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Journal of the American Society of Brewing Chemists
The Science of Beer

ISSN: 0361-0470 (Print) 1943-7854 (Online) Journal homepage: http://www.tandfonline.com/loi/ujbc20

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To cite this article: Laurence Gijs, Philippe Perpète, Aurore Timmermans, Christine Guyot-Declerck, Perrine Delincé & Sonia Collin (2004) Assessment of Added Glutathione in Yeast Propagations, Wort Fermentations, and Beer Storage, Journal of the American Society of Brewing Chemists, 62:3, 97-102, DOI: 10.1094/ASBCJ-62-0097

To link to this article: https://doi.org/10.1094/ASBCJ-62-0097

Assessment of Added Glutathione in Yeast Propagations, Wort Fermentations, and Beer Storage

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ABSTRACT

J. Am. Soc. Brew. Chem. 62(3):97-102, 2004

Glutathione (GSH) is one of the most abundant low-molecular-weight thiols found in living cells. Concentrations varying from 8 to 37 nmol/mg of cells were measured in brewing yeast. While other studies emphasize the key role of GSH in the response of some genetically modified yeasts to different stresses, our results show that GSH added to the yeast propagation medium has no significant impact on the growth of most brewing yeast strains. In high-gravity primary fermentations, a slight positive effect was observed during the first week with only two (lager) yeast strains. Added to phosphate buffer or beer, GSH imparts a reducing activity similar to that of ascorbic acid, but it proves to be a much less powerful antioxidant than phenolic compounds. Whatever the chosen step for spiking, beers aged in the presence of GSH were characterized by strong sulfurous off-flavors, despite a mere 6% degradation of the tripeptide.

Keywords: Beer aging, Off-flavors, Reducing power

RESUMEN

Valoración de Lutathione Agregado en Propagaciones de Levadura, Fermentaciones de Mosto, y Almacenaje de Cerveza

Glutathione (GSH) es uno de los thiols de poco peso molecular más abundantemente encontrado en células vivas. Concentraciones de variación de 8 a 37 nmol/mg de células fueron medidas en levadura de cerveza. Mientras que otros estudios acentúan el papel dominante de GSH en la respuesta de algunas levaduras genéticamente modificadas a diversas tensiones, nuestros resultados demuestran que GSH agregado al medio de propagación de levadura no tiene ningún impacto significativo en el crecimiento de la mayoría de las cepas de levadura de cerveza. En fermentaciones primarias de alta-gravedad, se observo un efecto positivo leve durante la primera semana con solamente dos cepas de levadura (lager). Agregado al almacenador intermediario de fosfato o a la cerveza, GSH imparte una actividad de reducción similar a la de ácido ascórbico, pero demuestra ser un antioxidante de mucho menos alcance en cuanto a compuestos fenolicos. Sin importar el pasos elegido para agregar, las cervezas envejecidas en la presencia de GSH fueron caracterizadas por mal-sabores de azufre fuertes, a pesar de una degradación de solo 6% de tripeptide.

Palabras claves: Energía de reducción, Envejecimiento de cerveza, Mal-sabores

Glutathione (GSH) is one of the most abundant low-molecular-weight thiols found in living cells. It is present in most cells from microorganisms to man. In the yeast *Saccharomyces cerevisiae*, this thiol tripeptide (L-γ-glutamyl-L-cysteinyl-glycine) may account for 0.5–1% of the dry weight depending on growth conditions (4) and the yeast strain (15). Its biological significance is mainly related to the free sulfhydryl moiety of the cysteine residue, which allows protein thiolation and modification of protein structure and function (14). GSH also participates in numerous biochemical processes, including the 1) uptake and intracellular transport of various amino acids, peptides, and ions; 2) bioreduction of inorganic molecules and metabolites; 3) protection against reactive oxygen species (redox potential (E°) = –0.24 V for thiol-disulfide exchange); and 4) detoxification of xenobiotics and heavy-metal ions.

