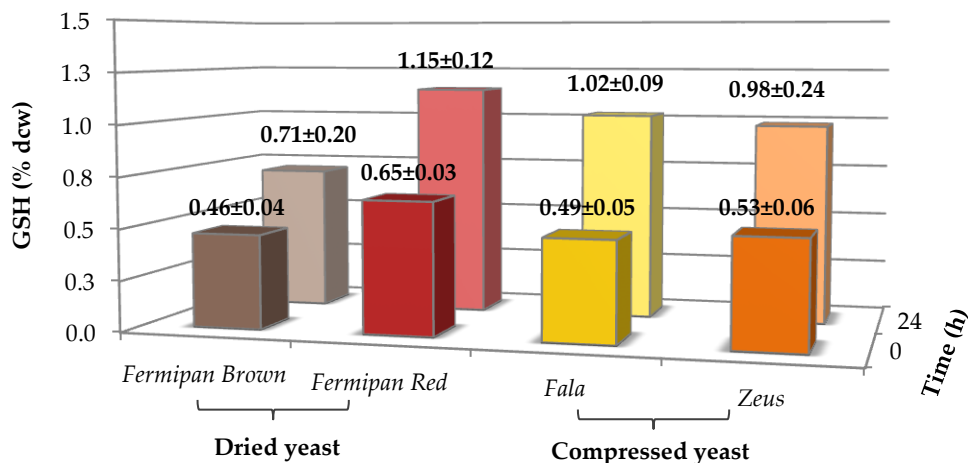
In the first part of the research trials were carried out to evaluate the influence of some factors on GSH accumulation, taking into account the different attitudes of compressed and dried yeasts, the influence of incubation temperature and the type of water employed. The aim of these preliminary trials was to screen different commercial yeasts and discard from the investigation irrelevant factors.

### Commercial baker's yeasts in compressed and dried form

Biosynthetic attitude of different commercial baker's yeast form to accumulate GSH was investigated, employing the compressed and dried forms.

GSH levels were evaluated at 24 h incubation time employing a named CYS-GLY mixture, considered as reference (control). This mixture is composed as follows (g/L): glucose 80, sodium citrate 10, ammonium sulphate 7,  $\text{KH}_2\text{PO}_4$  3.5, magnesium sulphate 0.5, cysteine 4 and glycine 4.

Results, reported in Fig 2.1, highlighted that the compressed yeasts *Fala* and *Zeus* were characterized by near the same initial GSH content (0.5%) and a similar GSH production attitude, with a 2-fold increase (about 1%) respect to the initial level. Otherwise the dried yeasts (*Fermipan Brown* and *Red*) with a not negligible different initial GSH levels (0.46 and 0.65% respectively), showed different activation behaviour, with the highest GSH levels (1.15%) reached by *Fermipan Red*. The sample *Fermipan Brown* showed a limited activation attitude and reached only 0.71% intracellular GSH content at 24 h incubation.



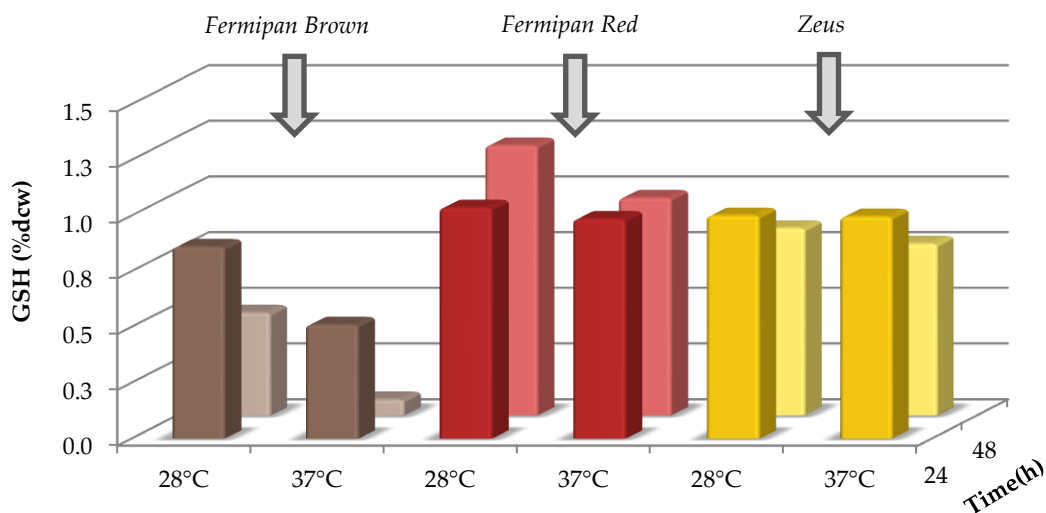
**Figure 2.1.** Intracellular GSH content (% dcw) of commercial compressed and dried baker's yeasts before and after activation (24 h incubation time).

In this set of trials the reproducibility of results was accurately verified. This aspect resulted important in the prosecution of the study, because not always the same type of yeast was found commercial available. This is the reason why part of the subsequent study was performed with *Zeus* yeast, while other with *Fala*.

### *Influence of incubation temperature: 28-37°C*

With the aim of evaluating the influence of temperature on GSH accumulation during the activation procedure, experiments were carried out by incubating samples at 28 and 37°C comparatively; GSH production was evaluated at 24 and 48 h incubation. In these trials the commercial yeast *Zeus* was employed as compressed yeast.

Highest yields were generally evidenced at 24 h incubation, while at 48 h a decrease occurred with exception of *Fermipan Red* yeast (at 28°C) that from about 1% at 24 h increased to 1.2% at 48 h incubation time (Fig. 2.2).



**Figure 2.2.** Intracellular GSH (% dcw) at 24 and 48 h activation (28-37°C) (to GSH content: *Fermipan Brown* 0.46%, *Fermipan Red* 0.65%, *Zeus* 0.49%). Data are means of three replicates, CV between 8 and 12%.

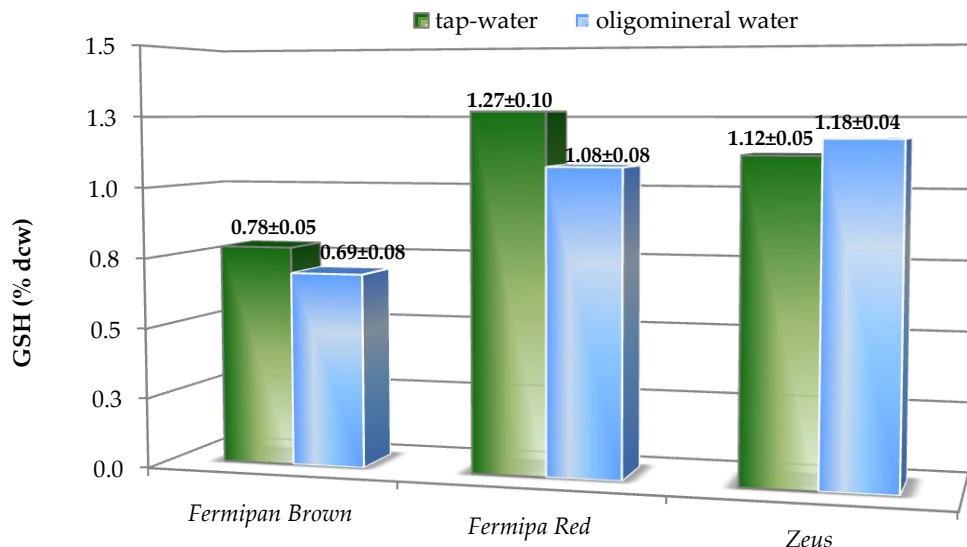
Results confirm that at 24 h the dried yeast *Fermipan Red* and the compressed *Zeus* had similar performances while the dried yeast *Fermipan Brown* had different and lower ability to accumulate GSH.

No significant evidences were obtained considering the different incubation temperature; only *Fermipan Brown* seemed to be negatively affected by a higher temperature.

### *Influence of water composition: tap- and oligomineral- water*

Ingredients present in CYS-GLY activation mixture were then dissolved both in tap- and oligomineral-water for comparative purposes, to evaluate the influence of minerals on yeast ability to accumulate GSH.

Results, reported in Fig. 2.3, underline that for each yeast sample no significant differences occurred in the two employed conditions. Furthermore results confirmed previous behaviour: *Fermipan Red* and *Zeus* had similar performances (1.1-1.3% dcw), while *Fermipan Brown* (0.7-0.8% dcw) had a lower GSH accumulation ability.



**Figure 2.3.** Intracellular GSH (% dcw) at 24 h; mixture CYS-GLY set up in tap- or oligomineral-water (to GSH content: *Fermipan Brown* 0.46%, *Fermipan Red* 0.65%, *Zeus* 0.49%).

### *Considerations*

Results obtained in this part of the research represented an interesting starting point for the prosecution of the study, in particular:

- *Fermipan Brown* dried yeast shows a low sensitive towards activation procedure, having reached GSH level of only 0.86±0.05% dcw at 24 h, starting from 0.46±0.04% dcw, so far it was not considered in subsequent trials;
- the two compressed yeasts had the same initial GSH level and also reached similar final GSH yields;
- *Fermipan Red* and *Zeus* presented similar performances, from 1 and 1.3%dcw, reaching highest yields at 24 h;



- temperature and water composition seem not to be relevant factors for GSH accumulation during activation. For this reason a temperature of 28°C and tap- water were selected for the prosecution of the research.

#### 2.4 Compressed and dried yeast: comparative experiments

*Fermipan Red* dried yeast and *Zeus* as compressed were comparatively employed.

##### *Influence of activation mixture formulation*

The aim of this set of trials was to evaluate the behaviour of the dried and compressed yeast according to the different activation mixtures employed. They had the same glucose and mineral (ammonium sulphate, sodium-citrate, magnesium sulphate and potassium phosphate) content as in the *control* mixture, but differed for amino acids, yeast extract and biotin content, as follows:

- tested amino acids: cysteine-CYS, glycine-GLY, glutamic acid-GLU, serine-SER;
- presence or absence of yeast extract (YE) and biotin (BIO);
- cysteine replacement with N-acetyl-cysteine (NAC).

The schematic organization of the trials is reported in Tab. 2.1.

**Table 2.1.** Organization of the trials to evaluate the influence of some ingredients (g/L) present in activation mixtures.

Activation mixture	GLY	CYS	SER	GLU	BIO*	YE	NAC
CYS-GLY	4	4	-	-	-	-	-
CYS-GLY-SER	1	1	2	-	-	-	-
CYS-GLY-GLU	4	4	-	4	-	-	-
CYS-GLY+YE	4	4	-	-	-	1	-
CYS-GLY-SER+YE	1	1	2	-	-	1	-
CYS-GLY-GLU+YE	4	4	-	4	-	1	-
CYS-GLY+BIO	4	4	-	-	0.01	-	-
CYS-GLY-SER+BIO	1	1	2	-	0.01	-	-
CYS-GLY-GLU+BIO	4	4	-	4	0.01	-	-
NAC-GLY	4	-	-	-	-	-	4
½NAC-½CYS	4	2	-	-	-	-	2

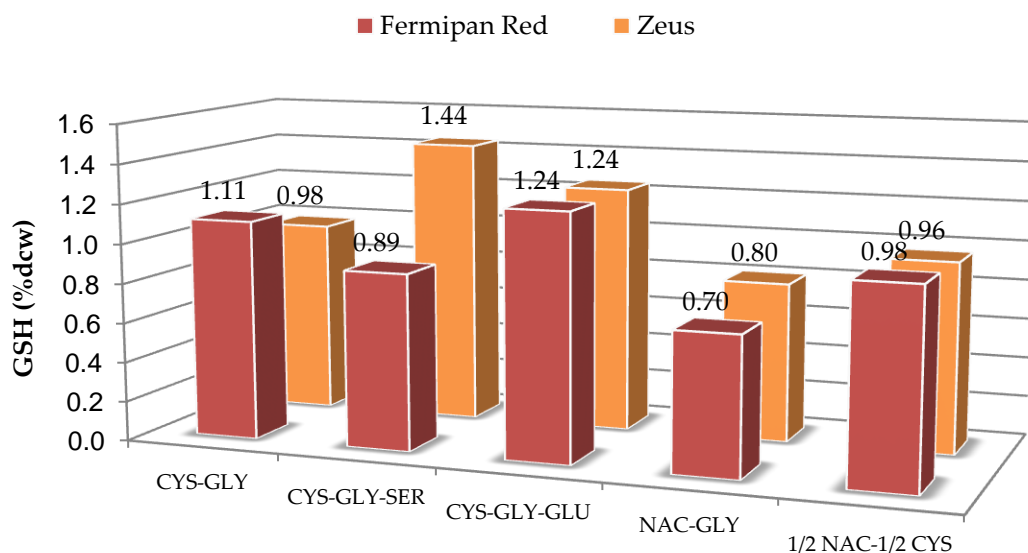
\*Biotin expressed as mg/L

Significant differences were evidenced in activation with dried and compressed yeasts, depending on amino acid combinations and yeast extract or biotin absence

(Fig. 2.4). Employing CYS-GLY-SER mixture, at 24 h *Fermipan Red* reached 0.89 GSH % dcw (with respect to 0.6-0.69% to), while *Zeus* 1.44% dcw (0.52-0.59% to). Employing CYS-GLY and CYS-GLY-GLU mixtures no significant differences were observed between the two yeasts (1-1.2% dcw) at 24 h incubation.

Furthermore for both yeasts the partial substitution of cysteine with N-acetyl-cysteine (1/2 NAC-1/2 GLY samples) did not affect GSH yields (about 1% dcw) respect to the control CYS-GLY, but the complete substitution of cysteine limited GSH accumulation (0.7-0.8% dcw).

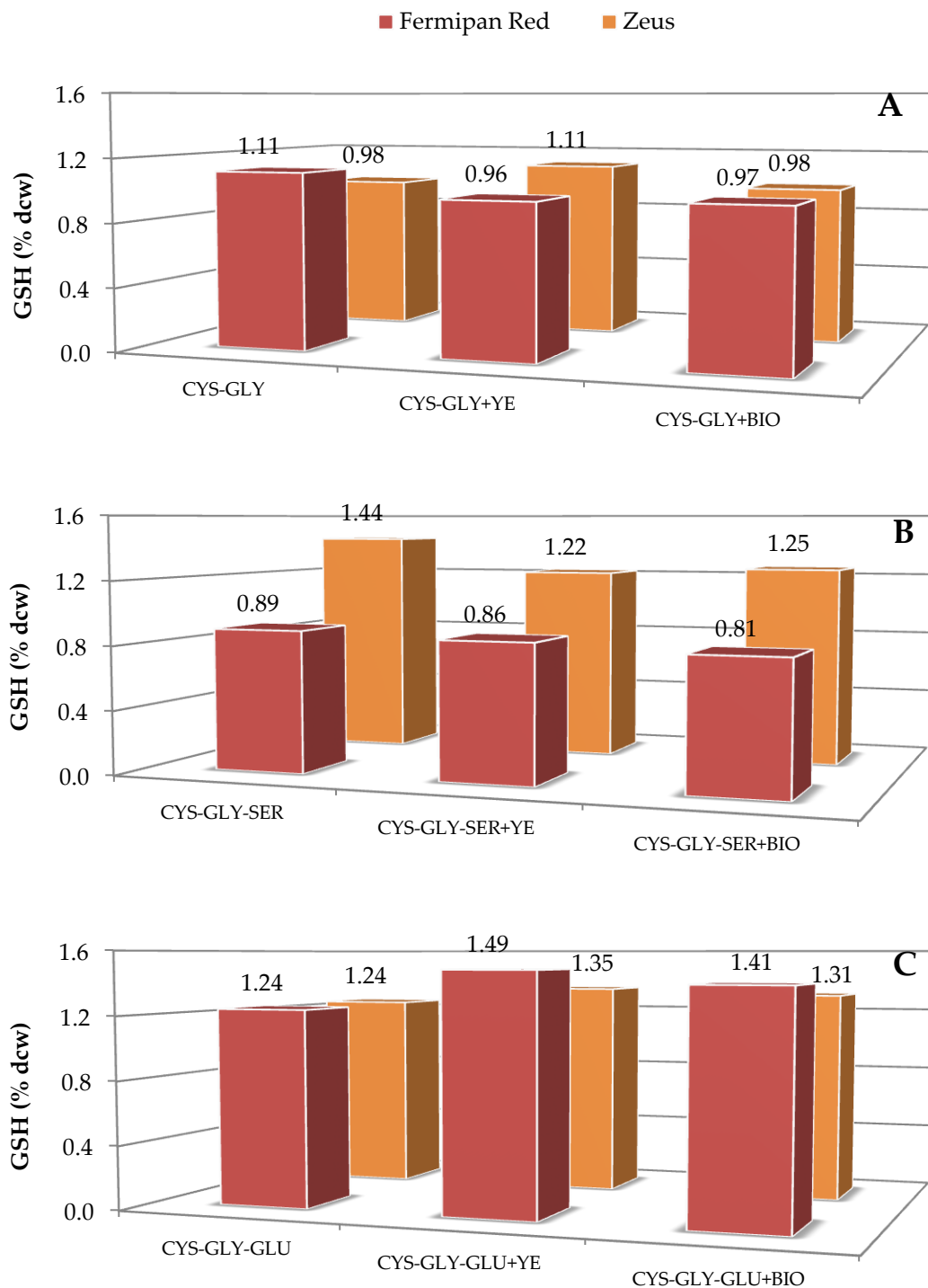
As expected, results obtained in this set of trials showed that amino acids composition affected in different extent yeast ability to accumulate GSH. Even if yeasts had similar initial GSH level of about 0.5-0.6% (Tab. 2.2), *Fermipan Red* accumulated the highest GSH level with the mixture CYS-GLY-GLU (1.24% dcw) while *Zeus* employing the mixture CYS-GLY-SER (1.44% dcw).



**Figure 2.4.** Intracellular GSH content (% dcw) at 24 h incubation in activation trials with amino acids differently combined, *Fermipan Red* and *Zeus* yeasts (to GSH content: *Fermipan Red* 0.60-0.69%, *Zeus* 0.52-0.59%).

**Table 2.2.** Intracellular GSH (% dcw) means and standard deviations at 0 and 24 h activation time employing mixtures with amino acids differently combined (*Fermipan Red* and *Zeus*).

Yeast	Mixture	GSH (% dcw)	
		t <sub>0</sub>	24 h
<i>Fermipan Red</i>	CYS-GLY	0.61±0.06	1.11±0.00
	CYS-GLY-SER	0.60±0.02	0.89±0.08
	CYS-GLY-GLU	0.66±0.06	1.24±0.08
	NAC-GLY	0.66±0.09	0.70±0.03
	½NAC-½CYS	0.69±0.05	0.98±0.05
<i>Zeus</i>	CYS-GLY	0.56±0.05	0.98±0.13
	CYS-GLY-SER	0.59±0.09	1.44±0.12
	CYS-GLY-GLU	0.69±0.04	1.24±0.10
	NAC-GLY	0.58±0.01	0.80±0.09
	½NAC-½CYS	0.52±0.08	0.96±0.13



**Figure 2.5.** Intracellular GSH content (%dcw) at 24 h incubation in activation mixture (A: CYS-GLY; B: CYS-GLY-SER; C: CYS-GLY-GLU) added with yeast extract (YE) or biotin (BIO). *Fermipan Red* and *Zeus* yeasts.

As regards the use of yeast extract (YE) or biotin (BIO), GSH yields were found not to significantly increase (Fig. 2.5 and Tab. 2.3), with respect to the control mixtures (CYS-GLY, CYS-GLY-SER, CYS-GLY-GLU). A slight GSH increase, due to YE and BIO addition, was only evidenced for *Fermipan Red* employing the mixture CYS-GLY-GLU. In these trials employing control mixture GSH yield was about 1.24% dcw, while with YE and biotin addition yields were about 1.49 and 1.41% dcw respectively. This increase however is marginal respect to the costs attributable to the introduction of these ingredients in the activation mixture formulation.

**Table 2.3.** Intracellular GSH content (% dcw): means and standard deviations at 0 and 24 h activation employing mixtures added with yeast extract (YE) or biotin (BIO), *Fermipan Red* and *Zeus*.

Yeast	Mixture	GSH (% dcw)	
		t <sub>0</sub>	24 h
<i>Fermipan Red</i>	CYS-GLY	0.61±0.06	1.11±0.00
	CYS-GLY-SER	0.60±0.02	0.89±0.08
	CYS-GLY-GLU	0.66±0.06	1.24±0.08
	CYS-GLY+YE	0.60±0.01	0.96±0.06
	CYS-GLY-SER+YE	0.64±0.06	0.86±0.01
	CYS-GLY-GLU+YE	0.72±0.09	1.49±0.15
	CYS-GLY+BIO	0.62±0.05	0.97±0.03
	CYS-GLY-SER+BIO	0.65±0.03	0.81±0.01
	CYS-GLY-GLU+BIO	0.66±0.01	1.41±0.01
<i>Zeus</i>	CYS-GLY	0.56±0.05	0.98±0.33
	CYS-GLY-SER	0.59±0.19	1.44±0.35
	CYS-GLY-GLU	0.69±0.04	1.24±0.30
	CYS-GLY+YE	0.66±0.06	1.11±0.01
	CYS-GLY-SER+YE	0.52±0.00	1.22±0.01
	CYS-GLY-GLU+YE	0.65±0.02	1.35±0.02
	CYS-GLY+BIO	0.76±0.00	0.98±0.13
	CYS-GLY-SER+BIO	0.69±0.02	1.25±0.09
	CYS-GLY-GLU+BIO	0.73±0.06	1.31±0.09

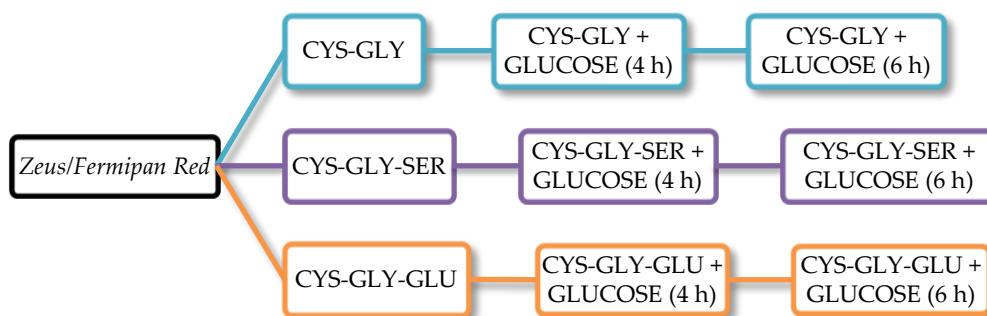
These trials led to assess different yeast sensitivity towards activation mixture composition, even if they had similarly initial GSH levels. In particular they showed different sensitivity to the presence of serine. This amino acid is not directly involved in GSH synthesis, but in sulphate metabolism of yeasts. Moreover both yeasts showed

better results with the mixture CYS-GLY-GLU, that contains all the GSH precursor amino acids, respect to the mixture CYS-GLY.

These results underline the relevance of amino acids for activation efficacy to accumulate intracellular GSH. This behaviour is in agreement with data reported in literature; in fact many authors consider amino acids, and in particular cysteine, as key ingredients for GSH synthesis (Alfajara et al., 1992; Wang et al., 2007; Wen et al., 2005; Liang et al., 2009).

### *Influence of carbon source*

The aim of these trials was to evaluate the influence of glucose addition during activation of dried and compressed yeast. For comparative purposes the mixtures CYS-GLY, CYS-GLY-SER and CYS-GLY-GLU were employed, and a shot of 50 g/L of glucose was added at 4 or 6 h incubation time. The general schematic organization of trials is reported in Fig. 2.6. Samples were collected at 0, 24 and 48 h activation.



**Figure 2.6.** General schematic organization of trials, employing *Zeus* and *Fermipan Red* yeasts.

In general at 48 h incubation, GSH yields were found of the same or lower levels than at 24 h (Fig. 2.7). Again, a different ability of the two employed yeasts was highlighted.

Only the *Zeus* strain appeared to be sensitive to glucose shots and this behaviour varied depending on the mixture employed. In samples with CYS-GLY mixture and glucose addition at 4 and 6 h (Fig. 2.7 A), at 24 h activation an interesting GSH increase to about 1.7-1.8% was obtained, with respect to 1.1% without glucose shots.

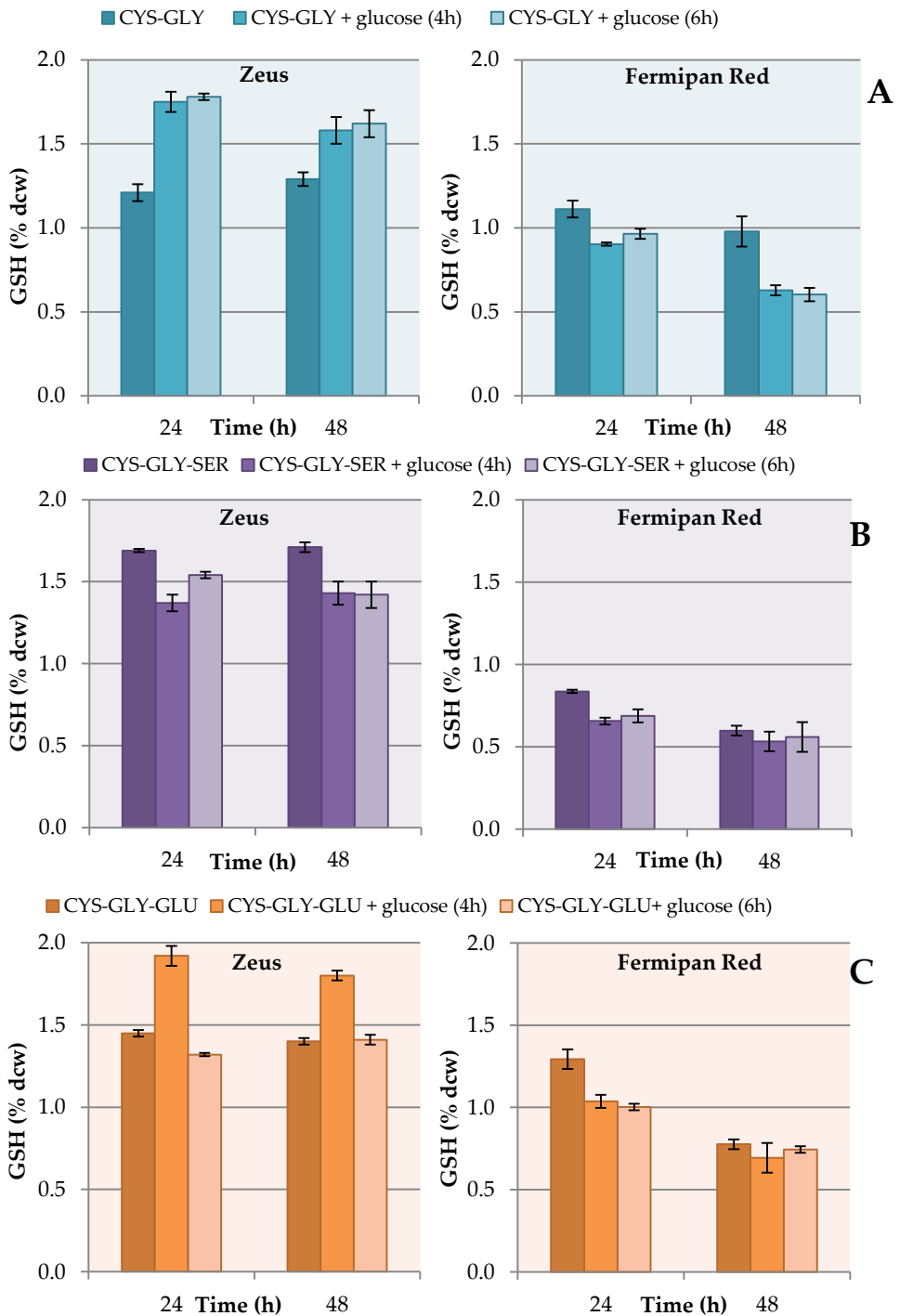
Employing CYS-GLY-SER mixture (Fig. 2.7 B), then in presence of serine, the sample without glucose reached about 1.7% already at 24 h, while glucose addition did not produce GSH increase in all tested samples (about 1.4-1.5%).

At 24 h, with CYS-GLY-GLU mixture and glucose added at 4 h, a GSH increase from 1.45% to 1.92% was observed (Fig. 2.7 C). To be noticed in this case that glucose addition at 6 h did not produce any GSH increase, neither at 24 nor at 48 h.

In general *Zeus* yeast accumulated much more than three times its initial GSH content (0.52% dcw).

As regards to *Fermipan Red*, glucose addition did not affect GSH accumulation neither at 4 nor 6 h, in all the tested activation mixture. Maximum GSH level gained was about 1.11% with CYS-GLY, 0.84% with CYS-GLY-SER and 1.29% with CYS-GLY-GLU mixtures, starting from an initial GSH level of about 0.6% (Fig. 2.7 A, B, and C).

These trials in general confirmed what previously obtained. Moreover it was possible to conclude that *Zeus*, yeast in compressed form, was generally able to produce GSH in higher yields with respect to *Fermipan Red*, yeast in dried form, and so subsequent research investigations were focused on compressed yeast applications.



**Figure 2.7.** Intracellular GSH (% dcw) at 24 and 48 h in presence/absence of glucose shots. Activation mixtures CYS-GLY (A), CYS-GLY-SER (B) and CYS-GLY-GLU (C); *Zeus* and *Fermipan Red*.