GSH seems to be involved in the response of yeast to different nutritional stresses (12,15,16). It can ensure cell maintenance when *Saccharomyces cerevisiae* is starved for sulfur or nitrogen nutrients. Vacuolar transport of metal-GSH derivatives provides resistance to metal stress. Formaldehyde produced by methylotrophic yeasts grown on methanol can be detoxified by a GSH-dependent formaldehyde dehydrogenase. Methylglyoxal biosynthesized during growth on glycerol can be detoxified by the glyoxalase pathway, in which GSH is a catalyst. The nucleophilicity of GSH explains how it can also increase cell resistance to α,β-unsaturated aldehydes, especially acrolein generated by lipid peroxide degradation (10). Unfortunately, most of these experiments have been carried out with haploid genetically modified yeasts in a specific media that is very different from a brewing complex wort.

Oxidative stress can also be controlled by GSH, through glutathione peroxidase that catalyses hydroperoxide breakdown, or by radical scavenging leading to glutathione disulfides (GSSG). Through its ability to reduce GSSG to GSH, glutathione reductase plays a key role by maintaining a high GSH/GSSG ratio (15,18). According to Vander Jagt et al (18), 4-hydroxynonenal and related α,β-unsaturated aldehydes issued from lipid oxidation are able to inactivate this enzyme, thus amplifying oxidative stress.

Breweries, like other food industries employing yeasts or filamentous fungi, are frequently confronted with environmental stress occurring before, during, or after fermentation. Particularly in high-gravity wort fermentations, yeasts are subject to osmotic stress and ethanol toxicity leading to insufficient viability and vitality. During propagation, yeast is particularly vulnerable to oxidative stress having disastrous consequences on its metabolism. To improve wort attenuation, the wort is often spiked with cations before fermentation.

Recently, Dillemans et al (2,3) proposed the use of a yeast peptide complex (YPC) to reduce the major negative effects of high-gravity brewing. YPC stimulates the fermentation rate, yeast growth, yeast performance, and yeast resistance to ethanol and osmotic stress, and stabilizes yeast performances during successive high-gravity fermentations. Amino acid analysis of YPC has revealed that it is composed mainly of aspartic acid, glutamic acid, glycine, and cystein. The last three mentioned are the amino acids found in the GSH tripeptide. Recently, Bourdaudhui et al (1) have described similar effects for another antioxidant yeast extract.

In the last decade, the effect of GSH on the stability of alcoholic beverages has also been investigated. The addition of

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Publication no. J-2004-0510-01R.

GSH to beer has been recommended to increase its flavor stability during storage and preserve the beverage against microorganisms (11). More recently, oenologists have become interested in GSH as a means of preventing wine color change, loss of varietal aroma, and atypical aging (8,13).

The aim of the current work was to investigate whether, and to what extent, wort spiking with GSH can improve yeast propagation and primary fermentation on a practical point of view. GSH antioxidant activity was further chemically quantified and compared with antioxidant references. Finally, sensory assessment of GSH stability was done on beers aged for 5 days at 40°C.

EXPERIMENTAL

Yeast Strains

All the *Saccharomyces cerevisiae* strains were from the BRAS collection of the Université catholique de Louvain (Louvain-la-Neuve, Belgium): BRAS12, BRAS24, BRAS26, BRAS33, BRAS40, BRAS41, BRAS51B, BRAS52A, and BRAS291 (lager yeasts); and BRAS212, BRAS214, BRAS223, BRAS240B, BRAS268, and BRAS270 (ale yeasts).

GSH Quantification in Yeast Cells

Yeasts were grown with shaking at 28°C on YPS medium (1% yeast extract, 5% peptone, and 10% sucrose) until the optical density measured at 660 nm reached 0.6.

Yeast cells were collected from 25- to 100-mL cultures (corresponding to 10 mg dry weight) by filtration on a 0.45-µm nitrocellulose filter. GSH was quantified in yeast cells with the 5,5′-dithiobis(2-nitrobenzoic acid) colorimetric method developed by Habeeb (6).

GSH Quantification in Beer or Phosphate Buffer

GSH was quantified in beer or phosphate buffer with the HPLC method developed by Fahey and Newton (5) with fluorescence detection of the bromobimane adduct.

Yeast Propagation in the Presence of GSH

Cultures were grown in YPS medium spiked with 0, 750, or 1,250 parts per million (ppm) of GSH (aqueous solution added through a sterile Acrodisc 32; Supor 0.45 µm, GelmanSciences, VWR, Leuven, Belgium). Propagations were carried out at 28 or 12°C with a pitching rate of 10^7 cells per milliliter. For the last experiment, cultures were grown in a 12°Plato (°P) industrial sweet wort spiked with 0 or 1,250 ppm of GSH.

TABLE I
Intracellular Concentration of Glutathione (GSH) in 15 *Saccharomyces cerevisiae* Brewing Yeast Strains

Brewing Yeast	Intracellular GSH (nmol/mg cells)
Lager yeast	
BRAS12	12.05
BRAS24	7.78
BRAS26	10.27
BRAS33	10.27
BRAS40	16.12
BRAS41	11.91
BRAS51B	12.11
BRAS52A	N.D.[a]
BRAS291	13.01
Ale yeast	
BRAS212	15.48
BRAS214	18.17
BRAS223	37.48
BRAS240B	29.45
BRAS268	14.99
BRAS270	9.61

[a] N.D. = not determined.

Primary Fermentation in the Presence of GSH

Yeast propagations were successively carried out with shaking at 28°C for 12 hr and 20°C for 24 hr in YPS medium (400 and 2,000 mL, respectively).

After collection, the yeast was pitched in 15.3°P industrial sweet wort. Fermentations were conducted in 3-L EBC tubes (pitching rate = 7.5×10^6 cells per milliliter) at 14°C for 12 days.

For BRAS291, the secondary fermentation was carried out for seven days at 5°C. Yeasts cells were removed from the beer by continuous centrifugation (15,000 rpm; Contifuge 17RS; Heraus Sepatech, Van der Heyden, Brussel, Belgium). The beer was aged at 40°C for five days in a dark room.

GSH Stability During Aging

A 250-mL bottled commercial lager beer was spiked with 0, 50, or 250 ppm of GSH (aqueous solution injected with a glass syringe through a silicone top; Vel n°5, VWR, Leuven, Belgium). The bottles were crown sealed, and the beers were aged for five days at 40°C in a dark room.

Two 250-mL phosphate buffer samples (50 m*M*, pH 4.2), (either bubbled beforehand with argon or not bubbled) were also spiked with 250 ppm of GSH and aged as described above.

Sensory Analyses of Beer Aged With or Without GSH

Nine panelists (three male and six female, aged 23–42 years) were selected for their ability to qualitatively and quantitatively discriminate odors and aromas in beer. Selection was conducted as advised by Issanchou et al (7).

To determine whether spiking with GSH (50 ppm) can modify odor and flavor, two of five tests were first conducted. Beer samples were presented in "Breughel" 500-mL glasses (Durobor, Belgium) covered with glass tops. The presentation order was the same for all panelists. Samples were assessed at room temperature in individual booths illuminated with red light. The panelists described then qualitatively each group of samples.

RESULTS AND DISCUSSION

Assessment of the Ability of GSH to Enhance Yeast Propagation

The efficiency of a GSH spiking was first assessed in YPS medium with five lager and two ale yeast strains. Taking into account that GSH quantification in our 15 *Saccharomyces cerevisiae* yeast strains revealed intracellular concentrations from 8 to 37 nmol/mg cells (2 to 12 µg of GSH/mg cells; Table I) and that levels close to 1–2 ppm are reported for unspiked worts and beers (17), 750 and 1,250 ppm were added in this experiment. As depicted in Figure 1A, (100% corresponds for each strain to growth in a medium without spiking), GSH has little impact on the level of cells measured after 24 hr at 28°C (optical density from 1.156 to 1.517 depending on the strain). The greatest increase (BRAS12) reached only 15%. Inhibition was even observed for BRAS291, especially when spiked with 1,250 ppm. Similar data were obtained for the five lager strains when propagation was conducted for 72 hr at 12°C (optical density from 1.064 to 1.518, depending on the strain; Fig. 1B). In this case, slight inhibition was observed with BRAS40.

The same experiment was applied to a peculiar lager strain, BRAS24, which shows a poor ability to ferment wort under usual conditions (the optical density reached only 0.064 after 24 hr of propagation at 28°C). Surprisingly, GSH addition proved here to be an excellent way to improve cell growth (optical density after 24 hr at 28°C = 0.223 and 0.393 with 750 and 1,250 ppm of GSH,

respectively). To be emphasized is the particularly low level of internal GSH in this strain (8 nmol/mg of cells).