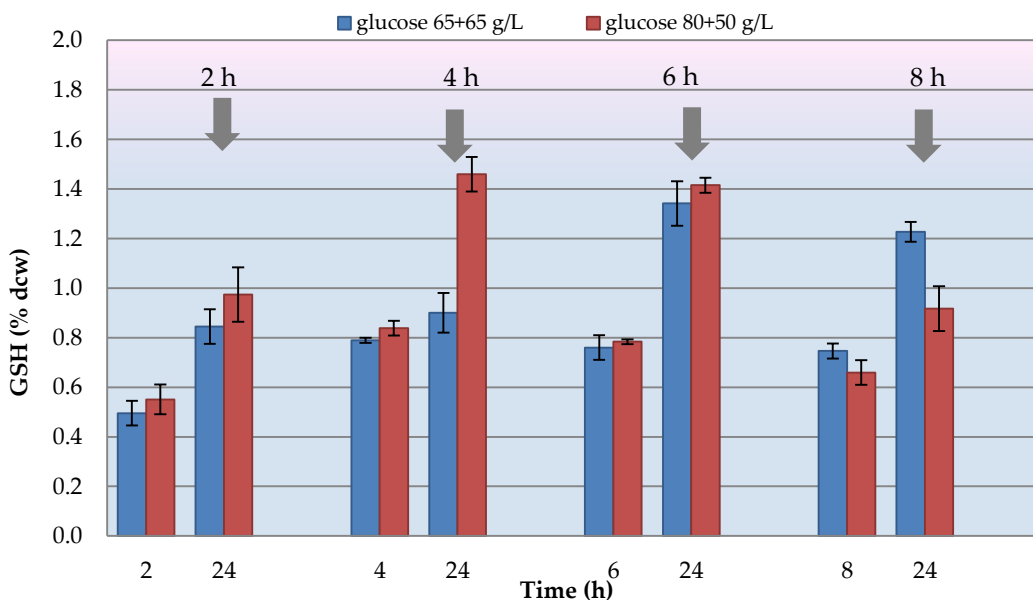


## 2.5 Yeast in compressed form: set up of the activation conditions

On the basis of previously obtained results the prosecution of the study was focused on the effect of glucose addition, during activation procedure, employing yeast in compressed form.

### Glucose addition at different incubation time (*Zeus yeast*)

In this part of the research trials were carried out by employing several combinations of glucose additions. In particular activations with CYS-GLY-80 (80 g/L glucose, reference condition) and CYS-GLY-65 (65 g/L glucose) mixtures were comparatively employed. Consequently, with the aim to supply to the yeasts 130 g/L total glucose during the process, shots with different glucose concentrations were done: 65 g/L for CYS-GLY-65 and 50 g/L for CYS-GLY-80, at individually 2, 4, 6 and 8 h incubation times. Samples were collected at 0, 24 h incubation times and before each glucose addition (2, 4, 6 and 8 h). Results are reported in Fig. 2.8.



**Figure 2.8.** Effect of glucose addition (2, 4, 6, 8 h) on intracellular GSH (% dcw) levels. 24 h incubation time, *Zeus yeast*.

Glucose additions at 2 and 8 h in any combinations were ineffective respect to control trials (without glucose shots) (GSH about 1-1.1% dcw).

On the contrary, additions at 4 and 6 h led, at 24 activation, to a GSH increase in CYS-GLY-80 mixture (control sample), to which 50 g/L were added; in these trials GSH

gained respectively 1.46 and 1.42%. In the CYS-GLY-65 mixture (65 g/L initial glucose) only addition at 6 h was effective and produced an interesting GSH yield of 1.34%.

Results obtained in these trials confirmed that *Zeus* compressed yeast was sensitive to glucose addition, above all at 4 and 6 h of incubation. The best condition was the activation in the mixture CYS-GLY with 80 g/L of glucose (control mixture) at the beginning and sequent shot of 50 g/L. It was noticed that generally in this series of trials obtained yields, about 1.45% dcw, were generally lower than previous one, about 1.8% dcw. Moreover this aspect could be attributable to the yeast characteristics that, as later verified, are dependent to storage conditions during market distribution and shelf life.

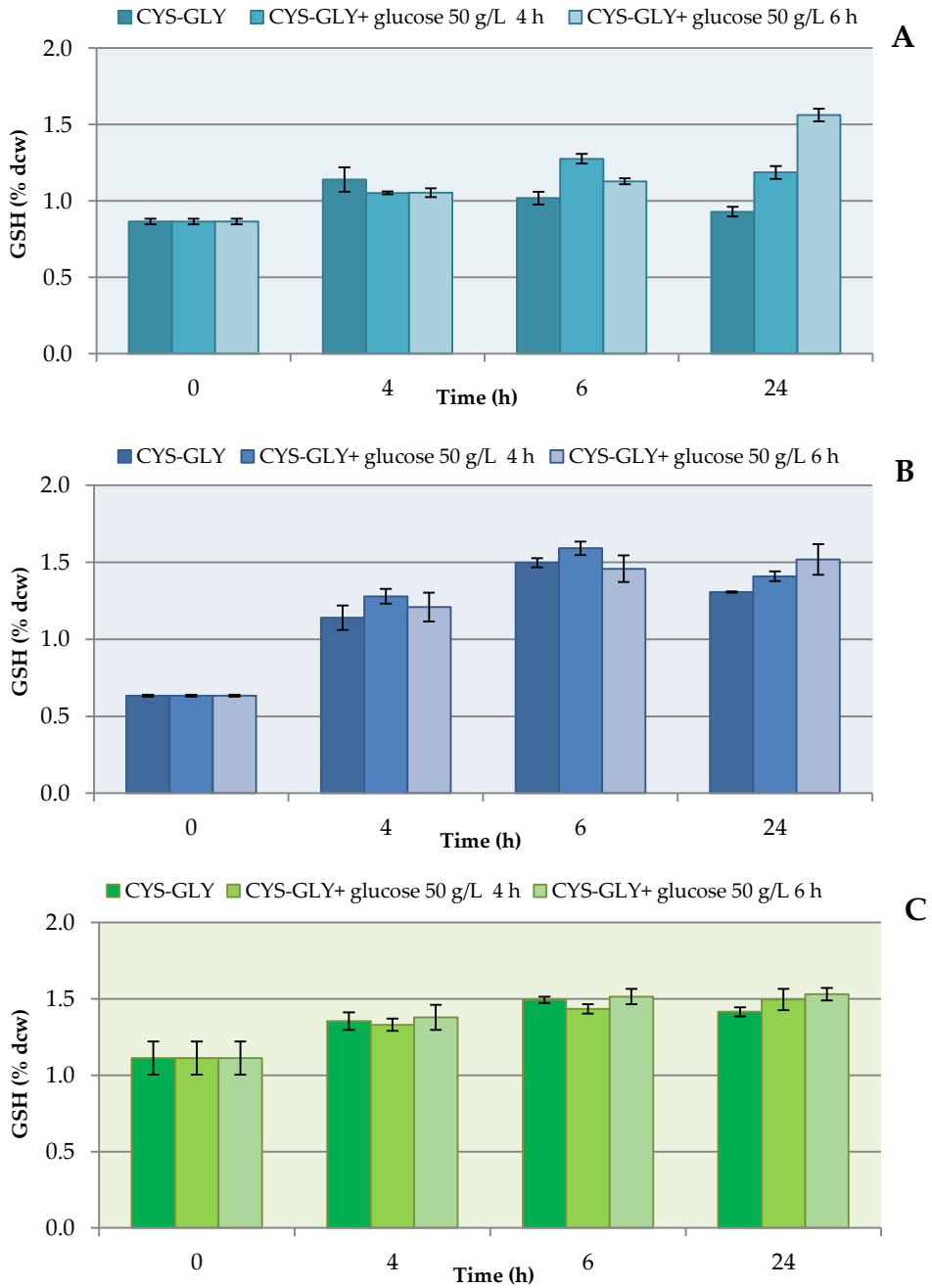
#### ***Comparison among trademarks performance***

With the aim to understand/evaluate experimental results variability, in subsequent experiments other commercial baker's yeasts were considered (*Fala*, *Primo* and *GB*).

Experiments were carried out with the CYS-GLY mixture (80 g/l glucose) in presence/absence of 50 g/L glucose shot, added at 4 and 6 h of incubation time. Samples were collected at 0, 24 h of incubation and before each glucose addition (4 and 6 h).

Results reported in Fig. 2.9 allowed to better understand the evidenced variability. First of all the three yeasts presented very different initial GSH levels: 0.86% *Fala*, 0.63% *Primo* and 1.11% *GB*. Moreover glucose additions seemed to be more effective for *Fala* yeast in which yields gained 1.19-1.56% in samples with glucose additions, with respect to 0.93% in the control mixture sample (without glucose shot).

In the other yeasts, GSH increase due to glucose shot was less evident at 24 h and higher GSH levels were generally evidenced at 6 h incubation time.



**Figure 2.9.** Intracellular GSH (% dcw) at 24 h in CYS-GLY mixture with/without glucose (50 g/L) shot (4 and 6 h). Yeasts: *Fala* (A), *Primo* (B), *GB* (C).

From the analysis of obtained results it appeared evident that in *Primo* and *Fala* yeasts the initial GSH level doubled, while for *GB* it increased of only about 30%; nevertheless the final levels in the best conditions were analogous, about 1.4-1.5%.

These results contradict some previous obtained data as for in these trials compressed yeasts presented different responses to activation procedure. This behaviour could be due to the physiological state of the yeast related to storage conditions, its technological production process and also shelf-life, as previous hypotheses. For this reasons this aspect was matter of the prosecution on the study.

**Activation mixture: amino acids combinations**

Three GSH precursor amino acids (cysteine, glycine and glutamic acid) were differently combined with the aim of investigating their influence on GSH accumulation. Samples were collected at 24 h incubation. The general schematic organization of trials and the corresponding yields are reported in Tab. 2.4. In general, no significantly different yields were found, with final GSH levels of about 1% dcw, 1.5-fold increase respect the initial GSH content. On the contrary, differences were evident when employing mixtures with/without glutamic acid (trials 5-6 and 1-4 respectively). In presence of glutamic acid higher yields were obtained, especially when all the precursors were set at 4 g/L, GSH level of 1.25% dcw was reached.

**Table 2.4.** Schematic organization of trials to evaluate the influence of amino acid combinations (g/L) in the activation mixtures and intracellular GSH (% dcw); means and standard deviations at 24 h activation time, *Fala* yeast.

<b>Trials</b>	<b>CYS</b>	<b>GLY</b>	<b>GLU</b>	<b>GSH (% dcw)</b>
1	4	4	-	0.96±0.09
2	2	4	-	1.02±0.08
3	4	2	-	0.93±0.05
4	2	2	-	0.93±0.01
5	4	4	4	1.25±0.05
6	2	2	2	1.12±0.02

It has to be noted that trials 1 and 5 correspond to the previously employed CYS-GLY and CYS-GLY-GLU mixtures, and obtained data confirmed the previous ones.

### *Energetic aspects*

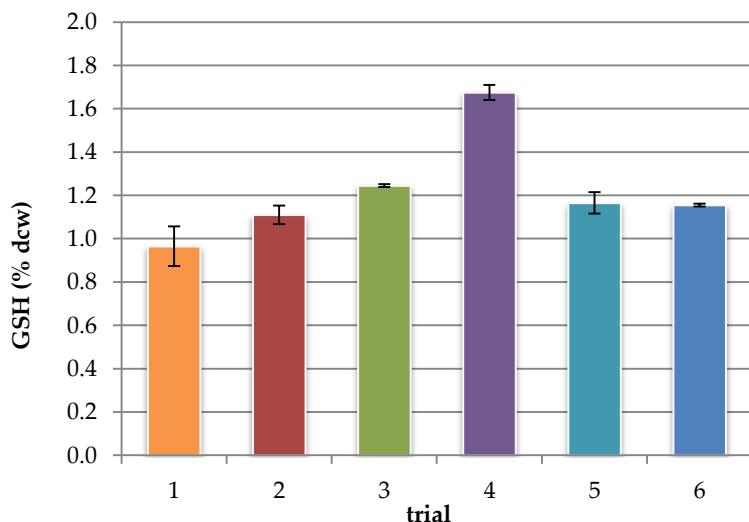
A critical analysis of the obtained results suggested some hypothesis. First of all it was evident that glucose addition might increase GSH accumulation. This aspect is probably attributable to an energy block limiting GSH synthesis, although precursor amino acids are present. However glucose addition did not always ensure high GSH yields.

Taking in account these considerations, trials were carried out for evaluating if GSH accumulation might be limited by an ATP deficiency. ATP regeneration was promoted adding adenine (ADE) or adenosine (ADO) and/or dithiotreitol (DTT) to biotransformation solution. Adenine and adenosine are directly involved in ATP regeneration while dithiotreitol is an adenosin-triphosphatase (ATP-ase) inhibitor.

Biotransformation trials were set-up employing CYS-GLY-GLU mixture added with adenine (0.5 g/L) or adenosine (1.5 g/L) and/or dithiotreitol (3 g/L) (Tab. 2.5). Results are reported in Fig. 2.10.

**Table 2.5.** Schematic organization of trials with addition (g/L) of adenosine (ADO), dithiotreitol (DTT) and adenine (ADE) to CYS-GLY-GLU mixture, *Fala* yeast.

trials	ADO	DTT	ADE
1	-	-	-
2	1.5	-	-
3	-	3	-
4	-	-	0.5
5	1.5	3	-
6	-	3	0.5



**Figure 2.10.** Intracellular GSH level (% dcw) at 24 h in CYS-GLY-GLU mixture added with ADO or ADE and/or DTT, *Fala* yeast. For legend refer to Tab 2.5.

Addition of the considered molecules led to a low GSH increase respect to control samples, with the exception of trial n°4.

The presence of adenine generally produced high GSH accumulation: 1.68% dcw, while the control CYS-GLY-GLU (trial 1) reached GSH level of 0.97%. Would it be possible to suppose that adenine together with precursor amino acids promote GSH accumulation? Is the presence of adenine the only factor influencing yeast performance? Some aspects are still unclear because this combination did not furnish reproducible results. In fact also in presence of adenine, in subsequent trials GSH yields were not so high and so different from the control. For these reasons some yeast metabolic characteristics were investigated.

#### ***Influence of yeast storage time on GSH production***

Commercial compressed baker's yeast shelf life is about 30 days if maintained at refrigerated temperature (+5 °C). Control experiments carried out in the first step of this study verified that fermentative ability of the tested yeasts was maintained until expiry. However in all trials performed for GSH accumulation commercial yeasts were employed no later than 15 days from the production.

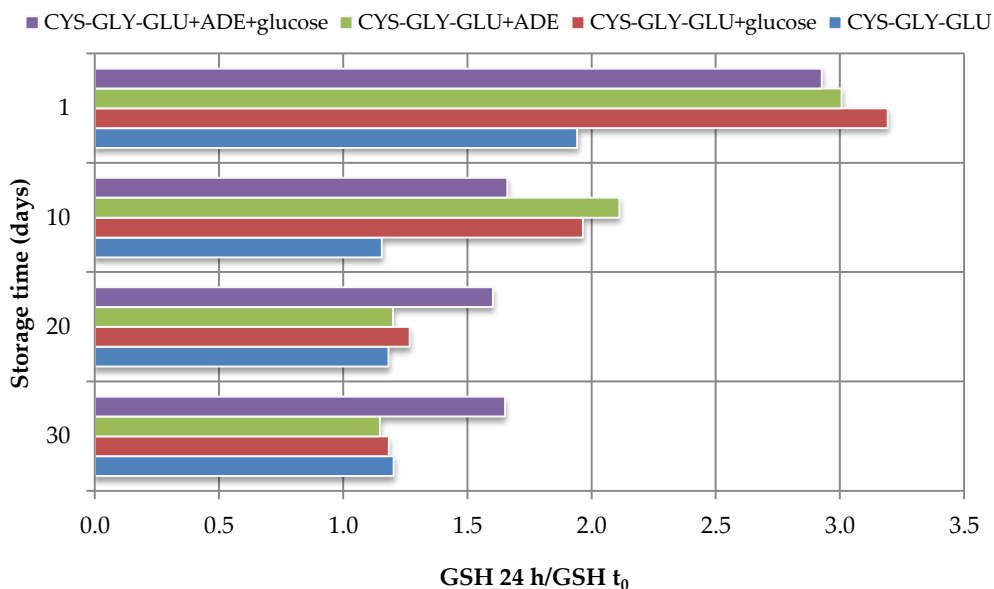
So far, it was supposed that during shelf-life modifications might took place inside yeast cells at molecules/characteristics levels, involved in stress resistances and influencing GSH metabolism (see 1.2).

Trials were then carried out with the CYS-GLY-GLU mixture (*control*) added with glucose (50 g/L at 4 h) and/or adenine (0.5 g/L), employing compressed yeast at different storage times (1, 10, 20 and 30 days). Samples were collected at 4, 8 and 24 h reaction incubation.

Results confirmed that not only the ability to accumulate GSH, but also its intracellular levels decreased during yeast storage. To better understand this behaviour, results have been reported as ratio between GSH obtained at 24 h and the initial content ( $t_0$ ) (Fig. 2.11). As expected, during storage the ability to produce GSH reduced progressively, reaching about a 1.2-1.5 ratio at 20 days storage; this condition was maintained until 30 days. The highest ratio of about 3 (i.e. a 3-fold GSH increase with respect to the initial content) was evidenced on fresh yeast (1 day storage) in activation carried out in presence of adenine, adenine-glucose and glucose; for the control CYS-GLY-GLU sample the ratio was 2. The situation changes from 10 days afterwards, when GSH ratio decreased from 3 to 2, a lower value of 1.2 being again obtained for the control CYS-GLY-GLU sample.

In general employing the mixture CYS-GLY-GLU with glucose added at 4 h a GSH increase was evident in all the samples. Yeast showed different attitude according to the mixture employed, however when prolonging its shelf life, a general decrease of GSH intracellular levels was found.

In this phase of the research a possible key role of trehalose (molecule involved in yeast stress resistance during fermentative process), one of carbohydrate molecules starved in the cells, was evaluated. In *Saccharomyces cerevisiae*, trehalose plays a double role: as carbon and energy reserve when in particular conditions, and as stress protectant (Lillie and Pringle, 1980; Attfield, 1997; Malluta et al., 2000). In fact, this molecule in some strains and as a consequences of defined cultural conditions employed during baker's yeast production, accumulates up to 15% dcw. Accumulated trehalose is utilized by cells during nutrient starvation and under stress conditions; moreover it protects biological membranes and proteins (Plourde-Owobi et al., 2000; Jules et al., 2008).

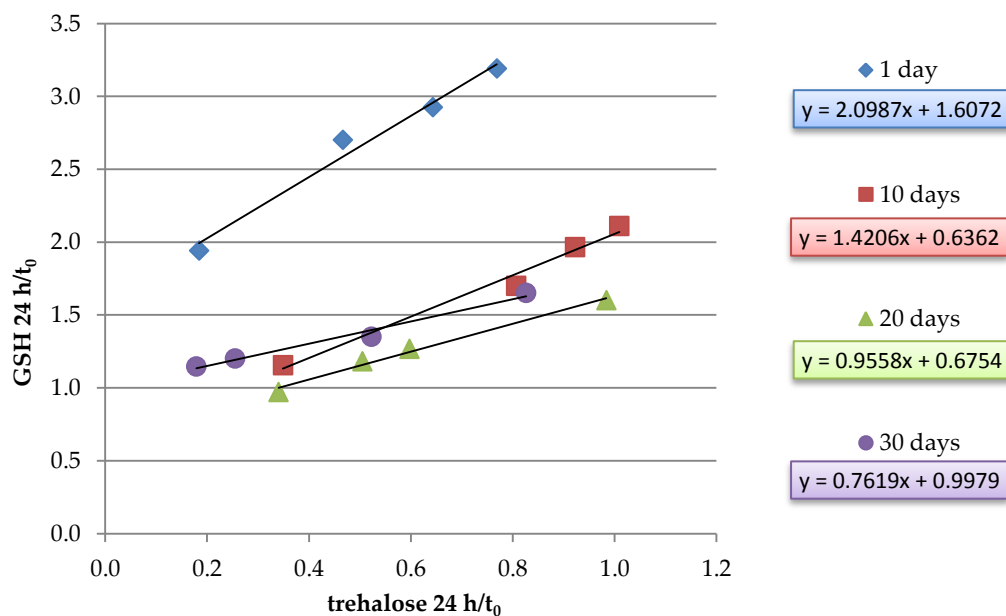


**Figure 2.11.** Ratio between GSH at 24 and initial  $t_0$  levels obtained with glucose (CYS-GLY-GLU+glucose), adenine (CYS-GLY-GLU+ADE), and adenine + glucose (CYS-GLY-GLU+ADE+glucose) added at 4 h to CYS-GLY-GLU control mixture. Sample at 1, 10, 20 and 30 storages days, *Fala* yeast.

Trehalose content was then determined in yeast cells during storage and activation reactions. A general trehalose decrease from 10.3% dcw at 1 day to 8.58, 7.81 and 7.73% dcw respectively at 10, 20 and 30 days of storage was evidenced. During activation, trehalose yeast content rapidly decreased in the first hours, to reach 3-5% dcw, to partially recover till 6-8% dcw (*regained trehalose*). Cell ability to regain trehalose levels was lost during yeast storage time.

With the aim to find out a correct correlation between GSH and trehalose levels, results were elaborated and compared with GSH accumulation ability (Fig. 2.12). The correlations between GSH ratio between 24 h and  $t_0$  and trehalose ratio at 24 h and  $t_0$  were always positive; anyway during yeast storage the coefficient estimate decreases from 2.0987 at 1 day to 1.4206, 0.9558 and 0.7619 respectively at 10, 20 and 30 days. These results are very important because they highlight that not only storage time, but also trehalose content is a very important factor for determining GSH accumulation ability of yeasts.





**Figure 2.12.** Correlations between GSH 24 h/t<sub>0</sub> and trehalose 24h/t<sub>0</sub> ratio during yeast storage (1, 10, 20 and 30 days).

## 2.6 Conclusions

From an overlook of the obtained results it can be concluded that activation was found an efficient procedure to obtain GSH enriched yeast.

Dried yeast led to only modest GSH yields; cells also do not seem to be sensitive to glucose addition during the activation procedure. Compressed yeast, instead, possesses different responses towards the applied activation procedure in relation to its energy storage conditions, its technological production process and also shelf-life.

All trials performed revealed that many factors are involved in the activation for GSH accumulation, as follows:

- yeast physiological condition
- type and amino acids concentration added during activation
- carbon and energy source added at the beginning or during the activation.

Applying different strategies (amino acids combinations, addition of ATP-related compound) high intracellular GSH levels can be obtained (1.6-1.9% dcw) with a 3-fold increase respect to initial GSH content.

## 2.7 References


- Alfajara CG, Kanda A, Shioi T, Shimizu H, Shioya S, Suga K.** 1992. Effect of amino acids on glutathione production by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 36:538–540.
- Attfield PV.** 1997. Stress tolerance: the key to effective strains of industrial baker's yeast. *Nature Biotech* 15:1351-1357.
- Jules M, Beltran G, François J, Parrou JL.** 2008. New insights into trehalose metabolism by *Saccharomyces cerevisiae*: NTH2 encodes a functional cytosolic trehalose, and deletion of TPS1 reveals ath1p-dependent trehalose mobilization. *Appl Environ Microbiol* 74:605-614.
- Liang G, Liao X, Du G, Chen J.** 2009. A new strategy to enhance glutathione production by multiple H<sub>2</sub>O<sub>2</sub>-induced oxidative stresses in *Candida utilis*. *Biores Technol* 100:350-355.
- Lillie SH, Pringle JR.** 1980. Reserve carbohydrate metabolism in *Saccharomyces cerevisiae*: responses to nutrient limitation *J Bacteriol* 143:1384-1394.
- Malluta EF, Decker P, Stambuk BU.** 2000. The Kluyver effect for trehalose in *Saccharomyces cerevisiae*. *J Basic Microbiol* 40:199-205.
- Plourde-Owobi L, Durner S, Goma G, François J.** 2000. Trehalose reserve in *Saccharomyces cerevisiae*: phenomenon of transport, accumulation and role in cell viability. *Int J Food Microbiol* 55:33-40.
- Wang Z, Tan T, Song J.** 2007. Effect of amino acids and feedback control strategies on the high-cell-density cultivation of *Saccharomyces cerevisiae* for glutathione production. *Process Biochem* 42:108-111.
- Wen S, Zhang T, Tan T.** 2005. Optimization of the amino acid composition in glutathione fermentation. *Process Biochem* 40:3474-3479.

### *3. Extracellular GSH production*


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### 3.1 Poster

Reported below is the poster exhibited during the Congress THIOI 2008, Glutathione and related thiols in microorganisms and plants. 26-29 August 2008, Nancy.



Departments Scienze e Tecnologia Alimentare e Microbiologia




UNIVERSITA' DEGLI STUDI DI MILANO

## APPLICATION OF AN EXPERIMENTAL DESIGN TO IMPROVE EXTRACELLULAR GSH PRODUCTION FROM *Saccharomyces cerevisiae*

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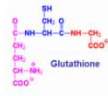


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#### INTRODUCTION

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol compound widely present in living organisms, from prokaryotes to eukaryotes. It is synthesised intracellularly by the consecutive actions of γ-glutamylcysteine synthetase, feedback inhibited by GSH, and GSH synthetase. This tripeptide's very low redox potential gives it the properties of a cellular redox buffer (Anderson 1996).

In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, xenobiotic and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism (Peminicko 2002). These characteristics make this active tripeptide an important aid and/or support for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, pancreatic inflammations and aging (Wu et al., 2004). In addition, GSH is of interest in the food additive industry and sports nutrition (Lomastro and Malone 1995). Yeasts, in particular belonging to the genus *Saccharomyces*, are the most commonly used microorganisms on an industrial scale for GSH fermentative production; however GSH contents of the wild-type strains are usually variable (0.1 – 1% dw) and always in intracellular form (Rollini et al. 2005; Rollini and Manzoni 2006).



Glutathione

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#### MATERIALS AND METHODS

##### Microorganisms

Two *S. cerevisiae* yeast strains (NCYC 2959 - National Collection of Yeast Cultures – and L288) were tested together with one commercial baker's yeast strains (Lievitale Spa).

##### Biotransformation conditions

Cells were suspended (5 % dw) in a solution setup as follows (g/L): glucose 80, sodium citrate 10, cysteine 4, glycine 4, serine 4, ammonium sulphate 7, KH<sub>2</sub>PO<sub>4</sub> 3.5, magnesium sulphate 0.5, tap water. Biotransformation trials were carried in 1000 mL Erlenmeyer flasks, each containing 100 mL reaction mixture. Incubation at 28°C on an alternative shaker (60 rpm, 4 cm run).

In order to obtain GSH in extracellular form, experiments were performed by adding Octyl glucoside or Sarcosine (0.5 g/L) to the reaction mixture. Lyophilised cells were also comparatively employed.

##### HPLC chromatography:

Purophor® RP-18 endcapped (250 × 4 mm) (Merck) 30 °C; eluent NaH<sub>2</sub>PO<sub>4</sub> 25 mM pH 3.5, flux 0.3 mL/min; UV 210 nm.

##### Experimental design

A full factorial design, with three variables tested at three levels and one at two levels (for a total of 54 trials) was carried out (Montgomery 2001). The tested variables were: type of yeast strain (NCYC 2959, L288 and commercial), energy source (glucose, ethanol and molasses), aminoacidic mixture (cysteine-glycine or cysteine-glycine-serine) and cell treatment (lyophilisation, sarcosine or octyl-glucoside). Results were analysed employing Design Expert® v. 7.0 (Statbase – Minneapolis) software.

##### Analytical procedures

Intracellular GSH was determined as follows: aliquots of the reaction mixture were centrifuged (10,000 rpm, 6 min); obtained cells were washed twice with H<sub>2</sub>O, and then thermally treated at 100 °C for 12 min. After cooling in ice bath, samples were centrifuged (12,000 rpm, 15 min) and on obtained supernatants intracellular GSH was evaluated. Extracellular GSH was instead determined on supernatants obtained from cell separation.

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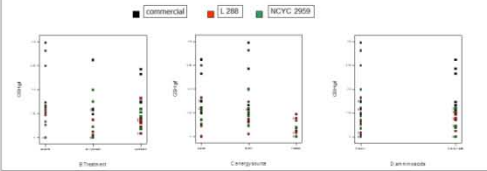
#### RESULTS

##### GSH intracellular (mg GSH/g dw) and extracellular (g GSH/L) yields obtained at 24 h reaction

ENERGY SOURCE	AMINOACIDIC MIXTURE	CELL TREATMENT	NCYC 2959 Intrac. (mg/g)	NCYC 2959 Extrac. (g/L)	L.288 Intrac. (mg/g)	L.288 Extrac. (g/L)	Commercial Intrac. (mg/g)	Commercial Extrac. (g/L)
Glucose	Cys-Gly	Sarcosine	0.6	1.4	1.4	1.4	1.4	1.4
Glucose	Cys-Gly-Ser	Sarcosine	1.2	1.4	1.4	1.4	1.4	1.4
Glucose	Cys-Gly	Octyl-glucoside	4	1.4	1.4	1.4	1.4	1.4
Glucose	Cys-Gly-Ser	Octyl-glucoside	1.4	1.4	1.4	1.4	1.4	1.4
Glucose	Cys-Gly	Lymphation	0.8	1.4	1.4	1.4	1.4	1.4
Glucose	Cys-Gly-Ser	Lymphation	1.2	1.4	1.4	1.4	1.4	1.4
Ethanol	Cys-Gly	Sarcosine	4.2	1.2	1.2	1.2	4.8	4.8
Ethanol	Cys-Gly-Ser	Sarcosine	1.2	1.2	1.2	1.2	1.6	1.6
Ethanol	Cys-Gly	Octyl-glucoside	3.2	0.2	0.2	0.2	1.4	1.4
Ethanol	Cys-Gly-Ser	Octyl-glucoside	0.8	1.0	1.0	1.0	1.4	1.4
Ethanol	Cys-Gly	Lymphation	1.0	1.0	1.0	1.0	1.6	1.6
Ethanol	Cys-Gly-Ser	Lymphation	1.4	1.0	1.0	1.0	3.2	3.2
Molasses	Cys-Gly	Sarcosine	-	-	0.8	-	-	-
Molasses	Cys-Gly-Ser	Sarcosine	-	-	1.0	-	-	-
Molasses	Cys-Gly	Octyl-glucoside	3	-	1.0	-	-	-
Molasses	Cys-Gly-Ser	Octyl-glucoside	-	-	3	-	-	-
Molasses	Cys-Gly	Lymphation	0.4	1	0.2	1	0.2	0.2
Molasses	Cys-Gly-Ser	Lymphation	0.4	1	0.2	1	0.2	0.2

\*: GSH yield < 1mg/g dw

##### GSH extracellular yields (g/L) in relation to the three tested variables (cell treatment, energy source and aminoacidic mixture)



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##### ANOVA analysis of extracellular GSH yields

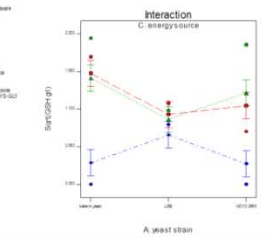
Source	Sum of Squares	df	Mean Square	F-value	p-value
Model	12.89	18	0.716	9.48	< 0.0001
A: Type of yeast	6.79	2	3.395	44.02	< 0.0001
B: treatment	3.08	2	1.54	20.29	< 0.0001
C: Energy source	0.89	4	0.223	2.92	< 0.0001
AB	2.25	4	0.563	7.41	< 0.0001
ABC	0.49	8	0.061	0.81	0.57
Con Total	17.37	33			

ANOVA highlighted the following most important factors:

- A:** yeast strain
- B:** treatment
- C:** energy source
- AC:** interaction yeast - energy source

The other terms were considered not significant (p-value > 0.1).

##### Interaction



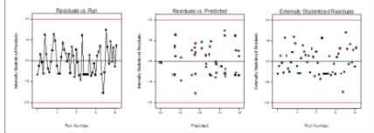
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The Model F-value of 9.48 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.

Final equation in terms of coded factors:

$$\text{Sqr}(\text{GSH g/L}) = 0.91 + 0.17 * A[1] - 0.099 * A[2] + 0.088 * B[1] - 0.19 * B[2] + 0.27 * C[1] + 0.28 * C[2] + 0.19 * A[1]C[1] - 0.14 * A[2]C[1] + 0.11 * A[1]C[2] - 0.29 * A[2]C[2]$$

Proceeding to the diagnostics, all terms appeared causal and no outliers were evidenced



#### CONCLUSIONS

From an overall look at the obtained results, it can be concluded that:

- baker's yeast in compressed form always produced the highest GSH extracellular levels
- glucose and ethanol furnished comparable yields, higher than those obtained with molasses
- the use of sarcosine and lyophilisation evidenced similar results
- the aminoacidic mixture Cys-Gly proved more effective than the mixture Cys-Gly-Ser

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#### REFERENCES

Anderson M.E. (1996) Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact.* 111-112: 1-14.

Lomastro D.M, Malone M. (1995) Glutathione in health and disease: pharmacotherapeutic issues. *Ann Pharmacother* 29: 1263-73.

Montgomery DC (2001) *Design and analysis of experiments*, 8th Edition. John Wiley and Sons Inc. McGraw-Hill, Milano.

Peminicko M. (2002) *An overview of glutathione in Saccharomyces versus non-conventional yeasts*. *FEMS Yeast Res* 2: 295-305.

Rullini M., Manzoni M. (2006) *Influence of different fermentation parameters on glutathione volumetric productivity by Saccharomyces cerevisiae*. *Process Biochem* 41: 1501-1505.

Rullini M., Pagani H., Ribicki S., Manzoni M. (2005) *Influence of carbon source on glutathione accumulation in methylotrophic yeasts*. *Ann Microbiol* 55: 199-203.

Wu Gu, Fang Y-Z, Lupton J.R., Turner K.D. (2004) *Glutathione metabolism and its implications for health*. *J Nutr* 134: 489-492.

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### 3.2 Published Paper

Here is reported the original submitted (*Process Biochemistry*) form of the paper because of copyright limitations.

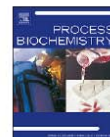
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## Production of glutathione in extracellular form by *Saccharomyces cerevisiae*

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#### ABSTRACT

The present research was aimed at inducing, in a post fermentative procedure (biotransformation) and by modifying cell permeability, glutathione (GSH) accumulation and subsequent release from cells of *Saccharomyces cerevisiae*. With the aim of limiting process costs, research considered the possibility of employing baker's yeasts (*S. cerevisiae*), inexpensive cells source available on the market, in comparison with a collection strain. The tested yeasts showed different sensitivity to the chemical/physical treatments performed to alter cell permeability. Modest effects were evidenced with Triton, active only on Zeus yeast samples (1.7 g GSH/l, near 60% of which in extracellular form). Lauroyl sarcosine showed an interesting action on GB Italy sample (2.8 g GSH/l, near 80% extracellular). Lyophilization evidenced good performance with Lievitalia yeast strain (2.9 g GSH/l, 90% extracellular). The possibility of obtaining GSH directly in extracellular form represents an interesting opportunity of reducing GSH production cost and furthering the range of application of this molecule.

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## PRODUCTION OF GLUTATHIONE IN EXTRACELLULAR FORM BY *Saccharomyces cerevisiae*

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## Abstract

The present research was aimed at inducing, in a post fermentative procedure (biotransformation) and by modifying cell permeability, GSH accumulation and subsequent release from *Saccharomyces cerevisiae* cells. ]. With the aim of limiting process costs, research considered also the possibility of employing different commercial baker's yeasts, available on the market at a reasonable price, in comparison with a collection strain. The tested yeast showed different sensitivity to the chemical/physical treatments performed to alter cell permeability. The use of Triton gave the lowest effects, being effective only with Zeus yeast samples (1.7 g GSH/l, near 60% of which in extracellular form). Sarcosine showed an interesting action on GB Italy sample (2.8 g GSH /l, near 80% extracellular). Lyophilisation evidenced good performance with Lievitalia yeast strain (2.9 g GSH/l, 90% extracellular). The possibility of obtaining GSH directly in extracellular form represents an interesting opportunity of reducing GSH production cost and furthering the range of application of this molecule.

Keywords: glutathione, *Saccharomyces cerevisiae*, extracellular metabolite, cell permeabilisation, baker's yeast, extracellular release

## 1. Introduction

Glutathione (GSH, L- $\gamma$ -glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol compound widely present in living organisms, from prokaryotes to eukaryotes [1]. It is synthesised intracellularly by the consecutive actions of  $\gamma$ -glutamylcysteine synthetase, feedback regulated by GSH content, and GSH synthetase. This tripeptide's very low redox potential gives it the properties of a cellular redox buffer [2]. In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, xenobiotic and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism [3,4].

These characteristics make this active tripeptide an important biochemical drug for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, pancreatic inflammations and aging. In addition, GSH is of interest in the food additive industry and sports nutrition [5,6]. GSH is widely used in pharmaceutical, food, and cosmetic industries, and the commercial demand for GSH is expanding [7].

*Saccharomyces cerevisiae* and *Candida utilis* are the most commonly used microorganisms on an industrial scale for GSH fermentative production; however GSH contents is usually variable among strains (0.1 – 1% dw) and always present in intracellular form [8,9]. Considering a single yeast strain, intracellular GSH content always remains at a stable level, since the first enzyme committed in its biosynthesis is feedback regulated by GSH [10]. Although GSH is widely distributed in nature, extraction of this tripeptide from yeast cells seems to be the only commercial available biotechnological production method to date [11]. Therefore, research projects have

been mainly focused on how to increase as much as possible, the intracellular GSH content of yeast through optimisation of biotechnological culture conditions [9,12,13].

Little has up to now been reported about extracellular GSH release from cells. Nie et al. (2005) studied the effect of low pH stress on GSH synthesis and excretion capability in growing cells of *Candida utilis* [14]. Wei et al. (2003) for the first time investigated the effects of surfactants (SDS and CTAB) on *S. cerevisiae* cell growth, intracellular GSH biosynthesis and extracellular release [15]. Results showed that cells growth was affected by the addition of high surfactant concentrations. Only when low concentrations of surfactants were added to the medium, total GSH concentration, taking into account both GSH synthesis and excretion, was increased.

The present research was aimed at inducing, in a post fermentative procedure (biotransformation), GSH accumulation and subsequent release from *Saccharomyces cerevisiae* cells, achieved by modifying cell permeability. In this paper the effect of different procedures, such as permeabilizing agent and lyophilisation, on yeast cells are reported. The possibility of obtaining GSH directly in extracellular form, avoiding the downstream cell extraction step, represents an interesting opportunity of reducing GSH production cost and furthering the range of application and utilization of this molecule [16]. With the aim of limiting process costs, research considered also the possibility to carry out the biotransformation by employing different commercial baker's yeasts, available on the market at a reasonable price. Baker's yeast are in fact obtained by strains in which mechanisms involved in stress response, as those based on GSH presence, are particularly efficient and reactive. Moreover, the use of surfactants on already pre-grown cells may avoid the risk of a detrimental effect of these molecules on cell enzymatic activity.

## 2. Materials and methods

### 2.1 Microorganisms

For GSH production *Saccharomyces cerevisiae* NCYC 2959 (National Collection of Yeast Cultures, Aberdeen, UK) was comparatively employed together with three commercial baker's yeast in compressed form (GB Italy, Lievitalia Spa and Zeus Industria Biologica Alimentare SpA).

As regards *S. cerevisiae* NCYC 2959, cells were pre-grown in MEB culture medium, having the following composition (g/l): malt extract 20 (Costantino, Turin), soybean peptone (Costantino, Turin) 1, glucose 20, pH adjusted to 5.8, sterilization at 118°C for 20 min. Cells production was carried out in 1000 ml Erlenmeyer flasks, each containing 100 ml of the culture medium MEB. Cultures were inoculated (10% v/v) with a 24 h-old culture prepared in the same medium, and then incubated at 28 °C for 48 h. The obtained culture was centrifuged at 10000 rpm for 10 min. Supernatant was discharged and separated cells were washed twice with distilled water and subsequently employed in biotransformation trials, after having determined cell dry weight.

## 2.2 Biotransformation conditions

Cells were suspended (10% dry weight) in a reaction solution having the following composition (g/l): glucose 80, sodium citrate 10, cysteine 4, glycine 4, ammonium sulphate 7,  $\text{KH}_2\text{PO}_4$  3.5, magnesium sulphate 0.5, tap water. Biotransformation trials were carried in 1000 ml Erlenmeyer flasks, each containing 100 ml reaction mixture, incubated at 28°C on an alternative shaker (60 spm, 4 cm run). Experiments involving modified cell permeability were performed by adding to the reaction mixture (0.2 - 0.5 g/l) CTAB, Digitonin, Mega 10, Octyl glucoside, Tweens (20, 40 and 80), Triton-X 100 or Lauroyl Sarcosine (Sigma), for comparison purposes. Lyophilised cells were also employed. Lyophilisation was carried out for 30 h at 25 °C and 1.33 Pa (Edwards Minifast MFD 01, UK).

## 2.3 Analytical procedures

Intracellular GSH was determined according to Rollini and Manzoni (2006) [8]. Samples at different incubation time were centrifuged (10,000 rpm, 6 min), and obtained cells were washed twice with  $\text{H}_2\text{O}$ , then thermally treated at 100 °C for 12 min. After cooling in ice bath, samples were centrifuged (12,000 rpm, 15 min) and on obtained supernatant fractions, intracellular GSH was evaluated. Extracellular GSH was directly determined on supernatants obtained from cell-culture separation, the first centrifugation step. GSH identification and quantification was carried out by HPLC, equipped with a UV detector (210 nm), at 30 °C using a (250 - 4) mm Purospher RP-18 endcapped column (Merck), eluted with 25 mM  $\text{NaH}_2\text{PO}_4$  pH 3.5, at 0.3 ml/min.

## 2.4 Transmission electron microscopy (TEM)

Samples (2 ml) obtained at different biotransformation reaction times were centrifuged (7000 rpm for 10 min) and the obtained cells were prepared for transmission electron microscopy as previously reported [8]. Ultrathin section (90 nm) were examined in a Leo912ab transmission electron microscope (Zeiss) at 80 kV using Omega filter. Digital images were acquired by Esivision CCD-BM/1K system.

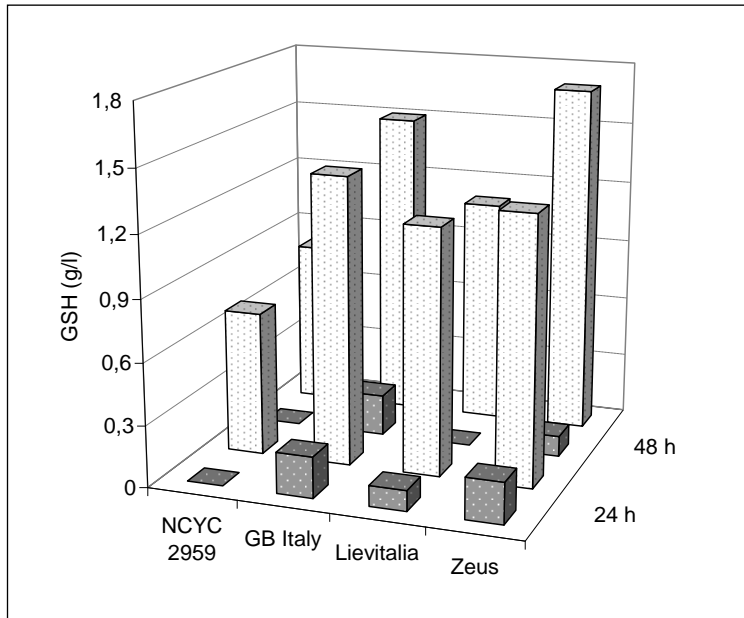
# 3. Results

## 3.1. Intracellular GSH production

In the preliminary part of the research, a set of biotransformation reactions were performed in order to evaluate GSH physiological accumulation by a *Saccharomyces cerevisiae* collection strain and commercial baker's yeasts, employing a post-fermentative procedure. Trials were performed suspending cells at a final concentration of 10% dw in an appropriate reaction solution, containing cysteine and



glycine, as GSH precursors, glucose as energy source, and ammonium and magnesium salts [16]. Figure 1 reports intracellular and extracellular GSH levels (g/l) obtained at 24 and 48 h incubation. For evaluation purposes of the equilibrium between intra- and extracellular GSH forms, intracellular GSH levels were expressed as concentration (g GSH/l biotransformation solution) and not as content percent of dry cell weight, as usually reported in the literature. The aim of this phase was to evaluate the ability of the tested yeast to accumulate GSH at highest levels, to be extracted for commercial purposes.



**Figure 1.** GSH production (g/l) by *S. cerevisiae* cells and commercial baker's yeast, at 24 and 48 h incubation time in post-fermentative conditions (dark bars indicate extracellular GSH, white bars intracellular GSH).

From obtained results GSH was mainly found as intracellular metabolite at interesting levels, in all yeast samples. The best results were obtained at 24 h reaction with GB Italy compressed baker's yeast (1.4 g/l, corresponding to 2.8 % dw), and at 48 h with Zeus (1.7 g/l, 3.4 % dw). For the reference collection *S. cerevisiae* strain, yields of 0.7-0.8 g GSH/l were obtained. Extracellular GSH was found always in the range 0-0.2 g/l, thus confirming GSH intracellular physiological nature. Results obtained in this phase were considered as reference data, and used to evaluate GSH release from the cells.

**Table 1.** GSH production levels (g/l) either as total, intra- and extracellular forms, obtained at 24 and 48 h employing Triton-X 100 (0.5 g/l) in the reaction mixture (data reported as mean  $\pm$  standard deviation).

Sample	Time (h)	GSH (g/l)		
		Total	Intracellular	Extracellular
NCYC 2959	24	0.7 $\pm$ 0.2	0.7 $\pm$ 0.1	0
	48	0	0	0
GB Italy	24	1.5 $\pm$ 0.1	1.2 $\pm$ 0.2	0.3 $\pm$ 0.1
	48	2.3 $\pm$ 0.2	1.7 $\pm$ 0.1	0.6 $\pm$ 0.1
Lievitalia	24	1.6 $\pm$ 0.1	0.9 $\pm$ 0.1	0.7 $\pm$ 0.2
	48	1.7 $\pm$ 0.2	0.4 $\pm$ 0.1	1.3 $\pm$ 0.2
Zeus	24	1.5 $\pm$ 0.2	1.2 $\pm$ 0.2	0.3 $\pm$ 0.1
	48	2.9 $\pm$ 0.3	1.2 $\pm$ 0.2	1.7 $\pm$ 0.2

### 3.2 Extracellular GSH production

The addition of surface-active agents to the reaction solution can be considered a strategy to alter cells properties, in particular the transport mechanisms across the structure surrounding the cell, and obtain metabolites release from yeast, without altering the metabolic pathways [17]. GSH release was investigated by adding different substances to the biotransformation mixture, with the aim of modifying membrane/wall permeability. CTAB, Digitonin, Mega 10, octyl-glucoside, Tweens (20, 40 and 80), Triton-X 100 and lauroyl sarcosine were employed for comparison purposes. CTAB is a cationic surfactant, acting as a membrane-modulating agent [18]. Octylglucoside, Mega -10 and Triton-X are mild non-ionic detergents used for solubilisation of cytoplasmatic membrane proteins. Digitonin, non-ionic detergent, permeabilises plasma membranes of eukaryotic cells by complexing with membrane cholesterol and other conjugates hydroxysterols. Lauroyl sarcosine does not precipitate and is commonly used instead of SDS for solubilisation of proteins [19-22].

Interesting GSH release levels were evidenced only in experiments carried out in presence of Triton (0.5 g/l) and lauroyl sarcosine (0.2 g/l). No interesting results were obtained with the other agents, as extracellular levels were found in the range 0-0.2 g/l, similar to those evidenced in reference samples (data not shown).

As regards the effect of Triton on the tested *Saccharomyces*, Table 1 reports GSH yields, as total, intra- and extracellular forms. The reference strain NCYC 2959 was found not to be influenced by the Triton presence. No GSH release was observed, moreover the GSH referred to the only intracellular form was 0.7 g/l, in accordance with that evidenced at 24 h in the first research phase, for untreated cells. At 48 h reaction, GSH was not present intracellularly anymore, but was completely consumed

presumably because of cell metabolism [23]. Commercial yeasts instead showed different behaviour, being all sensible to the presence of Triton. GSH in extracellular form was evidenced in levels ranging from 0.3 to 1.7 g/l, being the highest yield reached at 48 h incubation time. The best result was obtained employing Zeus, with 1.7 g GSH/l in extracellular form, about 60% of the total GSH produced.

By comparing total GSH produced, sum of the intra- and extracellular forms, GB Italy and Zeus samples evidenced that the GSH yields in presence of Triton resulted significantly higher at 48 h, 2.3 and 2.9 g/l, with respect to 24 h, about 1.5 g/l. Lievitalia total GSH remained almost constant during 24-48 h incubation time (1.6-1.7 g/l), but the compartmentation changed from mainly intracellular at 24 h, to mainly extracellular at 48 h. From the obtained results it was possible to highlight that in Lievitalia samples, no new GSH biosynthesis occurred from 24 to 48 h incubation time, while in GB Italy e Zeus cells, GSH increased with respect to untreated cells. This behaviour suggested the hypothesis that GSH extracellular release outside the cells allowed the same cells to become less sensible to the feed-back inhibition, mechanism controlling GSH biosynthesis and accumulation.

Table 2 shows GSH production levels obtained employing sarcosine. GSH was mainly detected outside the cells in all samples and the highest GSH levels were obtained at 48 h incubation time, similarly to experiments carried out with Triton.

**Table 2.** GSH production levels (g/l) either as total, intra- and extracellular forms, obtained at 24 and 48 h employing L-lauroylsarcosine (0.2 g/l) in the reaction mixture (data reported as mean  $\pm$  standard deviation).

Sample	Time (h)	GSH (g/l)		
		Total	Intracellular	Extracellular
NCYC 2959	24	1.3 $\pm$ 0.3	0.1 $\pm$ 0.1	1.2 $\pm$ 0.2
	48	1.1 $\pm$ 0.2	0	1.1 $\pm$ 0.3
GB Italy	24	2.1 $\pm$ 0.3	0.5 $\pm$ 0.2	1.6 $\pm$ 0.1
	48	3.2 $\pm$ 0.2	0.4 $\pm$ 0.1	2.8 $\pm$ 0.3
Lievitalia	24	1.5 $\pm$ 0.1	0.2 $\pm$ 0.1	1.3 $\pm$ 0.2
	48	2.2 $\pm$ 0.2	0.4 $\pm$ 0.1	1.8 $\pm$ 0.2
Zeus	24	1.5 $\pm$ 0.1	0.3 $\pm$ 0.1	1.2 $\pm$ 0.2
	48	1.9 $\pm$ 0.2	0.3 $\pm$ 0.2	1.6 $\pm$ 0.2

Among the tested yeasts, GB resulted the most sensitive, having obtained 2.8 g GSH/l in extracellular form, about 90% of the total GSH produced. The reference strain NCYC 2959, insensible to Triton action, employing sarcosine was found to release outside the cells almost all GSH produced, 1.1-1.2 g/l, even if at levels lower with respect those obtained for the other yeasts (1.6-2.8 g GSH/l). When comparing data as

total GSH produced, results evidenced that GB Italy yeast produced a total amount of 3.2 g GSH/l, while 2.2 and 1.9 g/l were obtained respectively from Lievitalia and Zeus.

The hypothesis that cells lyophilisation, carried out at temperature-controlled conditions, may produce weak alterations of cell structures, causing GSH release, without altering or interfering with metabolic activity, was considered as alternative to the use of surface-active agents. Generally, the use of lyophilised cells furnished good results (Table 3). Lyophilised cells were found to release GSH at interesting levels even at 24 h reaction, the best results being obtained by Lievitalia yeast, reaching 2.3 g extracellular GSH/l at 24 h and 2.9 g/L at 48. To be noted also the performance of NCYC 2959 strain, employing which GSH production gave higher extracellular GSH yield, 1.6-2.0 g/l, with respect to those evidenced in trials with Triton or Sarcosine (1.1-1.2 extracellular GSH/l). GB Italy and Zeus yeasts instead gave only modest GSH yield, both in term of total (0.8-1.4 g GSH/l) and extracellular GSH (0.6-1.3 g/l), with respect to results evidenced in presence of Triton and Sarcosine (Tables 1 and 2).

**Table 3.** GSH production levels (g/l) either as total, intra- and extracellular forms, obtained at 24 and 48 h employing lyophilised cells in the reaction mixture (data reported as mean  $\pm$  standard deviation).

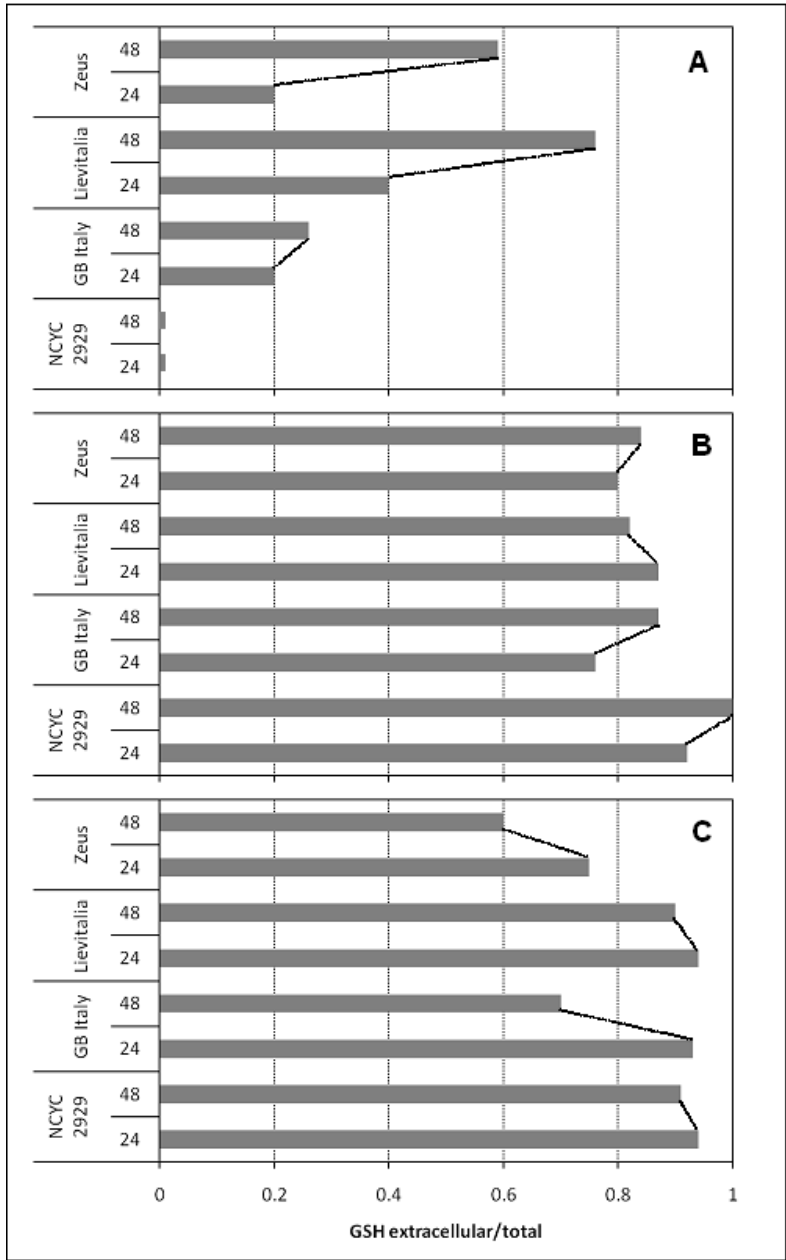
Sample	Time (h)	GSH (g/l)		
		Total	Intracellular	Extracellular
NCYC 2959	24	1.7 $\pm$ 0.3	0.1 $\pm$ 0.1	1.6 $\pm$ 0.2
	48	2.2 $\pm$ 0.2	0.2 $\pm$ 0.1	2.0 $\pm$ 0.2
GB Italy	24	1.4 $\pm$ 0.1	0.1 $\pm$ 0.1	1.3 $\pm$ 0.1
	48	1.0 $\pm$ 0.2	0.3 $\pm$ 0.1	0.7 $\pm$ 0.1
Lievitalia	24	2.5 $\pm$ 0.2	0.2 $\pm$ 0.1	2.3 $\pm$ 0.3
	48	3.2 $\pm$ 0.1	0.3 $\pm$ 0.2	2.9 $\pm$ 0.2
Zeus	24	0.8 $\pm$ 0.2	0.2 $\pm$ 0.1	0.6 $\pm$ 0.2
	48	1.0 $\pm$ 0.2	0.4 $\pm$ 0.2	0.6 $\pm$ 0.1

### 3.3. Relationships between extracellular and total GSH

Fig. 2 reports the extracellular/total GSH ratios determined from results obtained in experiments. From an overall look at the obtained data it is possible to notice a different cell susceptibility to the tested molecules/treatment, and consequently different degree in GSH release by the tested yeasts.

Employing Triton (0.5 g/l), the extracellular GSH fraction resulted limited with respect to the total GSH present in samples (Fig. 2A). Only for Lievitalia yeast a ratio of 0.76 was reached at 48 h. Moreover the reference *S. cerevisiae* NCYC strain resulted

insensitive to triton effect. Experiments carried out with decreasing or increasing Triton concentration did not produce any interesting results for all the tested strain (data not shown).



**Figure 2.** Extracellular/total GSH ratio obtained in biotransformation trials performed employing Triton X-100 (A), sarcosine (B) and lyophilized cells (C).

Sarcosine addition gave a meaningful increase of GSH release from cells (Fig. 2B). Generally, the extracellular/total GSH ratio was higher than 0.8, reaching 1, the maximum value, for the reference yeast strain, at 48 h incubation time. By considering both the GSH ratio, index of reached GSH release, and the GSH yield, production index, it is possible to conclude that GB Italy yeast coupled with sarcosine addition resulted a good starting point (0.9 extracellular/total GSH and  $2.8 \pm 0.3$  g GSH/l respectively) to develop an applied process to produce extracellular GSH.

The use of lyophilized cells gave fluctuant effects on GSH release, and did not prove to be a suitable procedure for the tested yeasts (Fig. 2C). Extracellular vs total GSH ratio was found higher at 24 h, ranging from about 0.7 (Zeus) to over 0.9 for the other yeasts. Lievitalia yeast maintained a similar value, 0.9, also at 48 h with a total yield of about 2.9 g GSH/l.

### 3.4. Electron microscopy

Fig. 3A shows ultrastructure of GB Italy cells after 48 h incubation in biotransformation experiments in which GSH was mainly found in intracellular form (1.5 g/l, about 90% of the total produced GSH) (sect 3.1). These cells represented the reference control sample. In this case GSH accumulation was related to the presence of several intracellular inclusion bodies, visible as dark vesicles as previously reported [8]. Fig. 3B shows again GB Italy yeast cells, after 48 h incubation in experiments carried out employing sarcosine (sect. 3.2). In this sample GSH resulted mainly extracellular, 2.8 g/l, with respect to the total GSH yield, 3.2 g/l, evidenced in this trial. Here cells surroundings appeared less defined and compact, with respect to the control samples, and inside the cell no inclusion body, associated to intracellular GSH production, was present.

The correlation between cellular structure and its integrity, related to extracellular GSH production are at present the object of ongoing investigation.