The impact of GSH was also investigated on propagations in wort instead of YPS medium. As shown in Figure 1C, addition of GSH has no positive impact on yeast growth whatever the strain (even BRAS12, which seems here to be rather inhibited).

BRAS24 proved inappropriate for investigation in wort because of insufficient growth.

Assessment of GSH Efficiency in Wort Primary Fermentations

To assess the impact of GSH in primary fermentation, wort was either spiked with 50 ppm of GSH or not spiked. As depicted in Figure 2, GSH had no impact on fermentations supported by BRAS26, BRAS52A, or BRAS291. In the case of BRAS40 and BRAS12, wort attenuation seemed to be slightly accelerated during the first week, but this advantage was almost completely lost by the end of the primary fermentation.

Assessment of GSH Antioxidant Activity

The level of antioxidant activity of GSH was determined with a reliable method recently developed by Liégeois et al (9). Linoleic acid oxidation is induced in an aqueous solution by the free radical initiator 2,2′-azobis(2-aminopropane)dihydrochloride. (AAPH). Production of conjugated diene hydroperoxides can be monitored at 234 nm. In the absence of an antioxidant, oxidation starts at a constant rate (R_0 in Fig. 3). When an antioxidant is added, oxidation is delayed and an inhibition time (T_{inh})

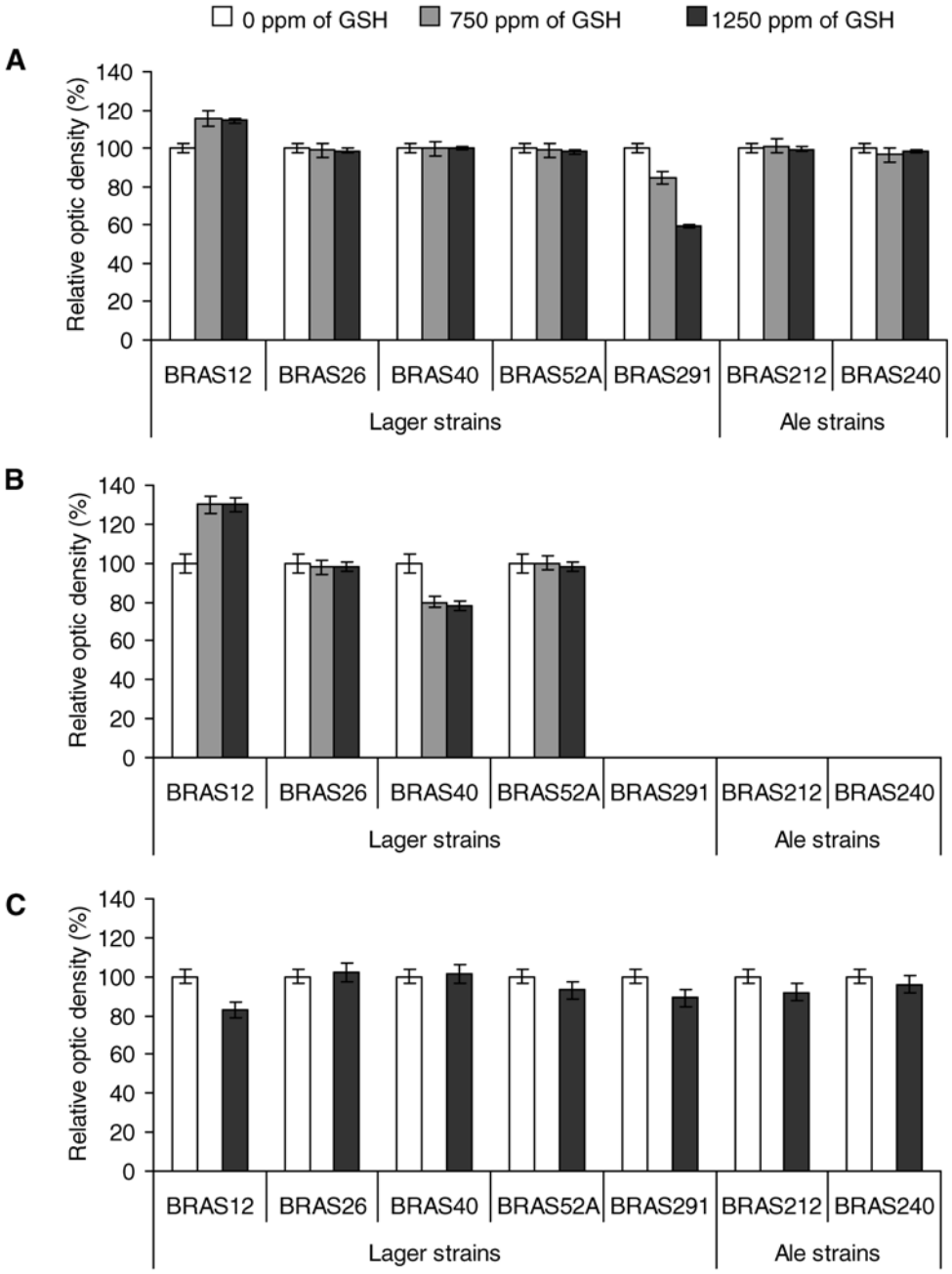

Fig. 1. Impact of the presence of 0, 750, or 1,250 parts per million (ppm) of glutathione on the relative optical density (660 nm; 100% expressed for the medium without spiking), obtained after 24 h propagation at 28°C in a YPS model medium (1% yeast extract, 5% peptone, and 10% sucrose) **(A)**, 72 h propagation at 12°C in a YPS model medium **(B)**, or 24 h propagation at 28°C in a 12°Plato industrial hopped wort **(C)**.