## 4. Discussion

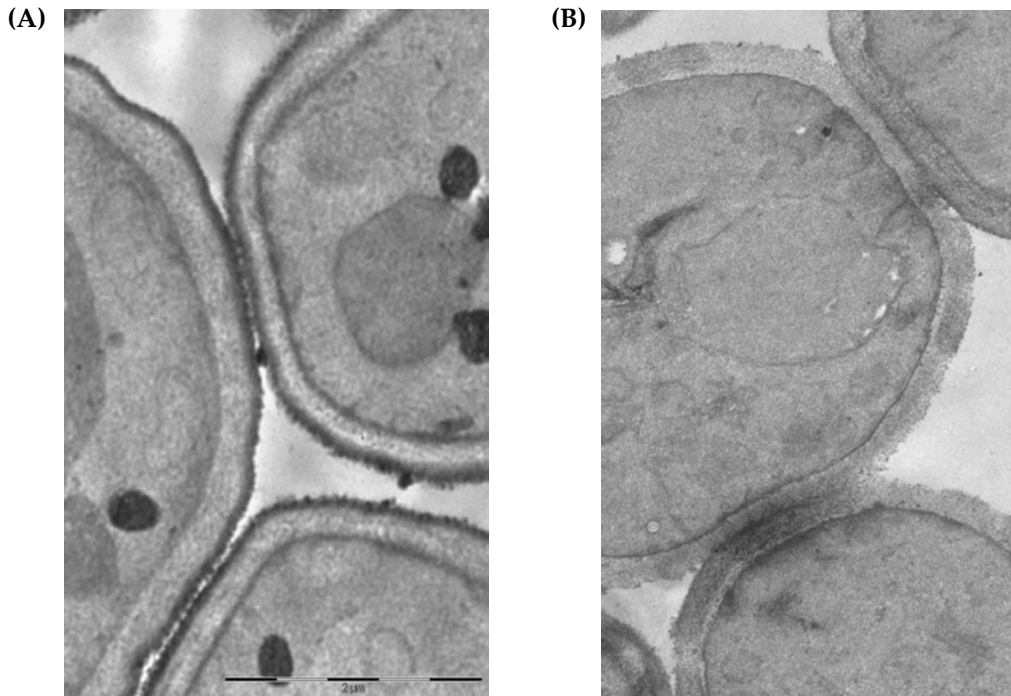
In growing cells or in post fermentative processes, GSH is physiologically accumulated intracellularly, in concentration generally not higher than 0.7% cell dry weight. In this context GSH release out of the cells may represent a strategy to by-pass the fed-back mechanism present in the cells, which controls the levels of intracellular GSH. This approach represented the starting point to develop a process to obtain GSH in extracellular form, in higher yields with respect to those obtainable by extraction from yeast cells. To obtain extracellular GSH in the research reported in this paper the application of chemical/physical procedures able to modify the cellular permeability was considered.

From an overall look at the obtained results it can be highlighted that tested yeast showed different sensitivity to chemical/physical treatments, performed to alter cell permeability. The use of Triton gave the lowest effects, being effective only with Zeus yeast samples (1.7 g GSH/l, near 60% of which in extracellular form). Sarcosine showed an interesting action on GB Italy sample (2.8 g GSH /l, near 80% extracellular). Lyophilisation evidenced good performance with Lievitalia yeast strain (2.9 g GSH/l, 90% extracellular).

Few papers are present in the literature related to the possibility of obtaining extracellular GSH. The effect of surfactants on extracellular GSH accumulation was investigated with *S. cerevisiae* in growing conditions [15]. Cell growth resulted not inhibited by the use of SDS and CTAB at low concentration, allowing the accumulation of extracellular GSH at levels up to 50 m/l. A research on GSH secretion studied the effect of low pH stress on GSH synthesis and excretion from *Candida utilis* [14]. In fed-batch production, 198 mg GSH/l were secreted into the medium, the total GSH concentration being 737 mg GSH/l.

Under physiological conditions, GSH intracellular content in yeast cells is kept at relative stable levels through a feed-back regulation of the first enzyme committed in its synthesis [10] . In feed-back regulated reactions, a common strategy to increase its production lies in product removal from the reaction mixture. In the present case, alteration of cell permeability causes GSH to be removed outside the cell, thus shifting the reaction equilibrium towards GSH synthesis. Results showed that when extracellular GSH was present, total GSH yields (max 3.2 g GSH/l) were found higher than those obtained without cell treatment (max 1.8 g GSH/l). This demonstrates that GSH release from yeast cells may represent an interesting way of by-passing the problem of feed-back regulation.

The present paper relates to the possibility of obtaining high extracellular GSH levels in a post-fermentative procedure employing commercial baker's yeast. Chemical or physical procedures can be applied to alter cell permeability, being different the sensitivity of *S. cerevisiae* yeast strains to treatments. Research trials are now in progress with the aim of understanding the meaning of such different sensitivity, that seems to be due to the fact that these strains possess difference in cell wall and/or membrane structures. The possibility of obtaining GSH in extracellular form at high levels (near 3 g/l) can represent a valid alternative to the extraction procedure from yeast cells. The achieved results are of great commercial interest because they will simplify GSH downstream procedures, thus lowering production cost and furthering the range of application of this molecule.



**Figure 3.** Transmission electron micrographs of ultrathin section of baker's yeast cells (GB Italy) obtained at 48 h incubation in post-fermentative GSH production trials: control sample (A) and in presence of sarcosine (B).

## References

- [1] Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem-Biol Interac* 1998; 111-112: 1-14.
- [2] Udeh KO, Achremowicz B. High glutathione-containing yeast *Saccharomyces cerevisiae*: optimization and production. *Acta Microbiol Polon* 1997; 46: 105-14.
- [3] Penninckx M. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res* 2002; 2: 295-305.
- [4] Wu, G., Fang, Y.-Z., Lupton, J.R., Turner, N.D. Glutathione metabolism and its implications for health. *J Nutr* 2004; 134: 489-492.
- [5] Lomaestro BM, Malone M. Glutathione in health and disease: pharmacotherapeutic issues. *Ann Pharmacother* 1995; 29: 1263-73.
- [6] Beutler, E. Nutritional and metabolic aspects of glutathione. *Annu Rev Nutr* 1989; 9: 287-302.
- [7] Mehdi K, Penninckx M. An important role for glutathione and  $\gamma$ -glutamyltranspeptidase in the supply of growth requirements during nitrogen starvation of the yeast *Saccharomyces cerevisiae*. *Microbiology* 1997; 143: 1885-1889.



- [8] Rollini M, Manzoni M. Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. *Process Biochem* 2006; 41: 1501-1505.
- [9] Li, Y., Wei, G., Chen, J. Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* 2004; 66: 233-42.
- [10] Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 1983; 52: 711-760.
- [11] Rollini, M., Pagani, H., Riboldi, S., Manzoni, M. Influence of carbon source on glutathione accumulation in methylotrophic yeasts. *Ann Microbiol* 2005; 55: 199-203.
- [12] Alfafara C, Kanda A, Araki K, Shimizu H, Shioya S, Suga K. Effects of amino acids on glutathione production by *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1992a; 36: 538-540.
- [13] Alfafara C, Miura K, Shimizu H, Shioya S, Suga K. Cysteine addition strategy for maximum glutathione production in fed-batch culture of *Saccharomyces cerevisiae*. *Appl Microbiol Biotechnol* 1992b; 37: 141-146.
- [14] Nie W, Wei G, Li Y, Chen J. Enhanced intracellular glutathione synthesis and excretion capability of *Candida utilis* by using a low pH-stress strategy. *Lett Appl Microbiol* 2005; 40: 378-384.
- [15] Wei G, Li Y, Du G, Chen J. Effect of surfactants on extracellular accumulation of glutathione by *Saccharomyces cerevisiae*. *Process Biochem* 2003; 38: 1133-1138.
- [16] Rollini M, Manzoni M. Glutathione release in extracellular form by *S. cerevisiae* strains. *J Biotechnol* 2007;131: S209.
- [17] Sekhar S, Bhat N, Bhat SG. Preparation of detergent permeabilized Baker's yeast whole cell catalase. *Process Biochem* 1999; 34: 349-354.
- [18] Ricu V, Gusbeth C, Anghel DF, Turcu G. Effects of cetyltrimethylammonium bromide (CTAB) surfactant upon the dielectric properties of yeast cells. *Biochim Biophys Acta* 1998; 1379: 7-15.
- [19] Laouar L, Lowe KC, Mulligan BJ. Yeast responses to nonionic surfactants. *Enzyme Microb Technol* 1996; 18: 433-438.
- [20] Abraham J, Bhat SG. Permeabilization of baker's yeast with N-lauroyl sarcosine. *J Ind Microbiol Biotechnol* 2008; 35: 799-804.
- [21] Presečki AV, Vasić-Rački D. Production of L-malic acid by permeabilized cells of commercial *Saccharomyces* sp. strains. *Biotechnol Lett* 2005; 27: 1835-1839.
- [22] Le Marie M, Champeil P, Moller JV. Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim Biophys Acta* 2000;1508: 68-81.
- [23] Penninckx M. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. *Enz Microb Technol* 2000; 26: 737-42.

#### *4. Copper enriched yeast*

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#### **4.1 Aim of the study**

This section of the research was aimed at investigating the possibility to obtain copper-enriched yeast cells and develop an efficient and non-toxic metal delivery system for human nutrition, according to recommended daily allowances (RDAs) (Annex I of the Directive 2008/100/EC amending Directive 90/496/EEC).

#### **4.2 Materials and Methods**

##### ***Samples***

Commercial baker's yeast *Fala* in compressed fresh form was employed. The yeast was maintained at 4 °C and tested at the same shelf life ( $10 \pm 1$  days).

HPLC-grade H<sub>2</sub>O was obtained through a Milli-Q A10 Gradient System (Millipore Corporation) as previously reported (1.2), EDTA, and HCl were supplied from Merck. GSH and copper acetate were obtained from Sigma.

##### ***Activation with copper acetate***

Bakers' yeast was suspended (5% dcw) in the CYS-GLY-GLU+ADE mixture (see section 2.3 for mixture composition) containing 0.3 mg/mL copper acetate. Experiments were performed in 100 mL and 1 L Erlenmeyer flasks, each containing respectively 10 and 100 mL reaction mixture, incubated at 28 °C and 200 rpm up to 48 h incubation time.

Determination of intracellular GSH and GSH-copper conjugate content was carried out on samples (1 mL), obtained at different incubation times as reported by Rollini et al. (2011) exceeding for washing procedure. Briefly, samples were centrifuged ( $10,600 \times g$  for 10 min) and collected cells were comparatively washed with distilled water twice (treatment A) or with 10 mM (EDTA) first and then with distilled water (treatment B). Cells were then suspended in a solution of ascorbic acid and thermally treated at 100°C for 10 min for achieving cell permeabilization; samples were then cooled in ice and subsequently centrifuged to eliminate cell residues.

##### ***Biomass treatments***

For comparative purpose ten different treatments were employed:

1. Control activation: cells suspended in the CYS-GLY-GLU+ADE mixture;
2. Activation with copper: cells suspended in the CYS-GLY-GLU+ADE mixture added with 0.3 mg/mL copper acetate;

3. Cells suspension in copper solution: cells suspended in 0.3 mg/mL copper acetate solution;
4. Copper addition at 24 h activation: as trial 1 (control) for 24 h, then addition of 0.3 mg/mL copper acetate
5. Copper addition to 24 h activated cells: as trial 1 (control) for 24 h, then cells were centrifuged, washed with distilled water and subsequently suspended in 0.3 mg/mL copper acetate solution;
6. Acid treatment and suspension of 24 h activated cells in copper solution: as trial 5, but collected cells were suspended for 1 h in 0.3% HCl before resuspension in copper acetate;
7. Acid treatment before activation with copper: as trial 2, but cells were pre-treated for 1 h in 0.3% HCl;
8. Acid treatment before activation: as trial 1, cells were pre- treated for 1 h in a 0.3% HCl;
9. Acid treatment before suspension in copper solution: as trial 3, cells pre-treated for 1 h in 0.3% HCl;
10. Acid treatment before activation and subsequent copper addition: as trial 4, cells pre- treated for 1 h in 0.3% HCl.

Determination of intracellular GSH and GSH-copper conjugate content was carried out on obtained samples (1 mL), at different incubation times as previously reported.

### ***Biomass copper content***

Results obtained in conjugate production were verified, in particular trial 4 (direct addition of copper acetate at 24 h), 5 (centrifugation and suspension in copper acetate) and 6 (centrifugation, acid treatment and resuspension in copper acetate). Samples collected at 28 h (that is 4 h after copper contact) and at 48 h incubation, were analysed for GSH and GSSG/GSH-Cu conjugate content. Cells were lyophilized for copper content determination.

### ***Analytical procedures***

Intracellular GSH and GSH-conjugate content was determined on supernatant samples by HPLC as reported by Rollini et al. (2011). Results were expressed in terms of percent dry cell weight (% dcw) as previously described (1.2).

Copper content was determined by inductively coupled plasma atomic emission spectroscopy (ICP-AES) after a microwave acid digestion. Samples of lyophilized yeast were placed in a Teflon vessel, treated with 1 mL of concentrate HNO<sub>3</sub> (14 mol/L) and 1 mL H<sub>2</sub>O<sub>2</sub> (30% v/v) and irradiated at 800 W (15 min at 180 °C) in the microwave

system. At the end of the digestion program, samples were filtered through Whatman No. 1 paper and made up to volume with water. Results were expressed mg/Kg dcw.

### 4.3 Results

#### *Activation with copper acetate*

From obtained results (Tab. 4.1) no significant differences were observed with respect to the washing treatment employed.

In general GSH-copper conjugate content at 24 h was about 1% dcw in trials carried out in 100 mL flasks and slightly higher, 1.3%, in 1 L ones. By prolonging incubation time to 48 h, GSH level was maintained in samples obtained in 100 mL flasks while it reached 1% in 1 L trials.

In this experimental phase each trial was carried out also in control conditions (data not shown), without copper acetate, to verify the effect of each treatment on yeast cells apart conjugate production. In trials activated with copper, cells accumulated higher GSH levels with respect to the control ones, with a GSH content reaching 1.1% at 24 h (starting from 0.8% dcw), that then decreased to 1% at 48 h incubation time.

**Table 4.1.** Intracellular GSH and GSH-copper conjugate content (% dcw) obtained in trials carried out with two different cells washing treatments. Yeast: *Fala* (data are means of three replicates, for GSH content Coefficient of Variation, CV, between 5 and 9%, and between 4 and 8% for conjugate).

Cells washed with H <sub>2</sub> O				
	24 h		48 h	
	GSH	Conjugate	GSH	Conjugate
100 mL flask	1.32	0.90	1.84	1.04
1 L flask	1.29	1.32	1.71	1.07

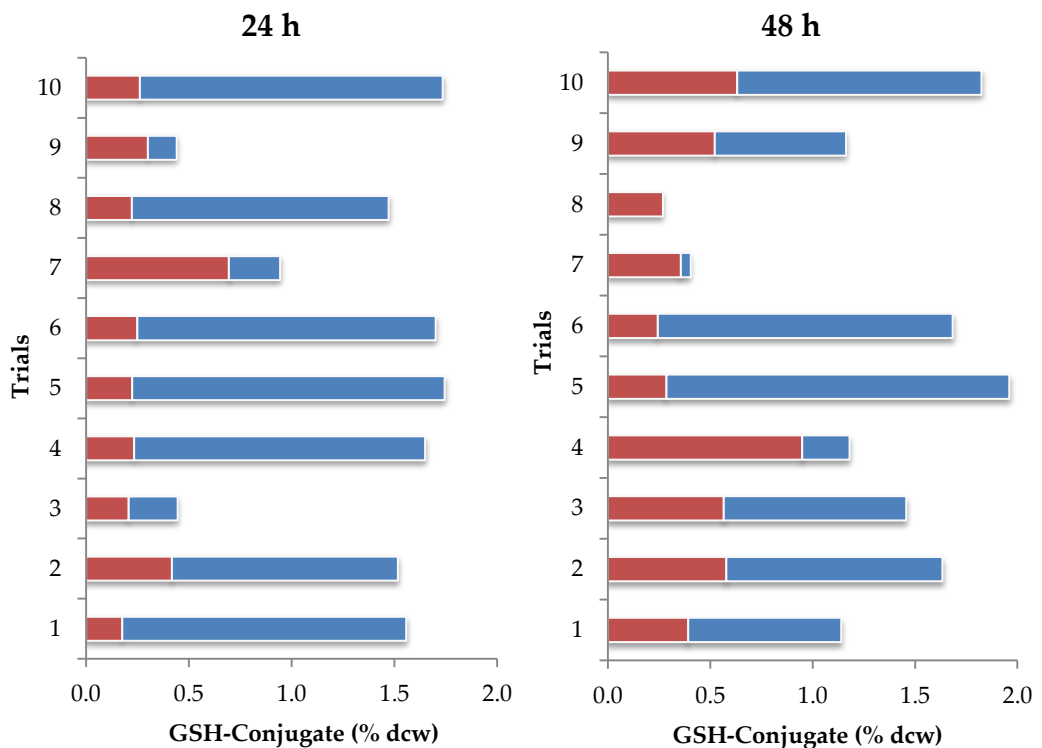
Cells washed with EDTA and H <sub>2</sub> O				
	24 h		48 h	
	GSH	Conjugate	GSH	Conjugate
100 mL flask	1.05	1.07	1.51	1.02
1 L flask	1.46	1.31	1.74	1.06

#### *Biomass treatment effects*

Samples from trials 1, 4, 5 and 6, carried out till 24 h in control conditions with CYS-GLY-GLU+ADE mixture, reached the same GSH and GSSG levels, of about 1.4% and 0.2% dcw, respectively. At 48 h incubation, GSH and GSSG levels in samples from

trials 5 and 6 remained unchanged, while in cells from trial 1, the control without any copper addition, GSH intracellular content decreased from 1.38 to 0.75%, while GSSG level doubled from 0.18 to 0.39% dcw.

Different behaviour was evidenced when, after 24 h activation, 0.3 mg/mL copper acetate were added (trial 4), where a significant GSH decrease was found together with a significant increase in HPLC of the peak attributable to GSSG/GSH-Cu conjugate. The acid pre- treatments with HCl performed in trials 7, 8, 9 and 10, did not produce significant differences at 24 h, with respect to the corresponding ones without the acid pre- treatment (trials 1, 2, 3 and 4). To be noticed that at 48 h incubation in cells obtained from trials 7 and 8, GSH no further accumulated, while in samples from trial 10, as well as trial 4, an increase of both GSSG/GSH-Cu conjugate level and GSH contents were found.



**Figure 4.1.** Intracellular GSH and GSSG/Conjugate content (% dcw) obtained in trials at 24 and 48 h employing *Fala* yeast (data are means of three replicates, CV for GSH content between 3 and 7%, and between 5 and 8% for conjugate).

### *Biomass copper content*

On samples from trial 4, (direct addition of copper acetate at 24 h), 5 (centrifugation at 24 h activation and suspension in copper acetate) and 6 (centrifugation at 24 h activation, acid treatment and resuspension in copper acetate) obtained at 24 (no copper contact), 28 (4 h copper contact) and 48 h (24 h copper contact), GSH and GSSG/GSH-Cu conjugate levels were determined. Results highlighted that at 24 h (data not shown) intracellular GSH levels were in the range 1.2-1.5% dcw, while at 28 and 48 h these levels decreased to 0.5-1% dcw.

To confirm the supposed presence of GSH-Cu conjugates in yeast cells, samples were analysed by atomic spectroscopy.

As regards intracellular copper levels, centrifugation and resuspension in the copper solution (treatment 5) allowed obtaining the highest copper intracellular levels; in particular at 24 h a content of 707 mg Cu/kg dcw was reached. Copper directly added to the samples at 24 activation time (treatment 4) led, at 24 h, to the lowest level, 183 mg Cu/kg dcw with respect to the other tested conditions (Tab. 4.2). These obtained results were in accordance with HPLC determination and allowed to evaluate GSH-Cu conjugate intracellular content.

**Table 4.2.** GSH, Cu-conjugate (% dcw) and Cu content (mg Cu/kg dcw) in samples obtained at 28 and 48 h incubation (4: direct addition of copper acetate at 24 h activation; 5. centrifugation at 24 h activation and suspension in copper acetate; 6. centrifugation at 24 h activation, acid treatment and resuspension in copper acetate). Data are means of three replicates, for GSH and Cu-conjugate CV between 5 and 9%, and between 2 and 5% for Cu determination.

Sample	Time	GSH	Cu-conjugate	Cu
	(h)	(% dcw)	(% dcw)	(mg/kg dcw)
4	24	1.04	0.19	183
	48	0.59	0.11	102
5	24	0.5	0.75	707
	48	0.38	0.63	589
6	24	0.53	0.41	390
	48	0.1	0.48	455

#### 4.4 Conclusions

Intracellular GSH-copper conjugate was obtained by applying a biotransformation procedure, in which cells are driven first to accumulate GSH, and then to conjugate it with copper.

Analysis performed by ICP-AES evidenced the presence of very interesting amount of intracellular copper, in particular when activated cell are separated and suspended in copper acetate (trials 5 and 6). In these conditions 390-707 mgCu/Kg dcw (corresponding to 0.4-0.7 mg/g dcw or 6-11  $\mu\text{mol}$  copper/g dcw) were accumulated. This intracellular copper level, linked to GSH, can be considered of great interest for the formulation of nutraceuticals based on yeast biomass: the 2008/100/EC Directive related to recommended daily allowances (RDAs) of food supplements, indicates 1 mg as RDA for copper.

Results obtained in subsequent experiments were the object of the paper reported in the following section.

#### 4.5 References

**AA. VV.** Council Directive 2008/100/EC amending Council Directive 90/496/EEC on nutrition labelling for foodstuffs as regards recommended daily allowances, energy conversion factors and definitions. Off J Eur Union 2008;L 285:9–12.

**Rollini M, Musatti A, Erba D, Benedetti A, Girardo F, Manzoni M.** 2011. Process for obtaining copper-enriched cells of *Saccharomyces cerevisiae*. Proc Biochem 46: 1417-1422.



## 4.6 Published paper

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### Process for obtaining copper-enriched cells of *Saccharomyces cerevisiae*

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#### ABSTRACT

The research was aimed at obtaining copper-enriched cells of *Saccharomyces cerevisiae*, developing an efficient metal delivery system for human nutrition and therapeutic treatments. To produce glutathione (GSH)–copper conjugates, four samples of commercial baker's yeasts (*S. cerevisiae*) were employed in biotransformation trials with copper acetate. GSH production was found variable, ranging from 0.15 to 1.2% cell dw. Trials carried out with copper-adapted cells resulted in GSH and conjugate limited biosynthesis. The highest copper conjugate level (1.7% dw) was instead obtained in experiments set up by employing not adapted cells. Cell disruption by sonication and subsequent analysis by atomic absorption evidenced the presence of very interesting amount of intracellular copper,  $1.8 \pm 0.2$  mg/g cell dw (i.e.  $28 \mu\text{mol copper/g cell dw}$ ). The proposed procedure can be considered an interesting opportunity to further the range of application of yeast cells culture, for the set up of a copper delivery system for therapeutic treatments.

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### PROCESS FOR OBTAINING COPPER-ENRICHED CELLS OF *Saccharomyces cerevisiae*

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## Abstract

The research was aimed at obtaining copper-enriched cells of *Saccharomyces cerevisiae*, developing an efficient metal delivery system for human nutrition and therapeutic treatments. To produce glutathione (GSH)-copper conjugates, four samples of commercial baker's yeasts (*S. cerevisiae*) were employed in biotransformation trials with copper acetate. GSH production was found variable, ranging from 0.15 to 1.2 % cell dw. Trials carried out with copper-adapted cells resulted in GSH and conjugate limited biosynthesis. The highest copper conjugate level (1.7 % dw) was instead obtained in experiments set up by employing not adapted cells. Cell disruption by sonication and subsequent analysis by atomic absorption evidenced the presence of very interesting amount of intracellular copper,  $1.8 \pm 0.2$  mg/g cell dw (i.e. 28  $\mu$ mol copper/g cell dw). The proposed procedure can be considered an interesting opportunity to further the range of application of yeast cells culture, for the set-up of a copper delivery system for therapeutic treatments.

**Keywords:** glutathione, *S. cerevisiae*, copper conjugates, atomic absorption

## 1. Introduction

Glutathione (GSH, L- $\gamma$ -glutamyl-L-cysteinyl-glycine) is the most abundant non-protein thiol compound widely present in living organisms, from prokaryotes to eukaryotes [1,2]. It is synthesised intracellularly by the consecutive actions of  $\gamma$ -glutamylcysteine synthetase (GSH I), feedback inhibited by GSH, and GSH synthetase (GSH II). This tripeptide's very low redox potential ( $E'_0 = -240$  mV) gives it the properties of a cellular redox buffer [3,4]. In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, detoxification of xenobiotics as well as of endogenous toxic metabolites, enzyme activity and sulphur and nitrogen metabolism [5]. GSH is potentially produced in many body areas, especially in the liver, and is involved in defence systems [6]. GSH is thus considered to be a powerful, versatile and important self-defence molecule [7].

These characteristics make GSH an important biochemical drug for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, pancreatic inflammations, as well as in aging [8]. In addition, GSH is of interest in the food additive industry and sports nutrition [9, 10].

High GSH concentrations have been found in some yeasts species, where this molecule seems to be involved in cell-defence mechanisms against nutritional and oxidative stresses [11-13].

Heavy metals (Mn, Cu, Co, Ni, Zn, and Fe), even if toxic at relatively low concentration, have a great significance for living cells, as they are essential in minute amounts and their deficiency can be associated with a range of pathological conditions

[14,15]. In particular zinc and copper ions, whether or not in combination with cysteine, are able to keep HIV-1 protease inactivated and are effective natural inhibitors. This enzyme is able to cleave serially linked virus elements that can combine to form new infectious virus particles. So far, zinc and copper ions are effective natural inhibitors of (AIDS) viruses, thereby preventing the development of chronic virus diseases that can lead to AIDS and autoimmune illness [16].

GSH facilitates the reduction of  $\text{Cu}^{2+}$  to  $\text{Cu}^{+}$  ions, and subsequently sequesters  $\text{Cu}^{+}$  ions under the form of copper-GSH conjugates. Several studies suggest that the copper-GSH complex plays a copper-transferring function by permitting a safe and efficient delivery of metal in the apo-form of cupro-enzymes (i.e. superoxide dismutase), copper storing (i.e. metallothioneins) and copper transporting (i.e. ceruloplasmines) proteins. In addition to playing a role in normal copper metabolism, GSH may also be of importance in defining the susceptibility of copper-overloaded cells to copper-associated toxicity [17].

The present research was aimed at investigating the possibility of obtaining copper-enriched cells of *Saccharomyces cerevisiae*, thus developing an efficient and less toxic copper delivery system for therapeutic treatments.

## 2. Material and Methods

### 2.1 Microorganisms and reagents

*Saccharomyces cerevisiae* commercial baker's yeasts in compressed fresh form were employed: Primapak (PRK, Pak Ihracat A.S., Turkey), Lievitalia (LVI, Lesaffre Italia S.p.A., Italy), La Parisienne (OS, Casteggio lieviti s.r.l., Italy), Lievito di Vienna (VIE, Lallemand GmbH, Austria). Commercial yeasts were maintained at 4 °C and tested at the same shelf life ( $10 \pm 1$  days).

HPLC-grade  $\text{H}_2\text{O}$ , EDTA, suprapur  $\text{H}_2\text{O}_2$  and HCl were supplied from Merck. Glutathione and copper salts were obtained from Sigma.

### 2.2 Biotransformation conditions

Bakers' yeasts were suspended (10% cell dry weight) in a reaction solution, as previously reported [18]. Experiments were carried out employing copper salts (acetate, carbonate and sulphate) at different concentration (0 – 2.5 mM), in 100 ml Erlenmeyer flasks, each containing 10 ml reaction mixture, incubated at 28°C and 200 rpm up to 72 h incubation time.

To obtain copper-adapted cells, yeast cells isolated from each commercial samples were transferred in Malt Extract Agar (MEA - composition in g/l: malt extract 20, glucose 20, soybean peptone 1, agar 15, pH 5.8) plates, containing increasing concentration of copper acetate, from 0.1 to 0.6 mg/ml. Colonies grown in presence of the highest copper concentration were isolated, and subsequently maintained on

copper-MEA medium (MEA added with 0.6 mg/ml copper acetate). To obtain biomass for biotransformation trials, these isolated strains were inoculated Malt Extract liquid (MEB – same composition as in MEA without agar) medium containing 0.3 mg/ml copper acetate (1.6 mM), and incubated at 28°C up to 72 h. Culture broth was then centrifuged (10600  $\times$  g for 15 min) and obtained cells employed in biotransformation trials.

Biomass dry weight was determined after drying the cells at 80°C for 24 h.

### *2.3 Analytical procedures*

Determination of intracellular GSH content was carried out on biotransformation samples (1 ml), obtained at different incubation times; samples were centrifuged (10600  $\times$  g for 10 min) and collected cells were washed twice with distilled water, suspended in 1 ml of 0.5 g ascorbic acid/l in ultrapure H<sub>2</sub>O, and thermally treated at 100°C for 6 min; samples were then cooled in ice and subsequently centrifuged (10600  $\times$  g for 10 min) to eliminate cell residues.

Intracellular GSH, GSSG and GSH- conjugate content was determined on supernatants by HPLC equipped with a UV detector (210 nm), at 30 °C using a (250  $\times$  4 mm) Purospher RP-18 endcapped column (Merck), eluted with 25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 3.5, at 0.3 ml/min [18,19].

### *2.4 Cell rupture by sonication*

Cells from samples obtained in presence of copper were broken through sonication to obtain the intracellular fraction, on which quantification of copper conjugates was carried out. Cell pellet obtained after centrifugation of biotransformation samples was suspended (final concentration 10% dw) in 5 ml solution having the following composition (mM): tris-HCl 20, MgCl<sub>2</sub> 10, ammonium sulphate 300, glycerol 5 % (w/v), pH 7.6. EDTA was added (1 mM final concentration) to chelate any non-conjugated copper that can interfere with the analysis. Cell rupture was performed by sonication (Soniprep 150 Plus MSE, 23 KHz frequency, 50 Hz), applying 4 cycles of 8 pulses for 45 s and 60 s interval, in ice. The level of disintegration (not less than 90%) was evaluated as the number of residual intact cells after sonication with respect to the initial cell content, determined either by direct total count and plate count. Samples were then centrifuged at 12000  $\times$  g for 15 min at 4 °C to remove cell debris.

### *2.5 Copper quantification by atomic absorption spectrometry*

Copper was determined by atomic absorption spectrometry (IL 551 Instrumentation Laboratory, Wilmington, MA, USA). After cell rupture, samples were centrifuged and intracellular copper was determined on supernatants. They were initially lyophilised,

incinerated overnight at 550 °C and finally suspended in 0.5 ml of 30% (v/v) suprapur H<sub>2</sub>O<sub>2</sub> for atomic absorption. Samples were dehydrated again at 100 °C for 5 h and again incinerated overnight at 550°C. Obtained samples were finally suspended in 0.5 ml of suprapur 30% (v/v) HCl and appropriately diluted with HPLC-grade H<sub>2</sub>O. Separated cell debris was analysed by atomic absorption to determine copper content accumulated in cell walls. They were incinerated overnight at 550 °C. The residue was treated with 0.5 ml of 30% (v/v) H<sub>2</sub>O<sub>2</sub> and then 1 ml 30% (v/v) HCl was added; samples were finally diluted to 10 ml with HPLC-grade H<sub>2</sub>O. Copper quantification was performed through comparison with a standard curve obtained with copper solutions in the range 0.5-3 µg/ml.