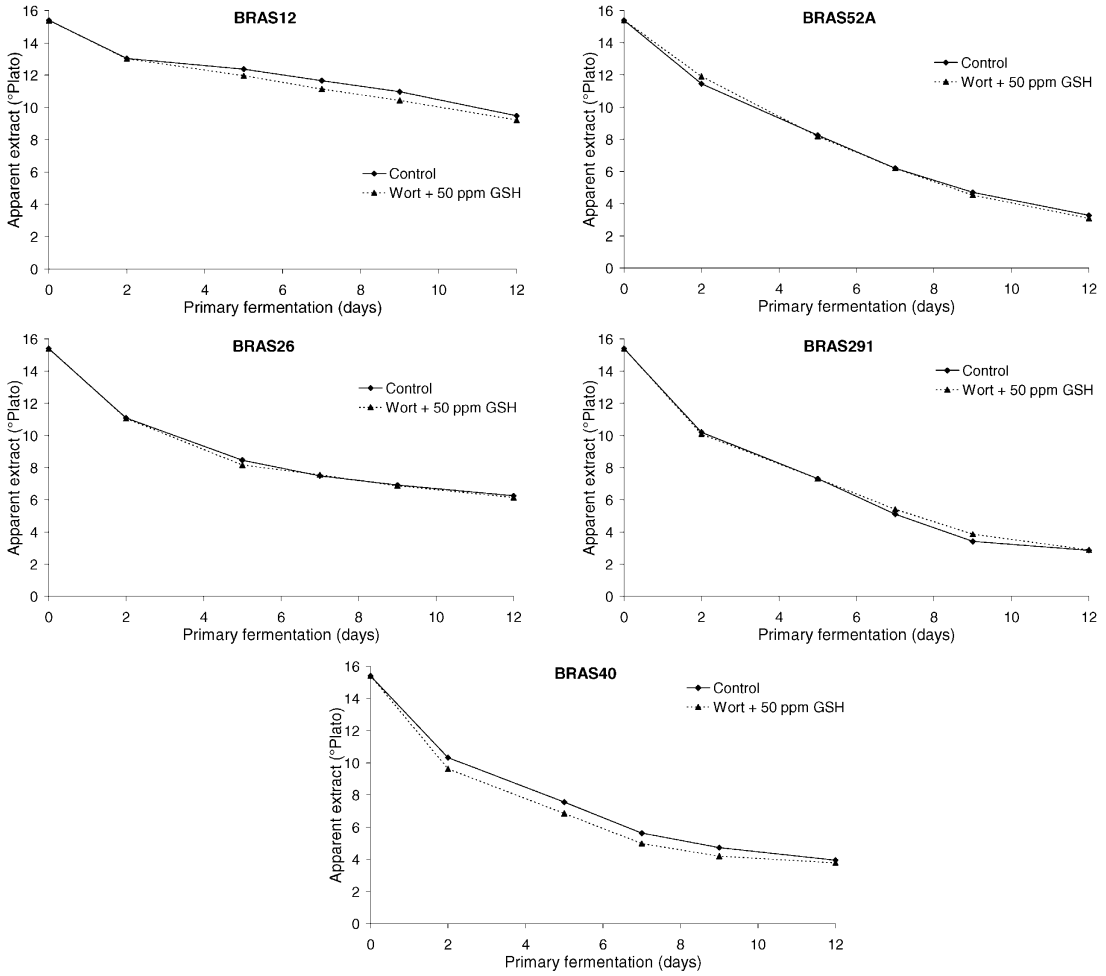

Fig. 2. Impact of the presence of 0 or 50 parts per million of glutathione on the evolution of the apparent extract, in °Plato (°P), during a primary fermentation conducted at 14°C on a 15.3°P industrial hopped wort.

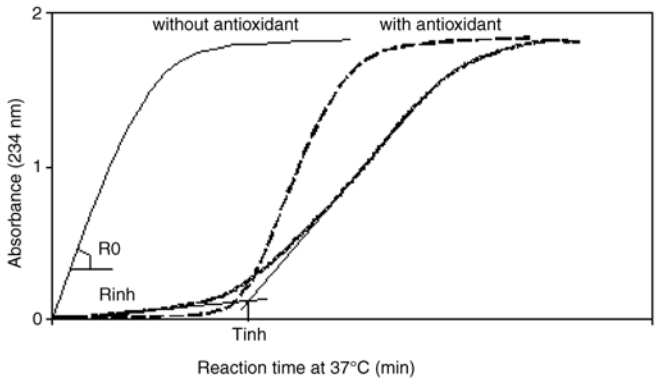

Fig. 3. Kinetics of conjugated diene formation during AAPH-induced linoleic oxidation, measured as the absorbance at 234 nm. Kinetic rate without antioxidant (R_0), kinetic rate (R_{inh}), and inhibition time (T_{inh}) in presence of antioxidant.

proportional to the concentration of the antioxidant can be determined. It is important to stress that this method measures the efficiency with which antioxidants scavenge free radicals without taking into account potential prooxidant activity (9).

Table II shows the inhibition times obtained with increasing concentrations of GSH (2, 3, and 4 µ*M*) in a 50-m*M* phosphate buffer (pH 7.4). The value obtained (4.2 min/µ*M*) is comparable to the 4.8 min/µ*M* measured with ascorbic acid. On the other hand, GSH cannot be considered very potent as compared with phenolic compounds (T_{inh}/µ*M* above 20 min) (9).

Similar results were obtained in beer. Probably because of the presence of endogenous polyphenols, beer gave a relatively high T_{inh} value (37.8 min before spiking; Table II). In the presence of GSH, T_{inh} increased by 4.4 min/µ*M*, as compared with 4.5 min/µ*M* with ascorbic acid.

Assessment of GSH Stability During Beer Aging

A commercial lager beer was enriched with 0 or 50 ppm of GSH and aged for five days at 40°C. In the fresh beer, two of five tests indicated that this spiking had no significant influence on odor, taste, and aroma. After beer aging, an olfactory two of five tests revealed significant differences ($P < 0.001$) between beers aged with or without GSH. The odor of the beer aged in the presence of the tripeptide was described as sulfurous, sewer like, and greeny by all the panelists (Table III). Similar descriptors were obtained after aging with beers fermented on a laboratory scale (BRAS291) in the presence of 50 ppm of GSH (Table III). Our data clearly indicate that GSH cannot reasonably be used as an antioxidant instead of ascorbic acid.

Such strong off-flavors can be surprising if we take into account that only 6% of the GSH is degraded after five days at 40°C (Table IV). The reducing power of the beer (partially

TABLE II
Comparison of the Level of Antioxidant Activity, Measured as Inhibition Time (T_{inh}) of the AAPH-Induced Linoleic Acid Oxidation, Obtained for Increasing Concentrations of Glutathione or Ascorbic Acid in a Phosphate Buffer or Beer

		Phosphate Buffer (50 mM, pH 7.4)		Beer	
Antioxidant	**Antioxidant Concentration**	**T_{inh}[a] (min)**	**$T_{inh}/\mu M$, min/μM (r^2)[b]**	**T_{inh}[a] (min)**	**$T_{inh}/\mu M$, min/μM (r^2)[b]**
Glutathione	0 µM	N.D.[c]		37.8	
	2 µM	10.1	4.2	44.2	4.4
	3 µM	14.4	(0.9996)	52.2	(0.9623)
	4 µM	18.4		55.4	
Ascorbic acid	0 µM	N.D.[c]		37.8	
	2 µM	9.4	4.8	N.D.[c]	4.5
	3 µM	15.8	(0.9613)	51.2	(1.0000)
	4 µM	18.9		55.7	

[a] Variation coefficient <2%.
[b] r^2 = Coefficient of determination.
[c] N.D. = not determined.

TABLE III
Odors Perceived by Panelists in Aged (5 days at 40°C) Beers

Spiked	0 ppm of GSH	50 ppm of GSH
Before aging[a]	Cereal	Boiled cereals, oatmeal
	Honey, sweet, vanilla	…
	Porto, cider, Madeira	…
	Floral	…
	Fruity, banana	…
	…	Green plants
	…	Sulfurous
	…	Lime kiln, plaster
		Stale, stagnant water, sewer, nauseating, stinking
Before fermentation[b]	Apple	Apple
	Caramel	Caramel, honey, cane sugar
	Unpleasant	Unpleasant
	Stale, oxidized	Stale, oxidized
	Rose, lilac	…
	…	Sulfurous, potato

[a] Beers spiked with 0 or 50 parts per million (ppm) of glutathione GSH before aging.
[b] Beers produced at laboratory scale from an industrial wort spiked before fermentation with 0 or 50 ppm of GSH.

TABLE IV
Residual Concentration[a] and Ratio of Glutathione After the Aging (40°C for Five Days) of a Beer or Phosphate Buffer[b]

	Residual Glutathione	
Sample	**Concentration (ppm)**	**Ratio (%)**
Phosphate buffer		
Unprotected with argon	131	53
Protected with argon	215	87
Beer	232	94

[a] Residual concentration = parts per million (ppm).
[b] Bear and phosphate buffer (bubbled with argon or not bubbled) spiked with 250 ppm of tripeptide.

imparted by polyphenols) is sufficient to significantly protect GSH from oxidation. In phosphate buffer, on the other hand, only 50% of the GSH is recovered after aging (90% in the presence of argon). It can be concluded that traces of sulfur compounds with very low-sensorial thresholds are probably responsible for the unsuitable off-flavors found after beer aging. Our research currently focuses on identifying all these aromas by gas chromatography olfactometry.

CONCLUSIONS

Issued from sulfate metabolism, GSH is naturally present in brewing yeast at concentrations varying from 8 to 37 nmol/mg of cells. Addition of GSH to the YPS or wort medium has no significant impact on the growth of most yeast strains (it even slightly inhibits growth of BRAS291 when added at 1,250 ppm). Only BRAS24, characterized by unusually slow growth and a low internal GSH pool, showed a strong growth-rate dependence on GSH.

In high-gravity primary fermentations, a slight positive effect was observed during the first week with only two (lager) yeast strains.

Added to phosphate buffer or beer, GSH imparts a reducing activity similar to that of ascorbic acid, but it proves to be a much less powerful antioxidant than phenolic compounds.

Whatever the chosen step for spiking, beers aged in the presence of GSH were characterized by strong off-flavors described as “sulfurous”, “sewer like”, or “greeny”, despite a mere 6% degradation of the tripeptide.

ACKNOWLEDGMENT

L. Gijs, A. Timmermans, and C. Guyot-Declerck are grateful to Réalco S.A. (Louvain-la-Neuve, Belgium) and the Région wallonne for financial support (BIOVAL-Entreprise convention 3997).

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[Received August 22, 2003. Accepted March 3, 2004.]