### *2.6 Transmission electron microscopy (TEM)*

Samples obtained at different incubation times were centrifuged and obtained cells prepared for transmission electron microscopy as previously reported [19]. Ultrathin sections (90 nm) were examined in a Leo912ab transmission electron microscope (Zeiss) at 80 kV using Omega filter. Digital images were acquired by Esivision CCD-BM/1K system.

## **3. Results and discussion**

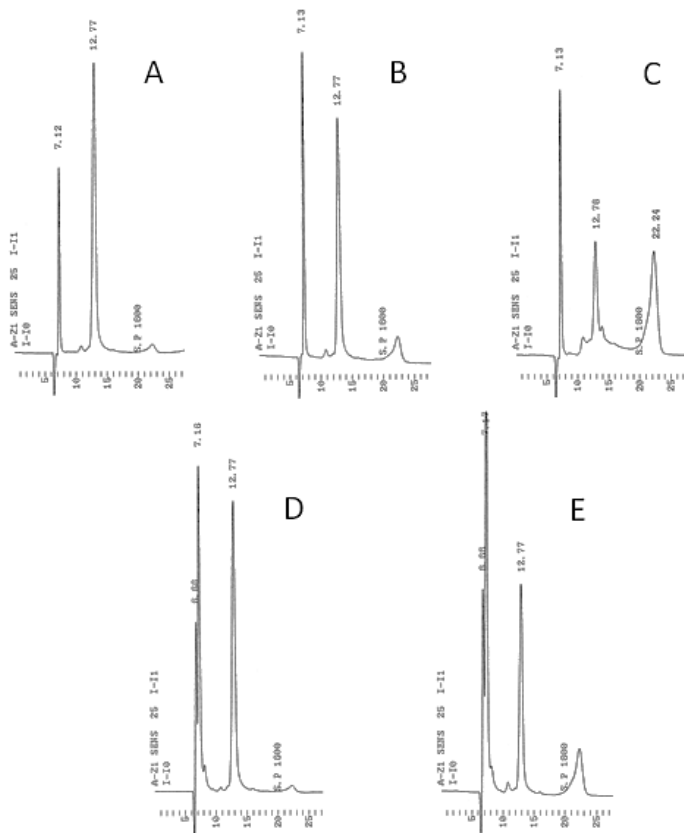
### *3.1 Formation of GSH-copper conjugates*

Preliminary investigations were carried out to identify the optimal conditions for the formation of GSH-copper conjugates. Different copper salts (acetate, carbonate and sulphate) were tested for their ability to form complexes with GSH. These salts (concentration range from 0.05 to 5 mM) were added to GSH standard solutions (concentration from 0.2 to 20 mM). Copper sulphate, having low solubility, produced an insoluble residue in all the tested conditions, as well as copper carbonate, producing the formation of a persistent opalescence, even at the lowest concentrations. Instead, copper acetate was soluble in the employed conditions and then chosen for the prosecution of the research.

Trials were then performed by adding copper acetate to GSH standard solution (0.2 mM), from copper excess (0.5 mM) to copper deficiency (0.005 mM). Copper added at low concentration only moderately increased a peak with Retention time (Rt) of about 22 min attributable to GSH-copper conjugate, with respect to GSH standard peak (Rt: 12 min) (Fig. 1a and b). When higher amount of copper was added to GSH standard solution, the conjugate level increased (Fig. 1c).

EDTA was then added to copper acetate solution before the addition of GSH. In these conditions, no increase of the peak having Rt 22 min was observed (Fig. 1d). This behaviour was directly attributable to the EDTA chelating effect on copper, that was no longer available to form conjugates with GSH. On the contrary, when EDTA was

added after copper and GSH mixing, the peak related to GSH-copper conjugate was present (Fig. 1e). This result is related to the fact that copper primarily reacts with GSH, and not with EDTA.



**Figure 1.** HPLC chromatograms: 0.2 mM GSH (a), 0.2 mM GSH and 0.005 mM Cu acetate (b), 0.2 mM GSH and 0.5 mM Cu acetate (c), 10 mM EDTA and 0.5 mM Cu acetate added to 0.2 mM GSH (d), 0.2 mM GSH and Cu acetate 0.5 mM added to 10 mM EDTA (e).

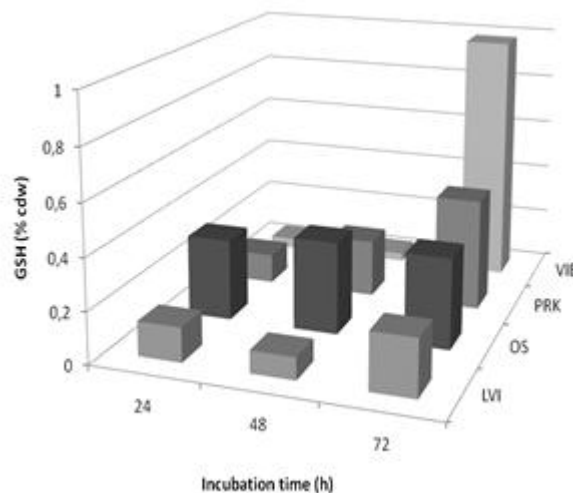
### 3.2 GSH production in baker's yeast samples

The possibility to employ *S. cerevisiae* as GSH source was considered because intracellular GSH accumulation in high levels is a specific metabolic characteristic of this specie [3, 5, 7, 12].

Four samples of baker's yeast in compressed form were tested for their ability of accumulating GSH. They were used at the same shelf life ( $10 \pm 1$  days) from production, considering the commercial life (generally 30 days).

Yeast biomass was suspended in an appropriate saline solution, containing glucose, glycine and cysteine, aminoacids present in GSH structure, as previously reported [18,19]. GSH time course was monitored for up to 72 h, time after which no increase of GSH levels was observed.

Results highlighted that intracellular GSH accumulation is a variable characteristic among yeasts, ranging generally from 0.15 to 0.7 % cell dw; only the commercial yeast VIE resulted in GSH accumulation up to 1% dw, with a 6-fold increase with respect to the physiological initial content (Fig. 2). Oxidised glutathione (GSSG) content, not an interesting compound so far for the present research, but index of cell stress level, was revealed only in LVI and PRK samples, with levels ranging from 0.1 to 0.2 % dw.



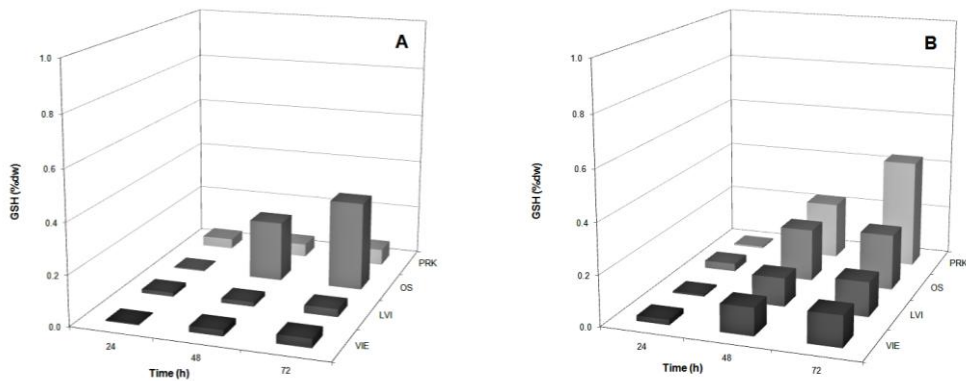
**Figure 2.** Time course of intracellular GSH content (% cell dry weight) in biotransformation trials employing the four tested baker's yeast strains.

### 3.3 Formation of GSH-copper conjugates with adapted cells

Yeast cells were isolated from each considered commercial bakers' yeast sample, and transferred in solid media containing increasing concentration of copper acetate. In plates with low copper concentration (0.1-0.3 mg/ml), colonies appeared darker than the control creamy ones. In the highest copper presence, a low cell growth was observed, due to the toxic effect of copper on yeast metabolism during the growth phase [20].

Yeast colonies grown on copper were subsequently maintained on solid copper-MEA medium. To obtain biomass to be used in biotransformation trials, these isolated strains were then inoculated in liquid cultures, employing malt extract (MEB) medium containing 0.3 mg copper acetate/ml. Harvested copper adapted cells were then used

in biotransformation trials in absence (control samples) and in presence of copper, for comparison purposes. Biotransformation trials were carried out for 72 h. Samples without copper (control), did not evidence any significant GSH production (Fig. 3a); only OS strain accumulated GSH (0.2-0.3 % dw) at 48 and 72 h. Biotransformations in presence of copper evidenced low GSH formation (from 0.1 to 0.2 % dw) at 48 h, with the maximum level of 0.4% dw only for the copper-adapted PRK strain (Fig. 3b).



**Figure 3.** Time course of intracellular GSH content (% cell dry weight) of copper adapted yeast cells. Biotransformation trials carried out in copper absence (a) and presence (b) (0.3 mg copper acetate/ml).

As regards the production of GSH-copper conjugate, adapted cells showed different behaviour. In absence of copper in biotransformation phase, three strains did not produce conjugate; only the OS strain accumulated conjugate in a range of about 0.5-0.6% dw at 72 h. These results may be related to peculiar osmotolerance characteristics of this strain that can accumulate copper in growing phase due to its ability of growing in hyperosmotic conditions (Fig.4a).

Biotransformations carried out with copper evidenced limited levels of copper conjugate (0.1-0.2% dw); again only the OS strain resulted the best conjugate producer (0.6-0.7% dw) (Fig. 4b).

Results obtained applying this two-step procedure, in which cells were first grown in presence of copper and then subjected to biotransformation procedure, showed both limited GSH accumulation and GSH-copper conjugate levels. The analyses of this behaviour are still ongoing and more detailed experiments are needed to clarify the mechanisms involved in cell response to copper exposure. In particular *S. cerevisiae* seems to be sensitive to the toxic effect of copper when cells are in growing conditions rather than when they are kept in contact with copper only in a post-fermentative procedure. This situation may be the cause of GSH production in very low levels and consequently of no conjugate formation.



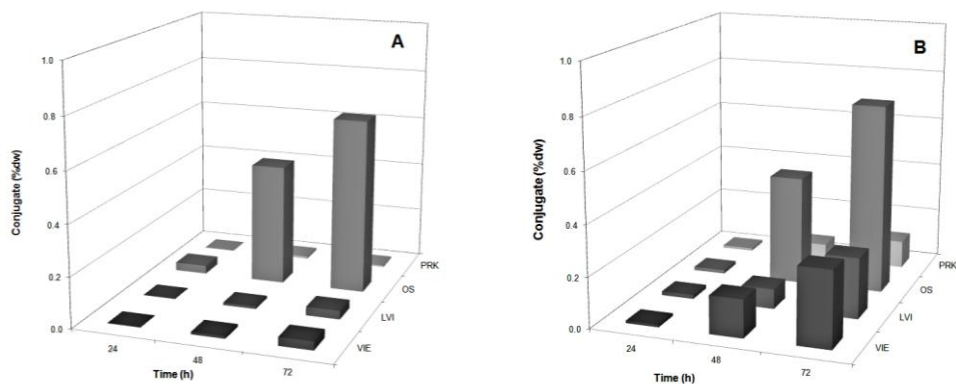


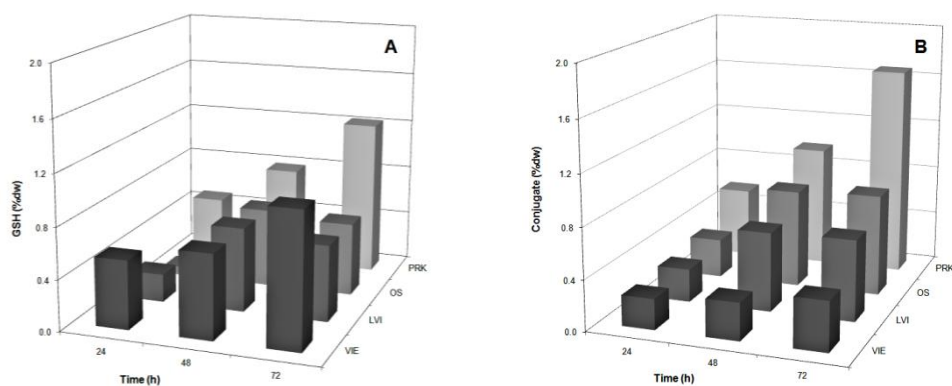
Fig. 4. Time course of intracellular GSH-copper conjugate (% cell dry weight) of four samples of copper adapted yeast cells. Biotransformation trials carried out in copper absence (a) and in presence (b) (0.3 mg copper acetate/ml).

### 3.4 Formation of GSH-copper conjugates with not adapted cells

As copper-adapted cells did not prove to be suitable for the formation of copper conjugates, experiments were then set up by employing not adapted cells. The aim of these experiments was to verify if yeasts, not adapted or not preliminary exposed to copper, produced a significant amount of conjugate.

At first, the most suitable copper concentration to be employed for the production of GSH-copper conjugates, was evaluated; the best results were obtained with 0.3 mg copper acetate/ml, corresponding to 1.6 mM (data not shown). Higher concentrations were found toxic for cells, as previously reported.

Baker's yeasts were suspended in biotransformations trials in which 0.3 mg copper acetate/mL were added, and reactions monitored for up to 72 h. As regards GSH content, all samples showed similar increasing time course, even if final levels reached were different (Fig. 5a). The maximum GSH level (1.2 % dw) was evidenced employing PRK yeast at 72 h. The same yeast was found to produce also the highest GSH-copper conjugate level (1.7 % dw) (Fig. 5b), while in the other samples this product accumulated in the range 0.4 - 0.7 % dw.



**Figure 5.** Time course of intracellular GSH (a) and GSH-copper conjugate (b) of four samples of not-adapted yeast cells. Biotransformation trials carried out with 0.3 mg copper acetate/ml.

### 3.5 Evaluation of copper conjugates by atomic absorption

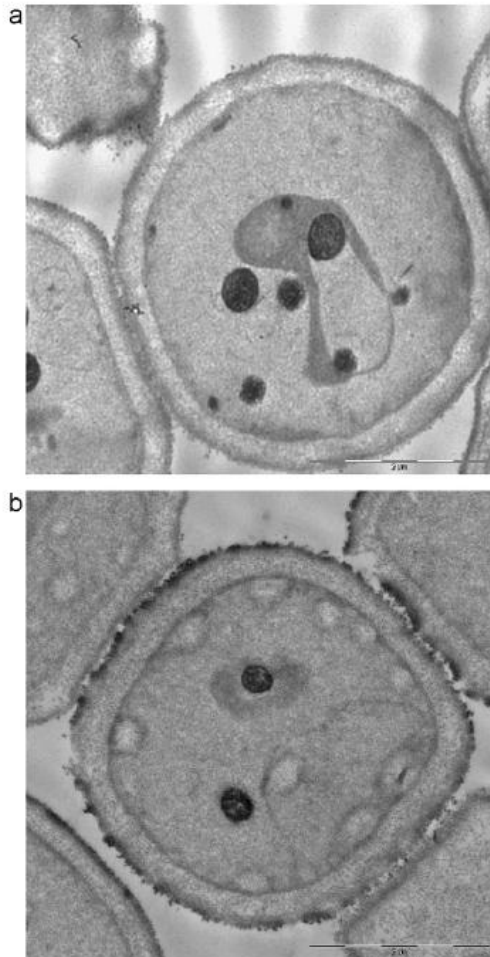
Samples of PRK yeast incubated for 72 h with 0.3 mg copper acetate/ml, in which the maximum conjugate formation was observed, were subjected to atomic absorption analysis.

In supernatants samples obtained by cell permeabilization procedure,  $1.2 \pm 0.1$  mg copper conjugate/g cell dw was evidenced, corresponding to  $19 \mu\text{mol}$  copper/g cell dw. However, morphological analysis of cells exposed to copper evidenced a more compact structure covering the cells, with respect to the control ones. These differences were confirmed by the transmission electron microscopy (TEM) (Fig. 6). This aspect was similar also in cell residues from the permeabilization procedure. Assuming that this behavior could be attributable to the presence of copper, which makes the cell recalcitrant to permeabilization procedure, a series of experiment on mechanical disruption of residual cells was set up. In the soluble fraction, separated by centrifugation after sonication,  $1.8 \pm 0.2$  mg copper conjugate/g cell dw was evidenced, corresponding to  $28 \mu\text{mol}$  copper/g cell dw. Analysis of cell debris proved the presence of residual copper, that was estimated about 0.17 mM.

The overall findings confirm data present in the literature, when *S. cerevisiae* cells are employed as heavy metal bioremediator [20]. Yeast metal uptake occurs in two steps: the first step (biosorption) is fast (it occurs in the first few minutes of contact with the metal), is independent of metabolism and happens in live and dead cells; the second step (bioaccumulation) is generally considered metabolism-dependent (it occurs only in live cells) and is attributed to intracellular metal uptake across the cell membrane. Using *S. cerevisiae*, authors reported a copper absorption of 7.6 and  $9.6 \mu\text{mol}$  copper/g cell dw for live and dead cells (inactivated at  $45^\circ\text{C}$ ), respectively [20]. In the present study a maximum of  $28 \mu\text{mol}$  copper/g cell dw was reached employing a post fermentative procedure.

Employing growing cells of *Candida intermedia*, the two steps procedure of metal cell surface adhesion and metal entrance inside cells was also highlighted, and a total amount of 1443  $\mu\text{g}$  copper/g cell dw was obtained (corresponding to 23  $\mu\text{mol}$  copper/g cell dw) [21].

As regards filamentous fungi, trials performed with *Trichoderma reesei* evidenced that copper accumulated on wall surface, and only the construction of a transformant strain led to copper accumulation (13 mg copper/g biomass) inside cell vacuols [20].



**Figure 6.** Transmission electron micrographs of ultrathin sections of *S. cerevisiae* PRK baker's yeast cells at 72 h incubation in biotransformation trials: control sample (a) and in presence of copper acetate (b).

#### 4. Conclusion

Samples of baker's yeast (*S. cerevisiae*) in compressed form were employed to obtain copper-enriched cells in biotransformation trials in presence of copper.

Cells adaptation through a two-steps procedure (grow adapted cells in presence of copper and then use this biomass in biotransformation trials to increase conjugates levels) demonstrated that cells are not stimulated to increase their intracellular GSH levels. On the contrary high GSH-copper conjugate levels (up to 1.7 % dw) were observed in experiments carried out employing not adapted cells. Cell disruption by sonication and analysis by atomic absorption evidenced the presence of very interesting amount (28  $\mu\text{mol}$  copper/g cell dw) of intracellular copper conjugate. In conclusion the applied procedure can be considered an interesting opportunity to further the range of application of yeast cell cultures for nutraceutical application in therapeutic treatments.

## 5. Acknowledgements

The authors would like to thank Dr. Nadia Santo for her excellent technical support in preparing TEM micrographs documentations.

## References

- [1] Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 1983;52:711-760
- [2] Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem-Biol Interac* 1998;111:1-14
- [3] Udeh KO, Achremowicz B. High glutathione-containing yeast *Saccharomyces cerevisiae*: optimization and production. *Acta Microbiol Polon* 1997;46:105-114
- [4] Monje-Casas F, Michan C, Pueyo C. Absolute transcript levels of thioredoxin- and glutathione-dependent redox system in *Saccharomyces cerevisiae*: response to stress and modulation with growth. *Biochem J* 2004;383:139-147
- [5] Penninckx M. An overview on glutathione in *Saccharomyces* versus non-conventional yeasts. *FEMS Yeast Res* 2002;2:295-305
- [6] Perricone C, De Carolis C, Perricone R. Glutathione: a key player in autoimmunity. *Autoimmun Rev* 2009;8:697-701
- [7] Li Y, Wei G, Chen J. Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* 2004;66:233-242
- [8] Wu G, Fang YZ, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;134:489-492
- [9] Beutler E. Nutritional and metabolic aspects of glutathione. *Annu Rev Nutr* 1989;9:287-302

- [10] Lomaestro BM, Malone M. Glutathione in health and disease: pharmacotherapeutic issues. *Ann Pharmacother* 1995;29:1263-1273
- [11] Penninckx M. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. *Enz Microb Technol* 2000;26:737-742
- [12] Rollini M, Pagani H, Riboldi S, Manzoni M. Influence of carbon source on glutathione accumulation in methylotrophic yeasts. *Ann Microbiol* 2005;55: 199-203
- [13] Wei GY, Wang DH, Chen J. Overproduction of glutathione by L-cysteine addition and a temperature strategy. *Biotechnol Bioprocesses Eng* 2008;13:347-353
- [14] Karlstrom AR, Levine LR. Copper inhibits the protease from human immunodeficiency virus 1 by both cysteine – dependent and cysteine -independent mechanisms. *Proc Natl Acad Sci USA*. 1991;88:5552-5556
- [15] Tang AM, Graham NMH, Saah AJ. Effects of micronutrient intake on survival in human immunodeficiency virus type 1 infection. *Am J Epidemiol* 1996;143:1244-1256
- [16] Sprietsma JE. Cysteine, glutathione (GSH) and zinc and copper ions together are effective, natural, intracellular inhibitors of (AIDS) viruses. *Med Hypoth* 1999;52:529-538
- [17] Jimenez I, Speisky H. Effect of copper ions on the free radical-scavenging properties of reduced glutathione: implications of a complex formation. *J Trace Elements Med Biol* 2000;14:161-167
- [18] Rollini M, Musatti A, Manzoni M. Production of glutathione in extracellular form by *Saccharomyces cerevisiae*. *Process Biochem* 2010;45:441-445
- [19] Rollini M, Manzoni M. Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. *Process Biochem* 2006;41:1501-1505
- [20] Machado MD, Janssen S, Soares HMVM, Soares EV. Removal of heavy metals using a brewer's yeast strain of *Saccharomyces cerevisiae*: advantages using dead biomass. *J Appl Microbiol* 2009;106:1792-1804
- [21] Fujs S, Gazdag Z, Poljsak B, Stibilj V, Milacic R, Pesti P, Batic M. The oxidative stress response of the yeast *Candida intermedia* to copper, zinc and selenium exposure. *J Basic Microbiol* 2005;45:125-135
- [22] Fu K, Liu L, Fan L, Liu T, Chen J. Accumulation of copper in *Trichoderma reesei* transformants, constructed with the modified *Agrobacterium tumefaciens*-mediated transformation technique. *Biotechnol Lett* 2010;32:1815-1820

## *5. Improvement of GSH post-growth production*

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## 5.1 Design of Experiments

In chapters 1 and 2 different parameters were evaluated for enhancing GSH intracellular accumulation. In summary the main important factors are GSH-precursor amino acids and energy source, as well as yeast physiology including its shelf-life. However nothing is known neither about the most amino acids influencing GSH accumulation, nor about possible interactions between amino acids and/or other factors.

As reported by Mandenius and Brundin (2008), the Design of Experiments (DoE) methodology provides powerful and efficient ways to optimize biotechnological processes using a reduced number of experiments. Its strength is that it also reveals how interactions between the input factors influence the output responses. These interactions are often difficult to discover and interpret with other methods. In optimization, the DoE methodology is clearly preferable to methods which vary one variable at a time.

The key elements of a DoE optimization methodology encompass planning the study objectives, screening of influential variables, experimental designs, postulation of mathematical models for various chosen response characteristics, fitting experimental data into these model(s), mapping and generating graphic outcomes, and design validation using model-based response surface methodology (Singh et al., 2005).

The selection of the variables and their levels is very important, since inappropriate choices will limit the usefulness of the results and making it necessary to carry out new experiments with other variables and levels. The reduced set of experiments can be described mathematically as  $2^{n-k}$ , where  $n$  is the number of factors to be investigated, 2 represents the low and high levels and  $k$  is the number of steps to reduce the experimental design. The screening will be further improved by replicates in the center point of the experimental domain: in the FCCD case, additional values of the variables are included in the surface central points between the corners of the experimental space. The reduction of factor experiments decreases the statistical quality and should consequently be applied with caution (Mandenius and Brundin, 2008).

For all these reasons the DoE methodology has been applied in this phase of process improvement.

## 5.2 References

**Mandenius CF, Brundin A.** 2008. Bioprocess optimization using design-of-experiments methodology. *Biotechnol Prog* 24:1191-1203.

Singh B, Kumar R, Ahuja N. 2005. Optimizing drug delivery systems using systematic "design of experiments." Part I: fundamental aspects. Crit Rev Ther Drug Carrier Syst 22:27-105.

### 5.3 Poster

Reported below is the poster exhibited during the 11<sup>th</sup> European Nutrition Conference, FENS, 26-29 Ottobre 2011, Madrid.

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Improvement Of Intracellular Glutathione Content In Baker's Yeast For Nutraceutical Application

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**INTRODUCTION**

**Glutathione** (GSH, L-γ-glutamyl-L-cysteinyl-glycine, Figure 1) is the most abundant non-protein thiol compound widely present in living organisms, from prokaryotes to eukaryotes. It is synthesized intracellularly by the consecutive actions of γ-glutamylcystine synthetase, feedback inhibited by GSH, and GSH synthetase (Figure 2). This tripeptide's very low redox potential gives it the properties of a cellular redox buffer (Anderson 1998). In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, xenobiotic and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism (Perninckx 2002). These characteristics make this active tripeptide of interest in the food additive industry and sports nutrition (Lomaestro and Malone 1995).

Yeasts, in particular belonging to the genus *Saccharomyces*, are the most commonly used microorganisms on an industrial scale for GSH fermentative production; however GSH contents in the wild-type strains are usually variable (0.1 – 1% dw) and always in intracellular form (Rollini et al. 2005; Rollini and Manzoni 2006). Being an intracellular product in yeast, a combination of high cell density and high GSH content leads to maximal GSH yield; however, increasing cell density can inevitably result in a lower GSH content. Addition of precursor amino acids was considered as a practical approach to enhance intracellular GSH content (Liang et al., 2009), in particular cysteine is considered as a key amino acid for GSH production. However the effect of amino acids addition during biotransformation process has not yet been investigated.

**The present study wants to investigate the influence of amino acids and/or their interactions for developing a simple and feasible procedure to obtain GSH enriched *S. cerevisiae* biomass for nutraceutical application.**

**Fig. 1 Glutathione**

**Fig. 2 Glutathione biosynthesis**

**MATERIALS AND METHODS**

**Experimental design and Biotransformation conditions**  
A *Saccharomyces cerevisiae* commercial baker's yeast in compressed form (Zeus IBA, Firenze), was employed. Cells were suspended (5 %dcw) in a solution having the following composition (g/L): glucose 80, sodium citrate 10, ammonium sulphate 7, KH<sub>2</sub>PO<sub>4</sub> 3.5, magnesium sulphate 0.5, tap water. Cysteine, glycine, serine and glutamic acid were differently combined according to the experimental design (Table 1). A Face Centered Central Composite Design (FCCD) with 4 variables tested at 2 levels (for a total of 30 trials) was applied. Each trial was performed in duplicate. Variables were cysteine (A-CYS), glycine (B-GLY), serine (C-SER), and glutamic acid (D-GLU), each tested between 0 ("level) and 4 ("level) g/L. Biotransformation trials were carried out in 100 mL Erlenmeyer flasks, each containing 10 mL reaction mixture. Samples were incubated at 28 °C on an alternative shaker (60 rpm, 4 cm run), collecting aliquots to analyze at 0 and 24 h. Results were analyzed employing Design Expert® v. 7.0 (StatEase – Minneapolis) software.

**Analytical procedures**  
Intracellular GSH was determined as follows: aliquots of the reaction mixture were centrifuged (7,000 rpm, 7 min); cells were washed with H<sub>2</sub>O, and then thermally treated at 100 °C for 10 min. After cooling in ice bath, samples were centrifuged (12,000 rpm, 12 min) and on obtained supernatants intracellular GSH was evaluated by HPLC. Results were expressed in terms of percentage of GSH content on dry cell weight (%dcw).

**HPLC chromatography conditions**  
Purospher® RP-18 endcapped (250 - 4 mm) (Merck) 30 °C; eluent NaH<sub>2</sub>PO<sub>4</sub> 25 mM pH 2.8, flux 0.3 mL/min, UV 210 nm.

**RESULTS**

The equation model was found significant (R<sup>2</sup> 0.9851, predicted R<sup>2</sup> 0.9599) and ANOVA analysis (Table 2) identified the significant the terms: A-cysteine, B-glycine, C-serine, D-glutamic acid, AC and BC interactions, as well as quadratic terms A<sup>2</sup> and D<sup>2</sup>.

$$GSH^{1.6} = +0.74 - 0.59 \times A - 0.15 \times B - 0.069 \times C - 0.093 \times D - 0.14 \times AC + 0.088 \times B \times C + 0.76 \times A^2 + 0.17 \times D^2$$

**Tab. 2 ANOVA response**

Block	SS	df	MS	F	p (sig)
Model	16.90	1	16.90	157.48	< 0.0001
A	0.16	1	0.16	1.51	< 0.0001
B	0.41	1	0.41	47.72	< 0.0001
C	0.085	1	0.085	0.85	0.0054
D	0.16	1	0.16	1.51	0.0054
AC	0.33	1	0.33	38.09	< 0.0001
BC	0.12	1	0.12	14.20	0.0013
A <sup>2</sup>	1.63	1	1.63	150.87	< 0.0001
D <sup>2</sup>	0.093	1	0.093	10.75	0.0039

**Fig. 3 3-D Surface of cysteine-serine (AC) interaction.**

**Tab. 1 Scheme of the experimental factorial design, and GSH production**

Trials	A (g/L)	B (g/L)	C (g/L)	D (g/L)	GSH (%dcw)
1	0	4	4	4	0.01
2	2	2	2	2	1.23
3	0	0	4	0	0.55
4	4	0	4	4	1.25
5	4	4	4	0	1.07
6	0	4	0	0	0.62
7	2	2	2	2	1.21
8	0	0	0	4	0.59
9	4	0	0	0	0.75
10	4	4	0	4	1.06
11	0	0	0	0	0.57
12	0	4	0	4	0.68
13	4	4	0	0	0.93
14	4	0	4	0	0.97
15	4	4	4	4	1.12
16	2	2	2	2	1.15
17	0	4	4	0	0.66
18	0	0	4	4	0.59
19	4	0	0	4	0.75
20	2	2	2	2	1.29
21	2	2	2	0	0.98
22	2	2	4	2	1.23
23	2	2	2	4	1.24
24	2	2	0	2	1.08
25	4	2	2	2	1.05
26	0	2	2	2	0.65
27	0	2	2	2	1.02
28	2	2	2	2	1.26
29	2	4	2	2	1.28
30	2	2	2	2	1.38

The lowest GSH yields were obtained in all the cysteine-free trials (Table 1) i.e. n° 1, 3, 6, 8, 11, 12, 17, 18 and 26, where GSH levels were not significantly different from the initial content (0.55±1 %dcw).

Cysteine led to the highest GSH levels when set at concentrations between 2-3 g/L.

The use of glycine significantly affected GSH yields (Figure 3), which were found higher when using 4 g/L of this amino acid.

The presence of serine, involved in sulphur metabolism of yeast cell, increased GSH synthesis when present at least at 2 g/L, above all when glycine was lower than 4 g/L.

The best combination of amino acids for high GSH accumulation is:

- Cysteine 2-3 g/L
- Glutamic acid 2-3 g/L
- Glycine and Serine >2 g/L

➔ GSH: 1.7 %dcw

**CONCLUSIONS**

The present study has confirmed cysteine relevance in GSH synthesis and results have revealed also the role of the other involved amino acids. Baker's yeast cells with increased intracellular GSH levels, can be obtained applying a post fermentative procedure with a proper combination of the amino acids. Yeast biomass with high GSH content for nutraceutical applications could be obtained up-scaling the biotransformation process.

**REFERENCES**

- Anderson M.E. (1998) Glutathione: an overview of biosynthesis and modulation. Chem-Biol Interact 111-112: 1-14.
- Lomaestro B.M. Malone M. (1995) Glutathione in health and disease: pharmacotherapeutic issues. Am J Pharmacotheor 29: 1263-73.
- Liang Q., Liao X., Du Q., Chen G. (2009) A new strategy to enhance glutathione production by multiple-H<sub>2</sub>O<sub>2</sub> induce oxidative stress in *Candida utilis*. Bioresour Technol 100: 350-355
- Perninckx M. (2002) An overview of glutathione in *Saccharomyces* versus non-conventional yeasts. FEMS Yeast Res 2: 295-305.
- Rollini M., Pagan H., Riboldi S., Manzoni M. (2005) Influence of carbon source on glutathione accumulation in methylotrophic yeasts. Am Microbiol 55: 169-203.
- Rollini M., Manzoni M. (2006) Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. Process Biochem 41: 1501-1505.

Poster Reference Number: 27/404

Area: Innovation in Food for Optimal Nutrition

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#### 5.4 Submitted Paper

Here is reported the original submitted (*New Biotechnology*) form of the paper because of copyright limitations.

#### **Post-fermentative production of glutathione by baker's yeast (*S. cerevisiae*) in compressed and dried forms**

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#### **Abstract**

The study was aimed at investigating the best biotransformation conditions to increase intracellular glutathione (GSH) levels in samples of baker's yeast (*S. cerevisiae*) employing either the commercially available compressed and dried forms. Glucose, GSH precursors amino acids, as well as other cofactors, were dissolved in a biotransformation solution and yeast cells were added (5% dcw). Two response surface central composite designs (RSCCD) were performed in sequence: in the first step the influence of amino acid composition (cysteine, glycine, glutamic acid and serine) on GSH accumulation was investigated; once setup their formulation, the influence of other components was studied. Initial GSH content was found 0.53 and 0.47% dcw for compressed and dried forms. GSH accumulation ability of baker's yeast in compressed form was higher at the beginning of shelf life, i.e. in the first week, and a maximum of 2.04% dcw was obtained. Performance of yeast in dried form was not found satisfactory, as the maximum GSH level was 1.18% dcw. When cysteine lacks from the reaction solution, yeast cells did not accumulate GSH. With dried yeast, the highest GSH yields occurred when cysteine was set at 3 g/L, glycine and glutamic acid at least at 4 g/L, without serine. Employing compressed yeast, the highest GSH yields occurred when cysteine and glutamic acid were set at 2-3 g/L, while glycine and serine higher than 2 g/L. Results allowed to setup an optimal and feasible procedure to obtain GSH-enriched yeast biomass, with up to 3-fold increase respect to initial content.

**Keywords:** *S. cerevisiae*, glutathione, cysteine, serine, response surface.

#### **Introduction**

Glutathione (GSH) is a biologically active tripeptide consisting of L-glutamate, L-cysteine, and glycine. It is the most abundant non-protein thiol compound widely

distributed in living organisms, from prokaryotes to eukaryotes [1, 2], and is synthesized intracellularly in two ATP-dependent steps by the consecutive actions of  $\gamma$ -glutamylcysteine synthetase ( $\gamma$ -GCS), feedback inhibited by GSH content, and glutathione synthetase (GS). GSH biosynthesis is thus closely related to precursors amino acids supply,  $\gamma$ -GCS activity and ATP availability [3, 4].

In living tissues, GSH plays a pivotal role in bioreduction, protection against oxidative stress, xenobiotic and endogenous toxic metabolite detoxification, enzyme activity and sulphur and nitrogen metabolism [5]. GSH is potentially produced in many body areas, especially in the liver, and is involved in defense systems [6].

These characteristics make GSH an important biochemical drug for the treatment of numerous diseases, such as HIV infections, liver cirrhosis, gastrointestinal and pancreatic inflammations, as well as neurodegenerative diseases and aging [7, 8]. Besides being extracted from some active tissues, GSH may be produced by chemical method, enzymatic reaction and microbial fermentation [9].

GSH microbial production using yeasts is currently the most common method employed on industrial scale [8, 10, 11], through *Saccharomyces cerevisiae* and *Candida utilis* [12-14]. Most of the works focused on GSH accumulation with growing cells, employing sugar materials as carbon and energy source, with or without precursor amino acids addition [15]. Employing growing cells, cysteine is considered a key amino acid for GSH production and, because of its growth-inhibiting nature, optimal time for its addition is at stationary phase [15, 16].

To date only Rollini et al [17] and Benedetti et al [18] have applied a post-growing procedure (biotransformation) for GSH accumulation, by employing commercial baker's yeasts in compressed form, an inexpensive cells source available on the market. In the present research the performance of baker's yeast in compressed and dried form was compared, and the influence of amino acids and the other components present in biotransformation mixture on GSH accumulation level was investigated. The research would pave the way to develop a simple and feasible procedure to obtain a GSH-enriched *S. cerevisiae* biomass to be used for nutraceutical applications.

## **Materials and Methods**

### *Microorganism and Biotransformation conditions*

Samples of commercial baker's yeast (*S. cerevisiae*) in compressed form, identified as Zeus (Zeus Industria Biologica Alimentare Spa, Firenze, Italy), and in dried form, identified as Fermipan red (GB Ingredients, Casteggio, Pavia, Italy) were employed in the research. Yeast cells were suspended (5 %dcw) in a biotransformation solution, set-up in tap water, containing glucose, sodium citrate, ammonium sulphate,  $\text{KH}_2\text{PO}_4$ , magnesium sulphate, cysteine, glycine, glutamic acid and in some cases serine, differently combined according to the detailed arrangement of each experiment, described below.

Biotransformation trials were carried out in 100 mL Erlenmeyer flasks, each containing 10 mL reaction mixture, incubated at 28 °C and 200 rpm. Samples aliquots were collected at 0 and 24 h.

Compressed baker's yeast was stored at 4 °C until expiring date (35-40 days shelf life at refrigerated temperature) and biotransformations performed on yeast samples of the same age, i.e. 1, 10, 20 and 30 days. Differently, dried yeast is considered stable for one year at room temperature, and samples were taken during the first 2 months of shelf-life.

#### *Experimental designs and data analysis*

Two experimental designs were performed; the response  $y$  was always defined as GSH content in yeast cells (% dcw), computed as ratio between intracellular GSH content in the permeabilized solution (g GSH/L) and dry cell weight (g/L) [19].

In the first step, the influence of amino acid presence and concentration (cysteine, glycine, serine, glutamic acid) was investigated employing both compressed and dried yeast samples. A response surface central composite design (RSCCD) (face centered) with 4 variables, each tested at 2 levels for a total of 30 trials, was performed (triplicate trials). Variables were: cysteine ( $X_1$ -CYS), glycine ( $X_2$ -GLY), serine ( $X_3$ -SER) and glutamic acid ( $X_4$ -GLU); they were tested between 0 (level -1) and 4 g/L (level +1). The other ingredients were set as follows (g/L): glucose 80, sodium citrate 10,  $(\text{NH}_4)_2\text{SO}_4$  7,  $\text{KH}_2\text{PO}_4$  3.5 and  $\text{MgSO}_4$  0.5. The complete scheme of the experimental design, with levels in natural units, is given in Table 1.

Subsequently, the influence of other components present in activation mix, i.e. glucose, sodium citrate,  $\text{MgSO}_4$  and  $(\text{NH}_4)_2\text{SO}_4$ , was evaluated. As before, a response surface central composite design (RSCCD) with 4 variables tested at 2 levels (for a total of 30 trials), with three replicates for each trial, was performed. Variables were (g/L):  $X_5$ -glucose tested between 80 (-1) and 120 (+1),  $X_6$ -sodium citrate between 10 (-1) and 15 (+1),  $X_7$ -magnesium sulphate between 0.5 (-1) and 1.5 (+1),  $X_8$ -ammonium sulphate between 7 (-1) and 9 (+1). Amino acids were all fixed at 3 g/L and  $\text{KH}_2\text{PO}_4$  at 3.5 g/L. The complete scheme of the design is given in Table 5. In this phase only yeast in compressed form was used.

Results were analyzed employing the Design Expert 7.0 (Statease, Minneapolis) software.

#### *Analytical procedures*

Intracellular GSH was determined according to Rollini et al [19]. Briefly, samples (1 mL) were centrifuged ( $10,600 \times g$ , 10 min) and collected cells washed with distilled water, suspended in 1 mL of 0.5 g ascorbic acid/L in HPLC-grade  $\text{H}_2\text{O}$  then thermally treated at 100°C for 10 min. After cooling in ice bath, samples were centrifuged ( $10,600 \times g$ , 15 min) to eliminate cell residues and, on obtained supernatant fractions, intracellular GSH was evaluated.

GSH identification and quantification were carried out by HPLC, equipped with a UV detector (210 nm), at 30°C using a (250 x 4 mm) Purospher® RP-18 endcapped column (Merck), eluted with 25 mM NaH<sub>2</sub>PO<sub>4</sub> pH 2.8 at 0.3 mL/min.

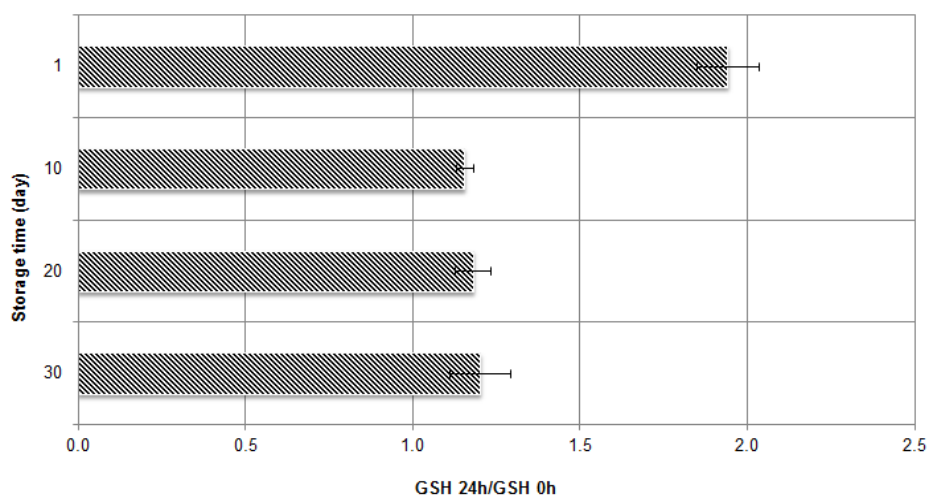
Standard GSH (reduced form) was purchased by Sigma and HPLC-grade H<sub>2</sub>O was obtained through a Milli-Q A10 Gradient System (Millipore Corporation).

Results were expressed in terms of GSH content as referred to dry cell weight (%dcw). Determination of dry cell weight was performed by drying cells at 105 °C (CEAL, Milano mod. SC4) to constant weight and samples were weighted using a thermobalance (Gibertini mod. TB2).

## Results

### *Influence of baker's yeast shelf life (compressed form) on GSH accumulation*

Time course of yeast performance was evaluated during shelf life of compressed yeast, that is usually between 35 and 40 days at refrigerated temperature. Biotransformation trials were performed employing a mixture containing GSH amino acids precursors that are cysteine, glycine and glutamic acid (CYS-GLY-GLU mixture).



**Figure 1.** Intracellular GSH ratio between 24 h and  $t_0$  during biotransformation trials, employing yeast in compressed form at 1, 10, 20 and 30 days of shelf life.

Data, reported in Figure 1, have been expressed as ratio between GSH content at 24 h reaction with respect to the initial level (GSH  $t_{24}/t_0$ ). Results highlighted that GSH accumulation is high at the beginning of shelf life, when yeast is able to double its intracellular GSH content (GSH ratio + 1.94±0.09), and decreases during storage, when cells retain GSH without further accumulation (ratio around + 1).

Differently, yeast samples in dried form can be considered an almost stable product with a shelf life of 1 year at room temperature. Nevertheless, for the present research, samples were used during the first 2 months in order to assure the best cell enzymatic activity/performance.

#### *Influence of amino acids*

The complete scheme of the experimental design performed with the two yeast forms, together with GSH production levels are shown in Table 1.

For both yeasts, the lowest GSH contents (0.55-0.68 and 0.51-0.59 %dcw for the compressed and the dried yeast, respectively) were obtained in all the cysteine-free trials, i.e. n° 1, 3, 6, 8, 11, 12, 17, 18 and 26, where GSH was not found significantly different from the initial content (0.53±0.06 and 0.47±0.05 %dcw respectively). It must be noted that the highest GSH yields were not obtained with the richest amino acid mixture, in fact trial n° 15 with all factors at +1 level furnished only 1.12 and 0.85 GSH %dcw employing compressed and dried yeast, respectively. Furthermore trial n° 10, corresponding to the CYS-GLY-GLU mixture, confirmed the ability of compressed yeast (see previous paragraph) to double its GSH intracellular level.

In the case of compressed yeast, the highest GSH levels (up to 1.38 %dcw), were obtained in trials where amino acids were set at 2 g/L (central points), i.e. n° 2, 7, 15, 20, 28 and 30. Otherwise, in the case of dried yeast, the highest GSH levels (up to 1.18 %dcw), were obtained when serine was absent and the other amino acids were set at 2 or 4 g/L (level 0 or +1), i.e. trials 10 and 24. In those cases both the yeasts were able increase GSH up to 2.5-fold their initial level.

The following second-order polynomial model (Equation 1) was used to describe the obtained results:

$$Y = \beta_0 + \sum_{j=1}^k \beta_j x_j + \sum_{i < j} \sum \beta_{ij} x_i x_j + \sum_{j=1}^k \beta_{jj} x_j^2 + \epsilon \quad (1)$$

In the case of compressed yeast with confidence intervals set at 95%, ANOVA analysis (Table 2) identified the following significant terms: X<sub>1</sub>-cysteine (p-value < 0.0001) and its quadratic term X<sub>1</sub><sup>2</sup> (p-value < 0.0001), X<sub>2</sub>-glycine (p-value < 0.0001), X<sub>3</sub>-serine (p-value 0.0054), X<sub>4</sub>-glutamic acid (p-value 0.0004) and its quadratic X<sub>4</sub><sup>2</sup> (p-value 0.0039), as well as interactions X<sub>1</sub>X<sub>3</sub> (p-value < 0.0001) and X<sub>2</sub>X<sub>3</sub> (p-value 0.0013).

**Table 1.** Scheme of the experimental factorial design RSCCD Face Centered: arrange of the experiments ( $X_1$  cysteine,  $X_2$  glycine,  $X_3$  serine,  $X_4$  glutamic acid) (in terms of g/L) and GSH production for compressed and dried yeast (GSH %dcw)

Trial number	Block	$X_1$	$X_2$	$X_3$	$X_4$	GSH *	
						Compressed	Dried
1	1	0	4	4	4	0.61±0.02	0.57±0.01
2	1	2	2	2	2	1.23±0.09	0.87±0.01
3	1	0	0	4	0	0.55±0.01	0.51±0.01
4	1	4	0	4	4	1.21±0.15	0.84±0.05
5	1	4	4	4	0	1.07±0.03	0.89±0.06
6	1	0	4	0	0	0.62±0.03	0.56±0.00
7	1	2	2	2	2	1.21±0.17	0.98±0.03
8	1	0	0	0	4	0.59±0.02	0.51±0.02
9	1	4	0	0	0	0.75±0.01	0.83±0.03
10	1	4	4	0	4	1.06±0.01	1.18±0.06
11	2	0	0	0	0	0.57±0.02	0.51±0.03
12	2	0	4	0	4	0.68±0.05	0.56±0.01
13	2	4	4	0	0	0.93±0.03	0.88±0.06
14	2	4	0	4	0	0.97±0.08	0.76±0.02
15	2	4	4	4	4	1.12±0.06	0.85±0.02
16	2	2	2	2	2	1.15±0.06	0.85±0.02
17	2	0	4	4	0	0.60±0.01	0.50±0.05
18	2	0	0	4	4	0.59±0.00	0.49±0.02
19	2	4	0	0	4	0.75±0.01	1.06±0.02
20	2	2	2	2	2	1.29±0.20	0.93±0.03
21	3	2	2	2	0	0.98±0.07	0.95±0.06
22	3	2	2	4	2	1.23±0.30	0.94±0.02
23	3	2	2	2	4	1.24±0.10	1.00±0.05
24	3	2	2	0	2	1.08±0.10	1.10±0.01
25	3	4	2	2	2	1.05±0.13	0.95±0.01
26	3	0	2	2	2	0.65±0.07	0.59±0.00
27	3	2	0	2	2	1.02±0.03	0.90±0.00
28	3	2	2	2	2	1.26±0.23	1.01±0.03
29	3	2	4	2	2	1.28±0.28	1.03±0.08
30	3	2	2	2	2	1.38±0.08	1.00±0.02

\*: mean ± standard deviation (three replicates)

**Table 2.** ANOVA response and effect estimates of the experimental design reported in Table 1 (compressed yeast) applying Equation 2 ( $\alpha$  out: 0.05)

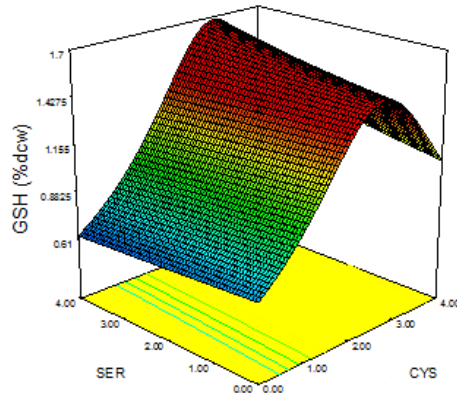
	Sum of Squares	df	Mean of Squares	F	p (p>F)
Block	2.18	2	1.09		
Model	10.90	8	1.36	157.48	< 0.0001
X <sub>1</sub> -CYS	6.16	1	6.16	711.79	< 0.0001
X <sub>2</sub> -GLY	0.41	1	0.41	47.72	< 0.0001
X <sub>3</sub> -SER	0.085	1	0.085	9.85	0.0054
X <sub>4</sub> -GLU	0.16	1	0.16	18.01	0.0004
X <sub>1</sub> X <sub>3</sub>	0.33	1	0.33	38.09	< 0.0001
X <sub>2</sub> X <sub>3</sub>	0.12	1	0.12	14.20	0.0013
X <sub>1</sub> <sup>2</sup>	1.83	1	1.83	210.87	< 0.0001
X <sub>4</sub> <sup>2</sup>	0.093	1	0.093	10.75	0.0039

The model was identified as significant and after power transformation ( $\lambda = -1.6$ ) recommended by the Box–Cox plot, the following equation (Equation 2) was identified (coded factors):

$$\text{GSH}^{-1.6} = +0.74 - 0.59X_1 - 0.15X_2 - 0.069X_3 - 0.093X_4 - 0.14X_1X_3 + 0.088X_2X_3 + 0.76X_1^2 + 0.17X_4^2 \quad (2)$$

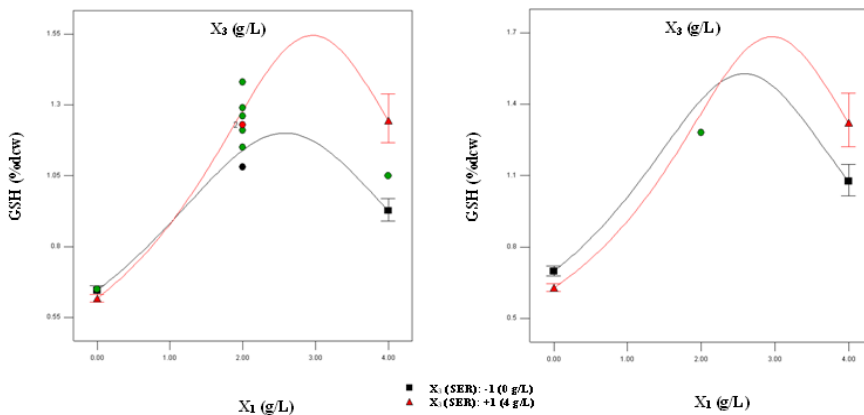
The software estimated model adaptation goodness ( $R^2$  0.9851, predicted  $R^2$  0.9599 and adjusted  $R^2$  0.9789), measures of variability (standard deviation 0.093, % confidence variation 7.15) and noise ratio (adequate precision 34.4897). Proceeding to diagnostics, attention was focused on Normal probability plot of studentized residuals for normality of residuals. Model acceptability was evaluated using studentized residuals versus predicted values plot, to evidence constant error. The analysis of these plots showed linearity of the data points on normal probability plot, as well as normality in the error term and a very good model prediction (data not shown).

Results evidenced that cysteine ( $X_1$ ) induced the highest GSH levels when set at concentrations between 2-3 g/L (Figure 2).



**Figure 2.** 3-D Surface of  $X_1X_3$  interaction (CYS-SER). Actual factors:  $X_2$ -GLY 4 g/L,  $X_4$ -GLU 2 g/L.

Also glycine ( $X_2$ ) significantly affected GSH yields, that were higher when using 4 g/L of this amino acid (Figure 3). The presence of serine ( $X_3$ ), that is not part of GSH but is involved in sulphur metabolism of yeast cell [20, 21], increased GSH synthesis when present at least at 2 g/L, above all when glycine was not at 4 g/L.



**Figure 3.**  $X_1X_3$  interaction (CYS-SER). a) actual factors  $X_2$ -GLY 2 g/L,  $X_4$ -GLU 2 g/L; b) actual factors  $X_2$ -GLY 4 g/L,  $X_4$ -GLU 2 g/L.

To confirm model robustness, other set of biotransformation trials were then performed. Results, shown in Table 3 confirmed that serine and glycine are important for obtaining high GSH levels; nevertheless, even if glycine is present at 4 g/L, serine cannot disappear from the formulation (see trial n° 4); glycine instead can be omitted but only if serine is present at 4 g/L (trial n° 1).



**Table 3.** Confirmation trials performed with compressed yeast: amino acid content (g/L), predicted (pred.) vs obtained (obt.) GSH yields, expressed as %dcw

Trials	CYS	GLY	SER	GLU	GSH pred.	GSH obt.*
<b>1</b>	2.5	0	4	2.5	1.45	<b>1.38±0.03</b>
<b>2</b>	2.5	4	0	2.5	1.24	<b>1.22±0.02</b>
<b>3</b>	2.5	4	4	2.5	1.72	<b>1.63±0.05</b>
<b>4</b>	4	4	0	4	1.07	<b>1.08±0.04</b>

\*: mean ± standard deviation (three replicates)

In the case of dried yeast with confidence intervals set at 95%, ANOVA analysis (Table 4) identified the following significant terms: X<sub>1</sub>-cysteine, and its quadratic term X<sub>1</sub><sup>2</sup>, X<sub>2</sub>-glycine, X<sub>3</sub>-serine and X<sub>4</sub>-glutamic acid.

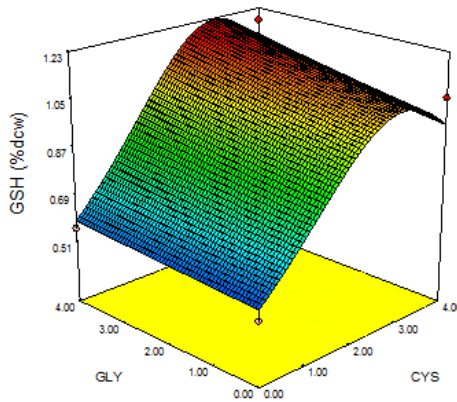
**Table 4.** ANOVA response of the experimental design reported in Table 1 (dried yeast) applying Equation 3 (α out: 0.05)

	Sum of Squares	df	Mean of Squares	F	p (p>F)
Block	0.82	2	0.41		
Model	3.59	5	0.72	145.44	<0.0001
X <sub>1</sub> -CYS	2.69	1	2.69	543.30	<0.0001
X <sub>2</sub> -GLY	0.068	1	0.068	13.80	0.0012
X <sub>3</sub> -SER	0.066	1	0.066	13.40	0.0014
X <sub>4</sub> -GLU	0.039	1	0.039	7.95	0.0100
X <sub>1</sub> <sup>2</sup>	0.74	1	0.74	148.75	<0.0001

The model was identified as significant and, after inverse transformation (lambda = -1), the following equation (Equation 3) was identified (as coded factors):

$$\frac{1}{\text{GSH}} = +1.08 - 0.39X_1 - 0.062X_2 + 0.061X_3 - 0.047X_4 + 0.39X_1^2 \quad (3)$$

Model adaptation goodness ( $R^2$  0.9706, predicted  $R^2$  0.9385 and adjusted  $R^2$  0.9640), variability (standard deviation 0.070 %, confidence variation 5.35) and noise ratio (adequate precision 30.585) were found satisfactory.



**Figure 4.** 3-D Surface:  $X_1X_2$  (CYS-GLY) interaction,  $X_3$ -SER 0 g/L,  $X_4$ -GLU 4 g/L.

3-D surface plot shows that, when employing dried yeast, cysteine ( $X_1$ ) induced the highest GSH levels when set at 3 g/L, while serine ( $X_3$ ) should be minimized (Figure 4). Also, the use of glycine ( $X_2$ ) and glutamic acid ( $X_4$ ) significantly affected GSH yield, that was found higher when using at least 4 g/L of these amino acids.

From an overlook at the results, it can be concluded that the use of baker's yeast in compressed form allowed to obtain the highest GSH intracellular accumulation levels (max 1.63 %dcw), so far this type of commercial yeast was chosen for the prosecution of the research.

#### *Influence of other mixture components*

The influence of the other activation mixture components on GSH production by compressed yeast is shown in Table 5.

**Table 5.** Scheme of the experimental factorial design RSCCD: arrange of the experiments ( $X_5$ : glucose,  $X_6$ : sodium citrate,  $X_7$ : magnesium sulphate,  $X_8$ : ammonium sulphate) (in terms of g/L) and results (GSH %dcw)

Trial number	Block	$X_5$	$X_6$	$X_7$	$X_8$	GSH *
1	1	80	15	1.5	9	1.57±0.05
2	1	120	15	0.5	9	1.46±0.14
3	1	120	15	1.5	7	1.24±0.05
4	1	120	10	0.5	7	1.44±0.13
5	1	100	12.5	1	8	1.47±0.14
6	1	80	10	0.5	9	1.38±0.08
7	1	100	12.5	1	8	1.49±0.14
8	1	120	10	1.5	9	1.33±0.15
9	1	80	15	0.5	7	1.35±0.34
10	1	80	10	1.5	7	1.47±0.10
11	2	120	15	1.5	9	1.38±0.07
12	2	80	15	1.5	7	1.38±0.27
13	2	80	15	0.5	9	1.67±0.02
14	2	120	10	1.5	7	1.37±0.04
15	2	120	15	0.5	7	1.49±0.15
16	2	120	10	0.5	9	1.52±0.16
17	2	80	10	1.5	9	1.65±0.10
18	2	80	10	0.5	7	1.73±0.03
19	2	100	12.5	1	8	1.64±0.01
20	2	100	12.5	1	8	1.56±0.11
21	3	100	7.5	1	8	2.04±0.00
22	3	100	12.5	2	8	1.43±0.05
23	3	100	17.5	1	8	1.66±0.11
24	3	100	12.5	1	8	1.51±0.02
25	3	100	12.5	1	6	1.28±0.10
26	3	100	12.5	0	8	1.28±0.06
27	3	60	12.5	1	8	0.98±0.04
28	3	100	12.5	1	8	1.61±0.03
29	3	100	12.5	1	10	1.43±0.04
30	3	140	12.5	1	8	1.01±0.03

\*: mean ± standard deviation (three replicates)

High GSH yields were generally obtained, with a maximum GSH level of 2.04 %dcw in trial 21. The lowest GSH levels (0.98-1.01 %dcw) were obtained in trials n° 27 and 30, with glucose present either at 60 or 140 g/L ( $-\alpha$  or  $+\alpha$  level).

With confidence intervals set at 95%, ANOVA analysis identified the following significant terms (Table 6):  $X_6$  – sodium citrate (p-value 0.03883) and its quadratic term  $X_6^2$  (p-value 0.0006) as well as  $X_5^2$  – glucose as quadratic term (p-value < 0.0001).

**Table 6.** ANOVA response of the experimental design reported in Table 5 (compressed yeast) applying Equation 4 ( $\alpha$  out: 0.05)

	Sum of Squares	df	Mean of Squares	F	p (p>F)
Block	0.099	2	0.049		
Model	0.78	4	0.19	13.93	< 0.0001
X <sub>5</sub> -GLUCOSE	0.035	1	0.035	2.48	0.1289
X <sub>6</sub> -SODIUM CITRATE	0.067	1	0.067	4.83	0.03883
X <sub>5</sub> <sup>2</sup>	0.38	1	0.38	27.16	< 0.0001
X <sub>6</sub> <sup>2</sup>	0.22	1	0.22	16.02	0.0006

The model was identified as significant and it allowed the identification of the following equation (Equation 4) (coded factors):

$$\text{GSH} = +1.2656 + 0.0557X_5 - 0.3752X_6 - 0.0003X_5^2 + 0.0140X_6^2 \quad (4)$$

Although the term X<sub>5</sub> (glucose) was not significant, it had been added into the model equation for hierarchical reason. In the present case, even if the model was identified as significant the software estimated a not nice model adaptation goodness (R<sup>2</sup> 0.7079, predicted R<sup>2</sup> 0.3124 and adjusted R<sup>2</sup> 0.6571), with a low predicted R<sup>2</sup>, not close to the adjusted R<sup>2</sup>, as a good model might expect. This behavior is due to the fact that the model confirms that the analyzed ingredients are less important than the previously investigated amino acids, and no strong influence had been evidenced. To maximize GSH yields, glucose should be set at 100 g/L (0 level) and sodium citrate at 10 g/L (-1), independently from magnesium and ammonium sulphate levels.

## Discussion and conclusions

*Saccharomyces cerevisiae* is one of the most studied microorganisms, and together with *Candida utilis* is the most commonly used microorganism on industrial scale for GSH fermentative production. Lots of strategies for enhancing GSH yields had been applied till now, but all employed growing yeast cells.

This study was instead aimed at investigating the best conditions to increase intracellular GSH levels present in samples of baker's yeast, employing already grown cells in a biotransformation procedure. GSH accumulation ability of baker's yeast in compressed form was found higher at the beginning of shelf life, i.e. in the first week, and a maximum of 2.04 %dcw was obtained. Performance of yeast in dried form was not found satisfactory, as the maximum GSH level was 1.18 %dcw.

To obtain high GSH levels, in the biotransformation solution the followings are needed: i) GSH precursors, i.e. cysteine, glycine, glutamic acid, but also serine, involved in sulphate metabolism and in cysteine synthesis; ii) cofactors such as

magnesium and ammonium sulphate, potassium phosphate, sodium citrate and iii) glucose as energy source, necessary for ATP regeneration in GSH synthesis. Results confirmed that cysteine is a key amino acid for GSH accumulation, not only during cell growth, as reported by Wen et al [16] and Nisamedtinov et al [11], but also in the presented post-growing procedure. Nevertheless, applying a post-growing procedure, cysteine negative effect as growth inhibitors, as reported by Wang et al [22], is undoubtedly countered.

Cysteine is not the only amino acid influencing GSH accumulation: all the tested amino acids were found significant and, in the case of compressed yeast, also cysteine-serine and glycine-serine interactions. Glycine is a direct precursor of GSH and also Wen et al [16] indicated it as the most important amino acid after cysteine in GSH synthesis.

Serine is not a direct GSH precursor but in *S. cerevisiae* two pathways exist for cysteine synthesis, and in both of them serine is involved [20, 21]. One route proceeds by serine acetylation to yield *O-acetylserine* (OAS), that is subsequently sulphhydrylated to cysteine; the second one involves the presence of homocysteine, through the cystathionine biosynthesis cycle. As suggested by Penninckx and Elskens [20], the presence of sulphates and serine may induce the yeast to incorporate sulphur present in excess into GSH, during cell growth. Results obtained in the present paper highlight that this mechanism seems to be present also in post-growing GSH accumulation, above all with compressed yeast.

All literature data related to GSH accumulation in yeasts have been collected employing growing cells. Wen et al. [16] found that while cysteine and glycine significantly affected GSH accumulation, glutamic acid did not. In our study, in biotransformation condition with already-grown cells, also glutamic acid showed to significantly affect GSH accumulation.

The second response surface CCD applied revealed that only glucose and sodium citrate showed to have significant effect on GSH accumulation, meaning that all the other parameters can be minimized with an economic advantage. Note that sodium citrate showed to have an inverse correlation towards GSH accumulation.

To minimize GSH production costs, future goals should be aimed at reducing biotransformation incubation time. This reduction would probably lead to a re-modulation of the concentration of amino acids added into the biotransformation reaction. Glucose substitution with others sugar matrix may led the process to be less expensive, even if it has to be taken in consideration that Cha et al [23], when comparing different carbon sources, found that in growing cells, glucose was the best substrate for GSH production. Also, as yeast has to be at the beginning of its shelf life, biotransformation step may be directly performed in baker's yeast production plant using yeast culture at the end of the biomass production step, before processing in the final compressed form, thus reducing GSH production costs.

The biotransformation procedure applied allowed to obtain high GSH levels, till 2.04 %dcw in the best case, and however, in general, levels higher than 1.5 %dcw at 24 h incubation. Considering an initial GSH intracellular level of 0.53 %dcw, a 3-fold

increase was obtained. Zhang et al. [10] reported GSH levels of 1.81 times higher than the control in terms of mg/L, but the increase in terms of intracellular content (%dcw) was lower. Cha et al [23] obtained high GSH levels (204 mg/L) that in terms of intracellular content means a 1.37-fold increase with respect to the control culture.

In conclusion we propose a post-fermentative biotransformation procedure as an economic alternative to the more traditional GSH biomass enrichment during cell growth; in this contest, we found the use of experimental designs, and in particular of CCD, an efficient approach for investigating and optimizing biotransformation processes. These results should be taken into account for a future up-scale of GSH-enriched yeast biomass production.

## References

- [1] Anderson ME. Glutathione: an overview of biosynthesis and modulation. *Chem Biol Interact* 1998;111:1–14
- [2] Meister A, Anderson ME. Glutathione. *Annu Rev Biochem* 1983;52:711–60
- [3] Liang G, Liao X, Du G, Chen J. Elevated glutathione production by adding precursor amino acids coupled with ATP in high cell density cultivation of *Candida utilis*. *J Appl Microbiol Biotechnol* 2008;105:1432–40
- [4] Liang G, Mo Y, Du G. Optimization of sodium dodecyl sulfate (SDS) addition coupled with adenosine triphosphate (ATP) regeneration for glutathione overproduction in high density cultivation of *Candida utilis*. *Enzyme Microb Technol* 2010;46:526–33
- [5] Penninckx M. An overview on glutathione in *Saccharomyces* versus non- conventional yeasts. *FEMS Yeast Res* 2002;2:295–305
- [6] Perricone C, De Carolis C, Perricone R. Glutathione: a key player in autoimmunity. *Autoimmun Rev* 2009;8:697–701
- [7] Wu G, Fang YZ, Lupton JR, Turner ND. Glutathione metabolism and its implications for health. *J Nutr* 2004;134:489–92
- [8] Li Y, Wei G, Chen J. Glutathione: a review on biotechnological production. *Appl Microbiol Biotechnol* 2004;66:233–42
- [9] Douglas KT. Chemical synthesis of glutathione and analogs. *Coenzymes Cofact* 1989;3:243–79
- [10] Zhang T, Wen S, Tan T. Optimization of the medium for glutathione production in *Saccharomyces cerevisiae*. *Process Biochem* 2007;42:454–8
- [11] Nisamedtinov I, Kevvai K, Orumets K, Rautio JJ, Paalme T. Glutathione accumulation in ethanol-stat fed-batch culture of *Saccharomyces cerevisiae* with a switch to cysteine feeding. *Appl Microbiol Biotechnol* 2010;87:175–83
- [12] Penninckx M. A short review on the role of glutathione in the response of yeasts to nutritional, environmental, and oxidative stresses. *Enzyme Microb Technol* 2000;26:737–42
- [13] Rollini M, Pagani H, Riboldi S, Manzoni M. Influence of carbon source on glutathione accumulation in methylotrophic yeasts. *Ann Microbiol* 2005;55:199–203
- [14] Wei GY, Wang DH, Chen J. Overproduction of glutathione by l-cysteine addition and a temperature strategy. *Biotechnol Bioproc Eng* 2008;13:347–53
- [15] Liang G, Liao X, Du G, Chen J. A new strategy to enhance glutathione production by multiple H<sub>2</sub>O<sub>2</sub>-induced oxidative stresses in *Candida utilis*. *Biores Technol* 2009;100:350–5

- [16] Wen S, Zhang T, Tan T. Optimization of the amino acid composition in glutathione fermentation. *Process Biochem* 2005;40:3474-9
- [17] Rollini M, Manzoni M. Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. *Process Biochem* 2006;41:1501-5
- [18] Benedetti A, Berardi E, Manzoni M, Nichele M, Pagani H, Rollini M. Process for producing glutathione. European Patent EP 1391517;2008
- [19] Rollini M, Musatti A, Erba D, Benedetti A, Girardo F, Manzoni M. Process for obtaining copper-enriched cells of *Saccharomyces cerevisiae*. *Process Biochem* 2011;46:1417-22
- [20] Penninckx MJ, Elskens MT. Metabolism and functions of glutathione in micro-organisms. In: *Advances in microbial physiology*, London, Academic Press Limited; 2009, p.239-301
- [21] Aitken SM, Kirsch JF. The enzymology of cystathionine biosynthesis: strategies for the control of substrate and reduction specificity. *Arch Biochem Biophys* 2005;433:166-75
- [22] Wang Z, Tan T, Song J. Effect of amino acids and feedback control strategies on the high-cell-density cultivation of *Saccharomyces cerevisiae* for glutathione production. *Process Biochem* 2007;42:108-11
- [23] Cha JY, Park JC, Jeon BS, Lee YC, Cho YS. Optimal fermentation conditions for enhanced glutathione production by *Saccharomyces cerevisiae* FF-8. *J Microbiol* 2004;42: 51-5

## *6. GSH bioavailability experiments*

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## 6.1 Bioavailability

The bioavailability of a compound involves different and subsequent processes: ingestion, release during digestion, absorption by intestinal epithelium and first pass metabolism. Bioavailability indicates the fraction of an ingest compound that is solubilized during gastrointestinal digestion and reaches the systemic circulation, following absorption from the gastrointestinal lumen (Calatayud et al., 2010).

Glutathione (GSH) bioavailability has been studied by several authors. Hagen et al. (1990) and Rahman & MacNee (1992) affirmed that plasma GSH concentration in rats increased from approximately 15 to 30 microM after administration of GSH, that can be absorbed intact increasing blood plasma GSH concentration. This indicates that oral supplementation may be useful to enhance GSH tissue availability. Likewise, Aw et al. (1991) reported that in mice oral GSH intake can increase GSH concentrations in several tissues following its depletion. On the contrary, Witschi et al. (1992) reported that in man systemic GSH availability is negligible and it is not possible to increase GSH circulating to a clinical beneficial extent by the oral administration of a single dose of 3 g.

The aim of this section was to better understand GSH bioavailability *in vitro*, employing model systems. Caco-2 intestinal cell line is one of the most commonly used models, established from human colon adenocarcinoma cells. These cells are able to differentiate spontaneously, giving rise to a monolayer possessing most of the functional and morphological features of mature human enterocytes. The differentiated cell monolayer is polarized, with microvilli on the apical border, intercellular tight junctions, secretion of enzymes inherent to the brush border membrane, expression of transporters, characteristic of the small intestine, in the apical and basolateral membranes (Maubon et al., 2007). For this reason, Caco-2 cell monolayer represents a valuable transport model system for the small intestinal epithelium (Hidalgo et al., 1989; Calatayud et al., 2010).

To further investigate the role of mucus on drug transport across the intestinal barrier, the human adenocarcinoma HT29-MTX model has been developed. This cell line is derived from the parental cell line HT29 and adapted to a medium containing  $10^{-6}$  M methotrexate for 6 months to acquire the morphological and mucin-producing characteristics of goblet cells (Lesuffleur et al., 1991; Pontier et al., 2001). To take advantage of this mucin-secreting ability of HT29-MTX cells, recent efforts have been focused on developing co-cultures of these two human intestinal cell lines (Caco-2 and

HT29-MTX) to produce a more physiological and real model mimicking the intestinal epithelium (Pontier et al., 2001; Laparra et al., 2009).

A further aim of this section was to verify if ingested GSH, together with GSH coming from the normal biliary efflux, may protect intestinal cells during oxidative stress conditions and contribute to normal intestinal functions (Valencia et al., 2001).

## 6.2 Materials and Methods

### *Samples*

Reduced and oxidized glutathione standards (GSH and GSSG, respectively) were supplied from Sigma. In these set of trials three different yeast samples were employed, as follows:

- *Control* - Commercial baker's yeast in compressed form *Fala*, with a low GSH content ( $0.45 \pm 0.05\%$  dcw);
- *Yeast A* - *Fala* yeast with a GSH content of  $1.09 \pm 0.13\%$  dcw, obtained after activation procedure employing CYS-GLY-GLU mixture (Tab. 2.1);
- *Yeast B* - *Fala* yeast with a GSH content of  $1.49 \pm 0.01\%$  dcw, obtained after activation procedure employing CYS-GLY-GLU+ADE mixture (Tab. 2.5).

All the three yeast samples were lyophilised and employed for gastrointestinal digestion test as well as transport and permeability experiments. Lyophilisation was carried out as follows: yeast cells were suspended in distilled water (20% dcw), placed in stainless steel trays as a thin layer, and then frozen at  $-40\text{ }^{\circ}\text{C}$  for 4 h; cell disidratation phase was carried out at  $25\text{ }^{\circ}\text{C}$  and 1.33 Pa for 30 h (Edwards Minifast MFD 01, UK) (maximum residual humidity 5–8% dcw).

### *In vitro gastro-intestinal digestion*

Aliquots of standard GSH (40-200 mg/L) and samples of lyophilised baker's yeast (1 g) were digested using a simulated digestion process proposed by Laparra et al. (2003) and modified for a yeast biomass.

The digestion comprises two stages: the gastric stage with pepsin at pH 2.0 and the intestinal step, with pancreatin and bile extract at pH 6. Samples were weighed, and cellular grade water (25 mL) was added. The pH was adjusted to 2.0 with 6 M HCl. Then pepsin solution (prepared in 0.1 M HCl) was added to provide 2 mg of pepsin/g sample. Water was then added to reach 30 g and obtained samples were incubated in a

shaking bath (120 strokes min<sup>-1</sup>) at 37 °C for 2 h. Afterward, pH was raised to 6.0 by addition of 1 M NaHCO<sub>3</sub>. Then the pancreatin-bile extract mixture (prepared in 0.1 M NaHCO<sub>3</sub>) was added to provide respectively 0.5 mg pancreatin/g sample and 3 mg bile extract/g sample; reaction mixtures were then incubated at 37 °C for 2 h. Digested samples were centrifuged (15000 rpm, 30 min, 4°C) to obtain the bioaccessible fraction; the soluble fraction was heat treated (100°C, 4 min) to inactivate enzymes and then cooled in an ice bath.

Cell culture grade water (B. Braun Medical, S.A.) was used throughout the *in vitro* digestion assay. Enzymes and bile salts for *in vitro* gastrointestinal digestion were purchased from Sigma: porcine pepsin (enzymatic activity 944 U/mg protein), porcine pancreatin (activity equivalent to 4 x US Pharmacopoeia specifications/mg pancreatin) and bile extract (glycine and taurine conjugates of hyodeoxycholic and other bile salts).

### ***Cell cultures***

GSH transport through the intestinal epithelium was carried out using the Caco-2 cell line model and a co-culture Caco-2:HT29-MTX (ratios 50:50 and 70:30). Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC, n. 86010202, Salisbury, UK), while HT29-MTX were kindly provided by Dr. T. Lesuffleur (Institut National de la Santé et de la Recherche Médicale, INSERM UMR S938, Fr).

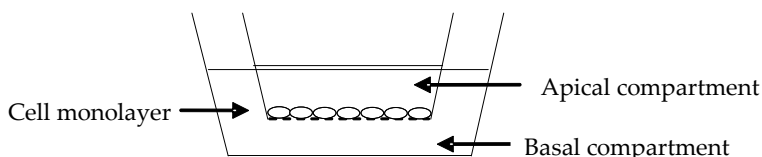
Caco-2 cell maintenance was performed in 75 cm<sup>2</sup> flasks to which 10 mL of Dulbecco's modified Eagle's medium (DMEM) with glucose (4.5 g/L) at pH 7.4 were added. The DMEM was supplemented with 10% (v/v) fetal bovine serum, 1% (v/v) non-essential amino acids, 1 mM of sodium pyruvate, 10 mM HEPES (N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid), 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.0025 mg/mL of fungizone, and (complete Dulbecco's modified Eagle's medium, DMEMc), as reported in Calatayud et al. (2010).

HT-29-MTX maintenance was performed in 25 cm<sup>2</sup> flasks to which 5 mL of medium was added, consisting of DMEM at pH 7.4 containing glucose (4.5 g/L) and supplemented with 10% (v/v) fetal bovine serum, 100 U/mL of penicillin, 0.1 mg/mL of streptomycin, 0.0025 mg/mL of fungizone, and 1 mM of sodium pyruvate (HT-DMEMc).

Both cell lines were incubated at 37 °C, in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>. The medium was changed every 2–3 days. When the cell monolayer reached 80% confluence, the cells were detached with a solution of trypsin (0.5 mg/L) and EDTA (0.22 g/L), followed by reseeding at a density of 5 x 10<sup>4</sup> cells/cm<sup>2</sup>. All the reagents used were purchased from PAA Laboratories GmbH (Germany).

### *Transport and permeability experiments*

Cell differentiation and transport tests were carried out in double chamber wells (24 mm diameter, pore size 0.4  $\mu\text{m}$ ; Transwell<sup>®</sup>, Costar Corp, NY) equipped with a porous support on which cell lines form monolayers, allowing the diffusion chamber to be divided into two compartments: apical (upper) and basal (lower) (Fig. 6.1).



**Figure 6.1.** Scheme of the double chamber wells employed for transport and permeability studies.

Cells were seeded at a density of  $6.5 \times 10^4$  cells/cm<sup>2</sup> in the following Caco-2/HT29-MTX proportions: 100/0; 70/30; 50/50; adding 1.5 mL of culture medium (DMEMc for Caco-2 and HT-DMEMc for co-culture) to the apical compartment and 2 mL to the basal side. Cells were then incubated at 37 °C in a humidified atmosphere of 95% air and 5% CO<sub>2</sub> replacing the medium every 2-3 days until cell differentiation was reached (13-14 days). All cell cultures were used between passages 20 and 40.

Transport experiments and apparent permeability ( $P_{app}$ ) tests were conducted in Hanks buffered solution salts (HBSS), supplemented with 20 mM *o*-2-(*N*-morpholine) ethanesulfonic acid (MES) (pH 5.5) in the upper chamber, and with 10 mM HEPES (pH 7.2) in the lower compartment. Digested samples of GSH and yeast were added of glucose (5 mM final concentration) and HEPES (50 mM final concentration) to facilitate cell viability, whereas water or NaCl (Merck, Barcelona, Spain) were added to adjust the osmolarity to  $310 \pm 10$  mOsm/kg using a freezing point osmometer (Osmometer Automatic type 15, Löser Messtechnik, Berlin, Germany). Samples were added to the acceptor compartment, apical or basal, according to the direction of the transport studied, i.e. basolateral or basolateral-apical, respectively.

To calculate apparent permeability coefficients, at appropriate intervals (15, 30, 60 min and 2, 3 and 4 h) samples (300  $\mu\text{L}$ ) were removed from the acceptor compartment and were replaced with an equal volume of fresh medium. GSH determination was carried out in the aliquots obtained at each time point as well as in the donor medium collected at the end of the experiment.

The apparent permeability coefficients ( $P_{app}$ ) were calculated from Equation 1:

$$P_{app} = (dC/dt)(V_r/AC_o) \quad \text{Eq.1}$$

where  $dC/dt$  is the flux ( $\mu\text{mol/s}$ ) determined by the slope of the cumulative concentration of GSH in the receptor chamber over time,  $V_r$  is the acceptor compartment volume (basal, 2 mL),  $A$  is the surface area occupied by the cell monolayer ( $4.67 \text{ cm}^2$ ), and  $C_o$  is the initial GSH concentration in the donor compartment.

When experiments regarded GSH transport at the final time, GSH determination was carried out in the aliquots obtained at the end of the experiment from the acceptor compartments and in the donor medium. GSH transport percentages were calculated with respect to the initial quantity of GSH added to the cell cultures.

During the period of growth and differentiation of the cultures on the Transwell<sup>®</sup> membranes, cell monolayer integrity was assessed daily from the sixth postseeding day onward by measuring the Transepithelial Electrical Resistance (TEER) using a Millicell<sup>®</sup>-ERS (Millipore Corporation, Madrid, Spain). During the tests of GSH cell transport in Transwell<sup>®</sup>, cell monolayer integrity was evaluated by measuring a) TEER at various points in the study, including the start and end times of the experiment, and b) the  $P_{app}$  of the paracellular transport marker lucifer yellow (LY), added at a concentration of  $100 \mu\text{M}$  to the apical compartment in the control wells and the wells treated with GSH. The fluorescence of the LY transported to the basal side was measured with a fluorescence microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany) at excitation/emission wavelengths of 485/520 nm. To evaluate possible interactions of LY with uptake and transport of GSH, parallel experiments were performed with and without paracellular marker, which demonstrated the absence of interference.

#### ***Antioxidant effect of GSH in intestinal epithelial cells***

Cell viability assays were performed employing sodium resazurin (7-hydroxy-3H-phenoxazin-3-one-10-oxide sodium salt, Sigma). The assay is based on the ability of viable, metabolically active cells to reduce resazurin to resorufin and dihydroresorufin, measurable by colorimetric methods. This conversion is intracellular, facilitated by mitochondrial, microsomal and cytosolic oxidoreductases (O' Brien et al., 2000; Rocha et al., 2011).

Caco-2 cells were inoculated at a density of  $6.25 \times 10^4$  cells/cm<sup>2</sup> in 96-well plates for 5 days and subsequently exposed to GSH standard solutions (3-10-30 mM prepared in HBSS), Yeast A and Yeast B (after *in vitro* digestion) for 1 h at 37°C. Afterwards, 2, 10 and 20 mM H<sub>2</sub>O<sub>2</sub> were added to cells and the reaction mixture incubated for further 2 h. After exposure, the medium was withdrawn and 150 µL of resazurin solution (10 µg/mL in MEM) were added. Well plates were incubated for 2 h at the same conditions. 100 µL for each reaction mixture were transferred to a 96-well plate and resazurin reduction was measured colorimetrically (570 and 600 nm) using a microplate reader (PolarSTAR OPTIMA, BMG-Labtech, Germany).

### ***Analytical procedures***

GSH identification and quantification in the soluble fraction obtained during *in vitro* gastrointestinal digestion and in samples collected during transport and apparent permeability tests, were carried out by HPLC as previously reported (1.2).

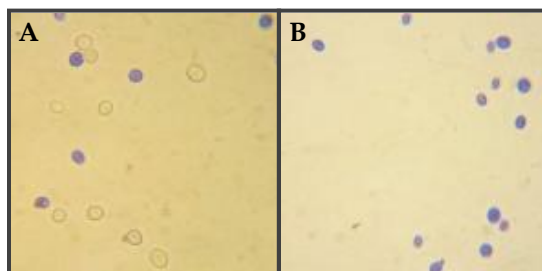
Intracellular GSH of lyophilised baker's yeast was determined according to Rollini & Manzoni (2006) as previously reported (1.2).

## **6.3 Results**

### ***Yeast lyophilisation***

Lyophilisation is a dehydration process consisting in freezing the material and reducing the pressure to allow the water to sublime directly from the solid phase to the gas phase.

Yeast samples (control, Yeast A, Yeast-B) were freeze-dried in order to faithfully reproduce the digestion of a yeast-based food supplement as normally present on the market, i.e. a lyophilised product. Samples were lyophilised suspending yeast cells in distilled water or in a solution containing skimmed milk (10% w/v) as cryoprotectant for comparative purpose.



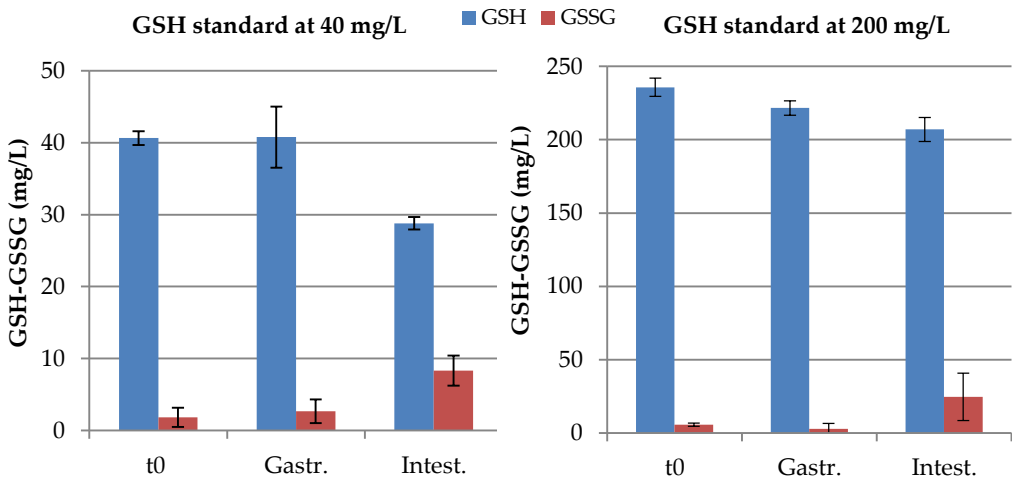
**Figure 6.2.** Microscope images of lyophilised yeast cells with cryoprotectant (A) and in water (B) colored by Trypan blue (0.4%) at 1000x.

Cell membrane was found to lose its integrity due to the lyophilisation treatment, in particular when no cryoprotectant was employed (Fig. 6.2). Microscope images of cells colored employing Trypan blue solution, confirmed that when cells were suspended in water the damage was increased (trypan blue colored all yeast cells) more than in presence of cryoprotectant.

From these observations it was hypothesized that GSH, previously accumulated intracellularly, could be released in the medium in which the yeast was suspended. For verifying this hypothesis intracellular and extracellular GSH were determined. Results (data not shown) confirmed that most of the GSH stored inside yeast cells after lyophilisation is released extracellularly and only in the case of yeast freeze-dried with cryoprotectant this release is lower.

#### ***In vitro gastro-intestinal digestion***

Two GSH standard solutions, 40 mg/L and 200 mg/L, were digested employing the previously described process. All trials were performed in triplicate. Results of GSH and GSSG contents in gastric and intestinal stage are reported in Fig. 6.3.



**Figure 6.3.** GSH and GSSG levels (mg/L) at different steps of the *in vitro* gastrointestinal digestion: t<sub>0</sub>, gastric and intestinal phases at 120 min. GSH standard solution at 40 mg/L and 200 mg/L.

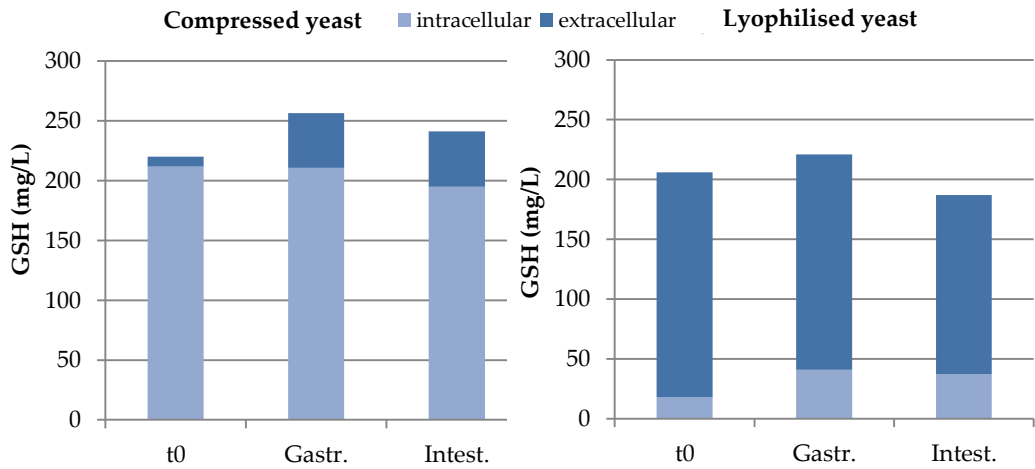
Results evidenced that in general GSH levels were not affected by the applied treatment: at the highest concentration (200 mg/L) a very limited amount of GSH (up to 10%) was found to oxidize to GSSG, while increased oxidation (up to 25%) occurred when GSH was used at 40 mg/L. This oxidation mainly occurred after the intestinal digestion phase and may be due to normal oxidation of GSH caused by temperature (37°C for 2+2 h).

At the end of the process, after the final heat treatment, a further oxidation occurred (data not shown) at both tested levels: 40% in the case of GSH at 40 g/L and 25% in the case of GSH at 200 g/L. Anyway, even if this is a necessary step during the *in vitro* procedure before use in cell culture studies, it cannot be considered a part of an *in vivo* gastrointestinal digestion.

Summarizing, by applying this simple simulated digestion process, an average of 75% of the ingested GSH (as reduced standard) may overcome the gastrointestinal barrier and reach the intestinal epithelium.

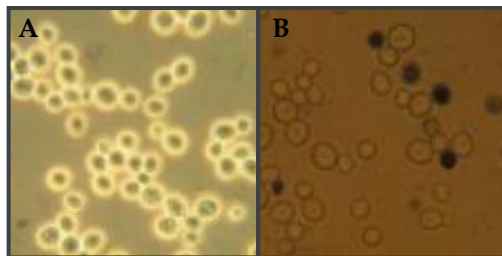


Trials were then performed employing both *Fala* yeast (control) in compressed form and after lyophilisation, with the aim of evaluating whether yeast structure influenced GSH bioaccessibility and stability during gastrointestinal digestion. All trials were performed in duplicate. Results showed that during gastrointestinal digestion GSH levels were found stable, both when GSH was mainly inside yeast cells (compressed yeast) and when most of the GSH was extracellular (lyophilised yeast) (Fig. 6.4).



**Figure 6.4.** GSH intracellular and extracellular levels (mg/L) at different steps of an in vitro gastrointestinal digestion: t<sub>0</sub>, gastric and intestinal phases at 120 min. *Fala* yeast in compressed and lyophilised form.

To be noticed, in the case of the compressed yeast, a limited amount of GSH partially released outside cells during the gastric phase of digestion (Fig. 6.4). This behaviour was probably due to the acidic pH (pH 2) of this phase that may have altered the membrane permeability and this is shown also in Fig. 6.5.

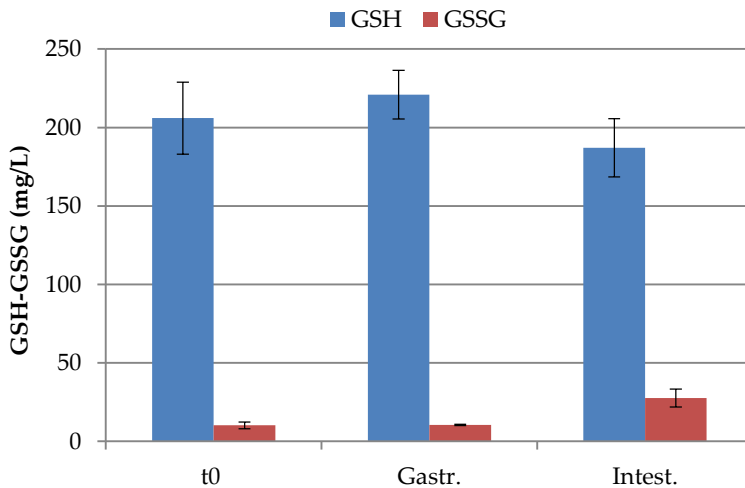


**Figure 6.5.** Microscope images of *Fala* compressed yeast colored by Trypan blue (0.4%) at 1000x at t<sub>0</sub> (A) and at 2 h of gastric digestion (B).

Regarding lyophilised yeast, as the cell structure was already damaged because of the lyophilisation process, GSH was not retained into the cell but released in the digestion solution. This explains the presence at the beginning of the digestion ( $t_0$ ) of most of the GSH in the digestion solution.

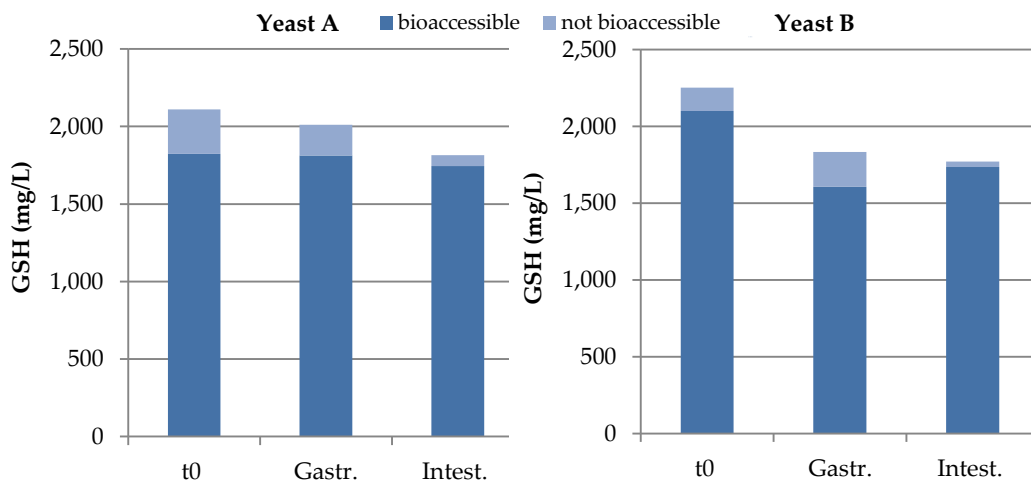
Due to the fact that lyophilised cells are commonly used to commercialize yeast-like food supplements, and that GSH, released from cells and thus considered bioaccessible, was found stable during digestion, *Fala* yeast sample, lyophilised in absence of cryoprotectant was selected for the prosecution of the research. The presence of skimmed milk as cryoprotectant had been considered an altering factor during GSH transport trials.

In detail, gastrointestinal digestion of lyophilised *Fala* yeast (control) produced a very limited GSH oxidation, above all during the intestinal step (Fig. 6.6). Results confirmed what previously evidenced for the 200 mg/L standard GSH solution (to be noted that GSH content in this yeast was found  $0.61 \pm 0.09\%$  dcw, corresponding to  $201 \pm 30$  mg/L).



**Figure 6.6.** GSH and GSSG levels (mg/L) at different steps of an *in vitro* gastrointestinal digestion:  $t_0$ , gastric and intestinal phases at 120 min; *Fala* lyophilised yeast.

Subsequently, two GSH- enriched yeasts were used, named A and B, having a GSH content of  $1.09 \pm 0.13\%$  dcw and  $1.49 \pm 0.01\%$  dcw respectively. These cells were enriched employing two different activation mixtures (CYS-GLY-GLU and CYS-GLY-GLU+ADE). All trials were performed in triplicate and bioaccessible (released in the medium) and not bioaccessible (retained inside cells) are reported in Fig. 6.7.



**Figure 6.7.** Bioaccessible and not bioaccessible GSH levels (mg/L) at different steps of an in vitro gastrointestinal digestion: t<sub>0</sub>, gastric and intestinal phases at 120 min. Yeast A and Yeast B.

Results showed that also in the case of GSH enriched-yeasts, lyophilisation damaged cell structure and GSH became bioaccessible (that is, released in the medium) already at the beginning of the process. Only a small amount of total GSH, between 6.5 and 13.5%, was not considered bioaccessible because of its intracellular nature (retained inside cells). Regarding oxidation, as previously seen only a limited amount of GSH was oxidized (data not shown).

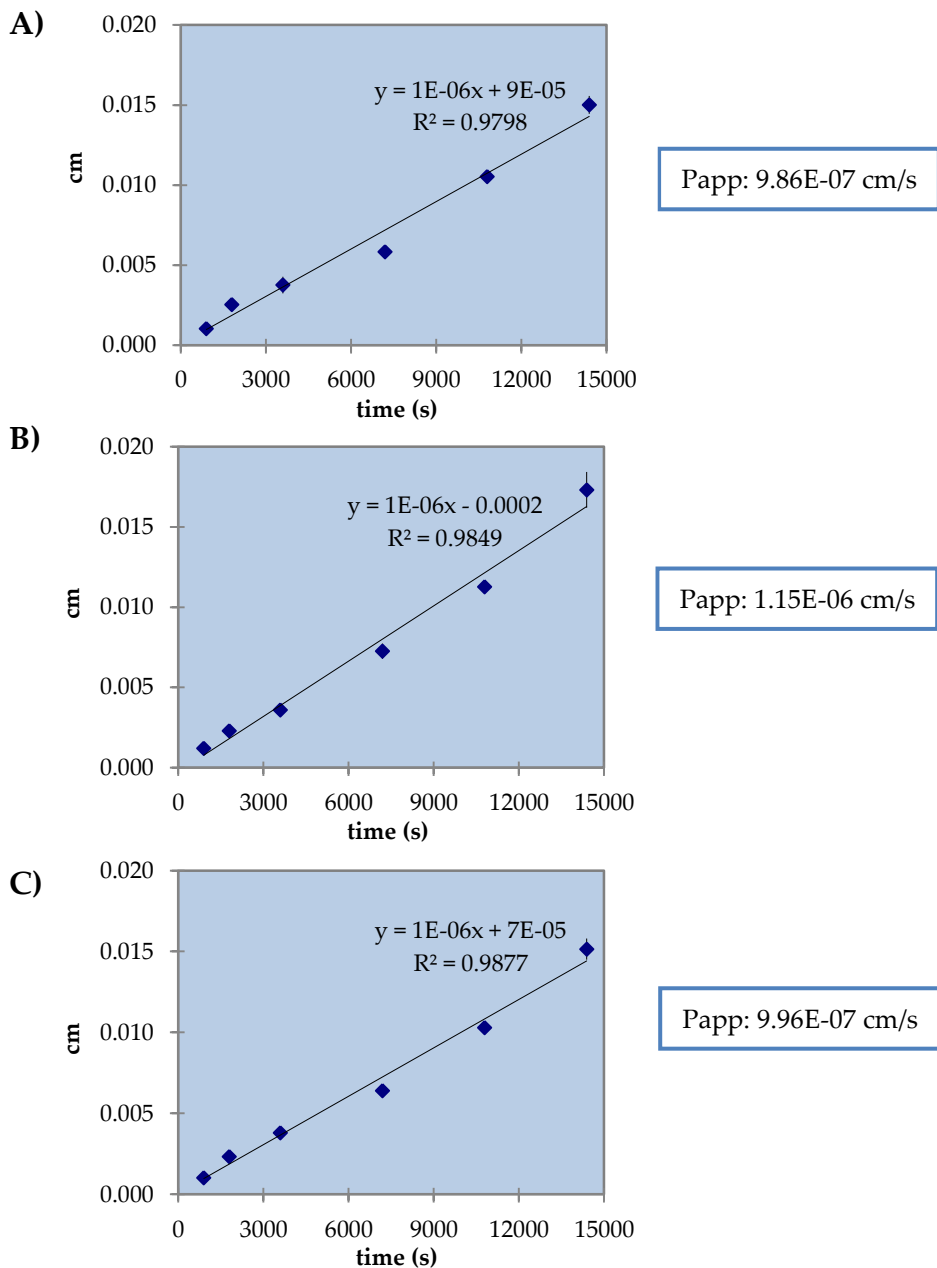
In conclusion, the applied treatment of gastrointestinal digestion did not affect GSH levels, neither when present as standard solution nor when it is accumulated inside yeast cells; also, a limited GSH oxidation was evidenced.

### ***GSH apparent permeability coefficient (P<sub>app</sub>)***

*In vitro* GSH transport trials through the intestinal epithelium were carried out to calculate apparent GSH permeability.

Using Caco-2 cell line, GSH was tested at three different levels (3, 10 and 30 mM). GSH solutions were loaded on the apical transwell chambers and from 15 to 240 min contact time, samples were collected from the basal compartments. GSH determination and P<sub>app</sub> coefficient were performed as previously reported (see section 6.2). Yee (1997) classified compounds as poorly absorbed (0-20%) when P<sub>app</sub> < 1 × 10<sup>-6</sup> cm/s, moderately absorbed (20-70%) when P<sub>app</sub> = 1-10 × 10<sup>-6</sup> cm/s and well absorbed (70-100%) when P<sub>app</sub> > 10 × 10<sup>-6</sup> cm/s. Results reported in Fig. 6.8 showed that GSH, at the 3 levels, is poorly absorbed, having an apparent permeability around 1×10<sup>-6</sup> cm/s, that

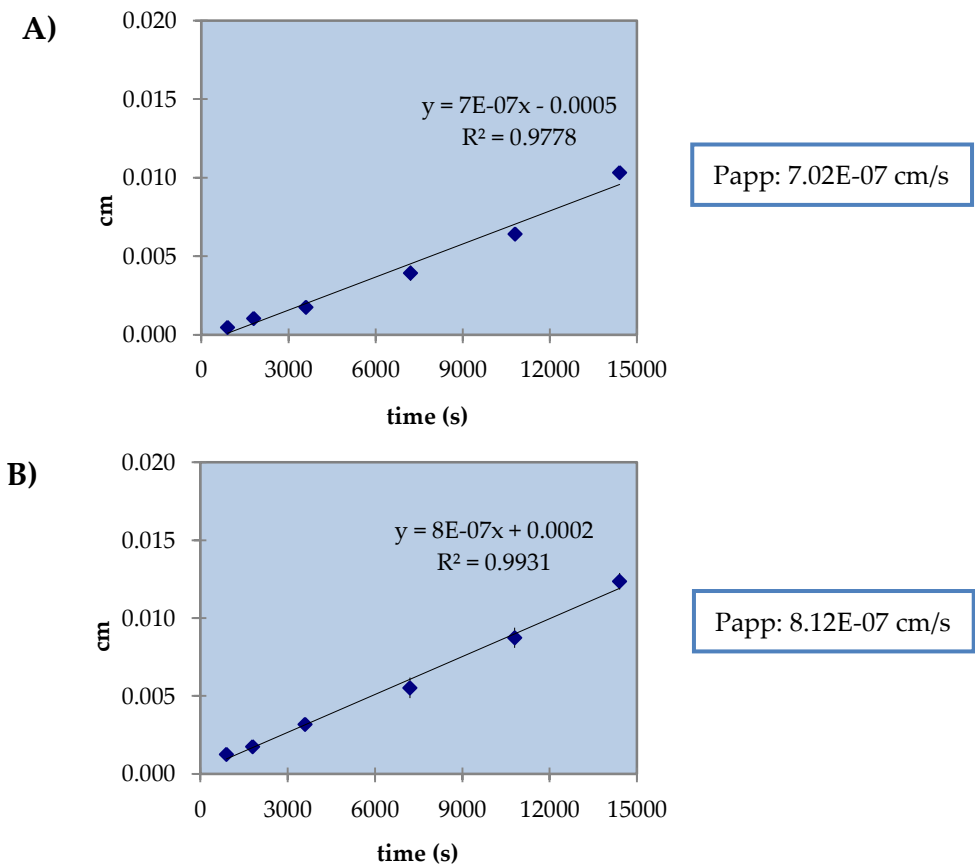
correspond to a percentage of GSH transported at 120 min of  $1.83 \pm 0.05$  when GSH 3 mM was employed,  $2.28 \pm 0.15$  with GSH 10 mM and  $2.00 \pm 0.01$  with GSH 30 mM.



**Figure 6.8.** Apparent permeability (Papp) of GSH at 3 (A), 10 (B) and 30 (C) mM calculated employing Caco-2 cell line.

Using co-cultures of Caco-2 and HT29-MTX (ratio 50:50 and 70:30), the apparent permeability of 10 mM GSH was evaluated. Again, results reported in Fig. 6.9 showed that GSH is poorly absorbed in both the co-culture cell employed, having an apparent permeability  $< 1 \times 10^{-6}$  cm/s. The percentage of GSH 10 mM transported at 120 min in co-cultures experiments were found  $1.23 \pm 0.15$  with ratio 50:50 and  $1.73 \pm 0.21$  with ratio 70:30.

The obtained Papp coefficients, calculated employing different cell cultures, were found of the same order and no significant differences were evidenced between Caco-2 cell and co-cultures.



**Figure 6.9.** Apparent permeability (Papp) of GSH at 10 mM calculated employing co-culture of Caco-2 and HT29-MTX at ratio 70:30 (A) and 50:50 (B).

From the obtained data, GSH transported in 120 min both employing Caco-2 cells and co-cultures was about 2%. Again data confirm that GSH is a poorly absorbed compound.

***GSH transport***

Further GSH transport trials through the intestinal epithelium were carried out with the Caco-2 cell line model and co-cultures of Caco-2:HT29-MTX (ratios 50:50 and 70:30). GSH standard solution, tested at three different levels (3, 10 and 30 mM) or enriched-yeast samples (Yeast-A and Yeast-B, characterized by GSH level of about 3 mM) were loaded on the apical transwell chamber. At the end of exposure (120 min), samples were collected from the apical and basal compartments and GSH content was evaluated. Data related to the percentage of GSH transported (%) from the apical to the basal chamber at final time of 120 min are reported in Tab. 6.1 (data are mean of three replicates).

**Table 6.1.** GSH transported (expressed as %) after 120 min from the apical to the basal chamber.

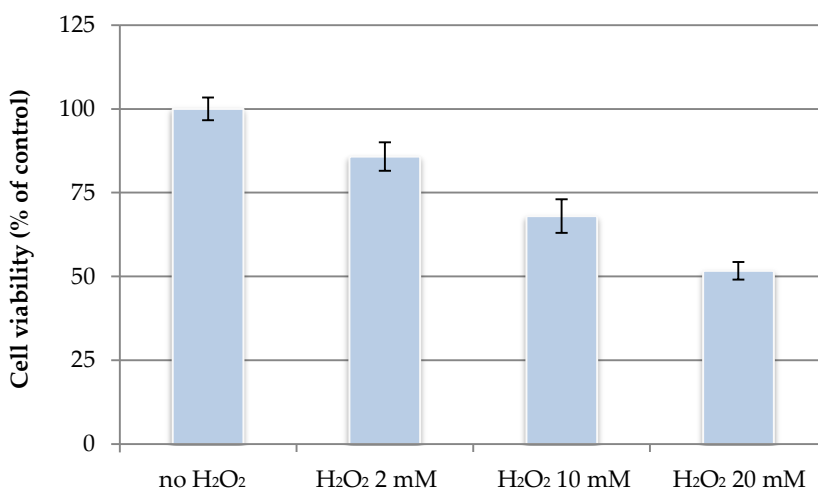
Cell line	GSH Transport (%)				
	GSH (3 mM)	GSH (10 mM)	GSH (30 mM)	Yeast-A	Yeast-B
Caco-2	2.16±0.06	2.33±0.18	2.10±0.13	1.11±0.11	1.23±0.05
Co-culture 70:30	3.25±0.31	3.35±0.33	3.82±0.06	4.16±0.31	3.92±0.36
Co-culture 50:50	6.96±0.40	7.58±0.25	6.68±0.23	5.20±0.53	7.93±0.84

Results showed that GSH transport from the upper to the basal compartment in 120 min is low, either when present in form of a standard GSH solution (between 2.10 and 2.33 %) and as enriched yeast (Yeast A 1.11 % and Yeast B 1.23 %). Nevertheless, it must be noted that in co-cultures, more similar that the monoculture of Caco-2 to the *in vivo* system, GSH transport was found higher than in Caco-2 cell line.

***Antioxidant effect of GSH in intestinal epithelial cells***

In this set of trials the possible protective role of GSH and GSH-enriched yeast on Caco-2 cells against oxidative stress was evaluated, studying cell viability with resazurin. The tested combinations were as follows:

- i. Cells, not added with GSH or GSH-enriched yeast, treated with 2-10-20 mM H<sub>2</sub>O<sub>2</sub>.
- ii. Cells added with standard GSH (3, 10 and 30 mM), and then treated with 2-10-20 mM H<sub>2</sub>O<sub>2</sub>. Trials should investigate any GSH protective role (as standard solution) on cells exposed to H<sub>2</sub>O<sub>2</sub>. A control trial was also set up with cells not treated with H<sub>2</sub>O<sub>2</sub>.
- iii. Cells added with GSH-enriched yeast (Yeast A or B) and then treated with 2-10-20 mM H<sub>2</sub>O<sub>2</sub>. Trials should investigate any protective role of GSH-enriched yeasts, on cells exposed to H<sub>2</sub>O<sub>2</sub>. A control trial was also set up with cells not treated with H<sub>2</sub>O<sub>2</sub>.



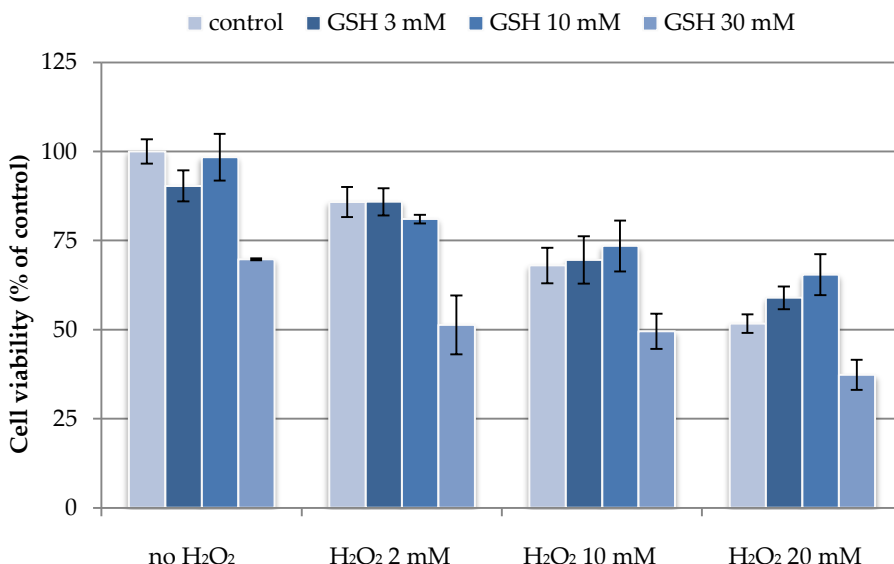
**Figure 6.10.** Viability of Caco-2 cells after H<sub>2</sub>O<sub>2</sub> different dose exposure (2-10-20 mM), measured using resazurin.

Results reported in Fig. 6.10 showed that Caco-2 cells viability decreased after H<sub>2</sub>O<sub>2</sub> exposure, and that this decrease is concentration dependent. In fact employing 2 mM H<sub>2</sub>O<sub>2</sub> cells viability was 85.77±4.22%, with 10 mM viability decreased to 68.02±4.98% and with 20 mM H<sub>2</sub>O<sub>2</sub> there was a viability decrease to 51.68±2.60%.

Relating to Caco-2 cells added with GSH aqueous solution before H<sub>2</sub>O<sub>2</sub> exposure (Fig. 6.11), when H<sub>2</sub>O<sub>2</sub> was employed at 2 and 10 mM, cell viability reduction was similar to those obtained in control sample (non added with GSH). On the contrary, cell viability reduction was significantly lower in samples where H<sub>2</sub>O<sub>2</sub> was present in high concentration (20 mM) and in these trials the GSH protective role was evident: the cell viability reduction of samples added with GSH 3-10 mM was found lower than those

obtained with control cells untreated with GSH (about 65% and 59% for GSH 3 mM and 10 mM respectively, 52% for control without GSH addition).

To be noted that the addition of 30 mM GSH to cell line caused a general decrease of cells viability, even when H<sub>2</sub>O<sub>2</sub> was not added (from 69.74±0.25% for control to 37.30±4.21% in presence of 20 mM H<sub>2</sub>O<sub>2</sub>).

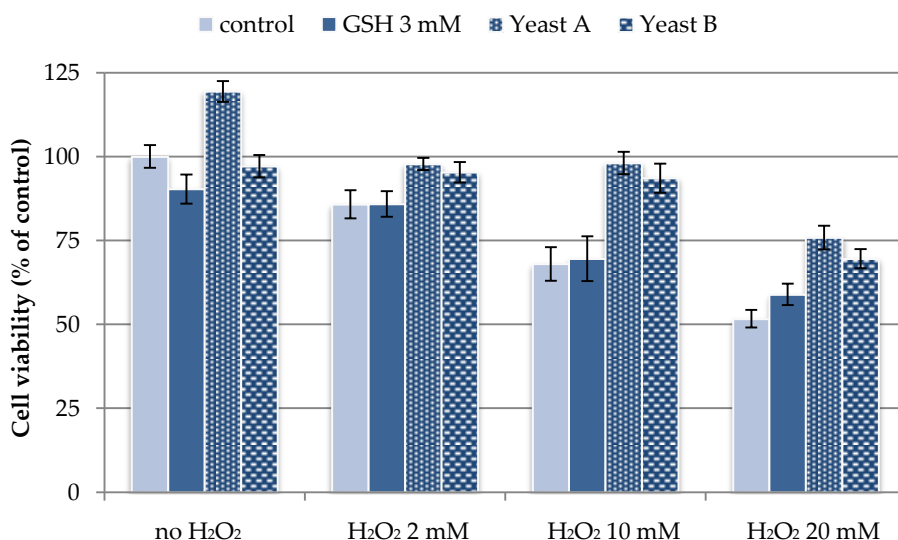


**Figure 6.11.** Viability of Caco-2 cells after 1h treatment with GSH (3, 10 and 30 mM) and subsequent H<sub>2</sub>O<sub>2</sub> different dose exposure (2-10-20 mM), measured using resazurin.

Fig. 6.12 reports the results obtained by adding GSH-enriched yeasts (1 h contact time), before H<sub>2</sub>O<sub>2</sub> addition; for comparison purposes a pre-treatment with 3 mM standard GSH solution was set up. Results showed that GSH-enriched yeasts pre-treatment did not negatively affect cells untreated with H<sub>2</sub>O<sub>2</sub>. Moreover, Yeast A pre-treatment increased cells viability up to to 119.41±3.07, probably not only for the GSH content but also because of the presence of other yeast components, such as vitamins...

When H<sub>2</sub>O<sub>2</sub> was added, Yeast A and Yeast B protected cells viability reduction much more than the equivalent GSH standard 3 mM. In particular, when 2-10 mM H<sub>2</sub>O<sub>2</sub> was added, cell viability with yeast pre-treatment was about 94-98% (*vs* 69-86% of the control), and when 20 mM H<sub>2</sub>O<sub>2</sub> was added, viability was found 70-76% (*vs* 52% of the control).





**Figure 6.12.** Viability of Caco-2 cells (measured using resazurin) after 1 h treatment with 3 mM GSH or GSH-enriched yeast (Yeast A and B) and subsequent addition of 2-10-20 mM H<sub>2</sub>O<sub>2</sub>.

#### 6.4 Conclusions

From an overlook of the results it can be concluded that gastrointestinal static digestion does not negatively affect GSH levels and only induces a limited oxidation (10-25%); thus, at least 75% of the hypothetical GSH ingested should arrive at gastrointestinal lumen for its absorption in the reduced form. The use of a lyophilised GSH-enriched biomass is crucial because the lyophilisation process contributes to GSH bioaccessibility. In fact lyophilised yeast cells have a damaged structure and their intracellular content, comprising GSH, can be readily solubilized in the medium.

Regarding GSH transport and Papp coefficient evaluation in model systems, results showed that GSH is poorly absorbed, having an apparent permeability around  $1 \times 10^{-6}$  cm/s in all the tested conditions (Caco-2, co-culture Caco-2: HT29-MTX at ratios 70:30 and 50:50). Although in a context of a limited absorption, the highest GSH transport was obtained at final time (2 h) in the co-culture Caco-2:HT29-MTX 50:50. Further studies should be performed both *in vitro* and *in vivo* for elucidating how GSH is transported and in which proportion is transported intact or degraded into its constituent amino acids.

Important results were obtained in experiments regarding the protective effect of GSH in intestinal cells exposed to an inducer of oxidative stress (H<sub>2</sub>O<sub>2</sub>). Cell viability

reduction was lower in samples added with GSH when employing H<sub>2</sub>O<sub>2</sub> at high concentration (10-20 mM); moreover the use of Yeast A and Yeast B was found to prevent cells viability reduction much more than the equivalent standard GSH (3 mM). In conclusion even if GSH can be considered a poorly absorbed compound, and a limited amount of the ingested GSH can reach the systemic circulation, however the ingested GSH, especially GSH-enriched biomass, could protect intestinal cells, particularly during oxidative stress conditions. Thus, ingested GSH may have a local effect acting together with the GSH coming from the biliary efflux, for important functions in the intestine, i.e. detoxification of fatty-acid hydroperoxides in intestinal epithelium, maintenance of the luminal thiol-disulfide balance and absorption of iron and other trace elements (Valencia et al., 2001).

## 6.5 References

- Aw TY, Wierzbicka G, Jones DP.** 1991. Oral glutathione increases tissue GSH in vivo. *Chem Biol Interact* 80:89-97.
- Calatayud M, Gimeno J, Vélez D, Devesa V, Montoro R.** 2010. Characterization of the intestinal absorption of arsenate, monomethylarsonic acid, and dimethylarsinic acid using the Caco-2 cell line. *Chem Res Toxicol* 23:547-556.
- Hagen TM, Wierzbicka GT, Sillau AH, Bowman BB, Jones DP.** 1990. Bioavailability of dietary glutathione: effect on plasma concentration. *Am J Physiol (Gastrointest Liver Physiol)* 22:259:G524-G529.
- Hidalgo IJ, Raub TJ, Borchardt RT.** 1989. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterol* 96:736-749.
- Laparra JM, Glahn RP, Miller DD.** 2009. Different responses of Fe transporters in Caco-2/HT29-MTX cocultures than in independent Caco-2 cell cultures. *Cell Biol Int* 33:971-977.
- Lesuffleur T, Barbat A, Luccioni C, Beaumatin J, Clair M, Kornowski A, Dussaulx E, Dutrillaux B, Zweibaum A.** 1991. Dihydrofolate reductase gene amplification-associated shift of differentiation in methotrexate-adapted HT-29 cells. *J Cell Biol* 115:1409-1418.
- Maubon N, Le Vee M, Fossati L, Audry M, Le Ferrec E, Bolze S, Fardel O.** 2007. Analysis of drug transporter expression in human intestinal Caco-2 cells by real-time PCR. *Fundam Clin Pharmacol* 21:659-663.
- O'Brien J, Wilson I, Orton T, Pognan F.** 2000. Investigation of the Alamar Blue (resazurin) fluorescent dye for the assessment of mammalian cell cytotoxicity. *Eur J Biochem* 267:5421-5426.

- Pontier C, Pachot J, Botham R, Lenfant B, Arnaud P.** 2001. HT29-MTX and Caco-2/TC7 monolayers as Predictive Models for Human Intestinal Absorption: Role of the Mucus Layer. *J Pharm Sci* 90:1608-1619.
- Rahman I, MacNee W.** 1992. Lung glutathione and oxidative stress: implications in cigarette smoke-induced airway disease. *Am J Physiol Lung Cell Mol Physiol* 77:L1067-L1088.
- Rocha RA, Gimeno-Alcañiz JV, Martín-Ibañez R, Canals JM, Vélez D, Devesa V.** 2011. Arsenic and fluoride induce neural progenitor cell apoptosis. *Toxicol Lett* 203:237-244.
- Rollini M, Manzoni M.** 2006. Influence of different fermentation parameters on glutathione volumetric productivity by *Saccharomyces cerevisiae*. *Process Biochem* 41:1501–1505.
- Valencia E, Marin A, Hardy G.** 2001. Glutathione-Nutritional and pharmacological viewpoints: Part III. *Nutrition* 17:696-697
- Witschi A, Reddy S, Stofer B, Lauterburg BH.** 1992. The systemic availability of oral glutathione. *Eur J Clin Pharmacol* 43:667-669.
- Yee S.** 1997. In vitro permeability across Caco-2 cells (colonic) can predict in vivo (small intestinal) absorption in man—fact or myth. *Pharm Res* 14:763-766.