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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.

1. Introduction

Glutathione (GSH, L-γ-glutamyl-L-cysteinyl-glycine), the most ubiquitous low molecular weight non-protein thiol compound, is widely distributed in living organisms, from prokaryotes to eukaryotes [1]. It is synthesized at intracellular level by the consecutive actions of γ-glutamylcysteine synthetase, feedback regulated by GSH content, and GSH synthetase [2].

The major functions of GSH can be summarized into three ways of acting, as antioxidant, immunity booster and detoxifier [3]. In particular, in living tissues, GSH plays a pivotal role in protecting DNA and other biomolecules against oxidative damage, preserves immune function, and accomplishes detoxification by linking to exogenous electrophiles [3,4].

As a powerful and versatile defence molecule, GSH has been widely used as medical treatment as well as in food and cosmetic industries [4–6]. Accordingly, the commercial demand for GSH is ever-increasing [7].

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 [7,8]. Some yeast strains, such as *Saccharomyces cerevisiae* and *Candida utilis* are commonly used microorganisms on an industrial scale for GSH fermentative production [8,9]. However GSH contents is usually variable among strains (0.1–1% dw) and always present in intracellular form [9,10]. Moreover, 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 [11].

Therefore, in these years 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 [10–13]. Little has up to now been reported about extracellular GSH release from cells. Wei et al. [14] for the first time investigated the effects of surfactants (SDS and CTAB) on *S. cerevisiae* cell growth, intracellular GSH biosynthesis and extracellular release [14]. Results showed that cell growth was greatly affected by the addition of high surfactant concentrations. In particular, when SDS and CTAB concentrations were higher than 0.5 and 0.25 g/l respectively, cell growth was completely inhibited. Instead, only when low concentration of surfactants was added to the medium, total GSH concentration, taking into account both GSH synthesis and excretion, was increased. Applying this procedure, a maximum of 50 mg GSH/l were obtained in extracellular form. Nie et al. [15] studied the effect of low pH stress on GSH synthesis and excretion capability in growing cells of *C. utilis*. The applied procedure allowed to obtain a total amount of 737.1 mg GSH/l, 197.3 mg/l of which in extracellular form.

The present research was aimed at inducing, in a post fermentative procedure (biotransformation), GSH accumulation and subsequent release from *S. cerevisiae* cells, achieved by modifying cell permeability. With the aim of limiting process costs, research considered also the possibility to carry out the biotransformation by employing different commercial baker's yeasts, inexpensive cells source available on the market [16]. The use of surfactants on already grown cells may also avoid the risk of

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a detrimental effect of these molecules on cell enzymatic activity, evidenced by Wei et al. [14].

In this paper the effect of different procedures, such as permeabilizing agent and lyophilization, 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.

2. Materials and methods

2.1. Microrganisms

For GSH production *S. cerevisiae* NCYC 2959 (National Collection of Yeast Cultures, Aberdeen, UK) was comparatively employed together with three *S. cerevisiae* 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 on an alternative shaker (60 spm, 4 cm run) for 48 h. The obtained culture was centrifuged at 10,600 × *g* 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, KH_2PO_4 3.5, magnesium sulphate 0.5, pH 5.3, 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 and lauroyl sarcosine (Sigma), for comparison purposes. Lyophilization was carried out as follows: yeast cells were suspended in distilled water (20% dw), placed in stainless steel trays as a thin layer, and then frozen at −40 °C for 4 h; cell disidratation phase was carried out at 25 °C and 1.33 Pa for 30 h (Edwards Minifast MFD 01, UK) (maximum residual humidity 5–8% dw).

2.3. Analytical procedures

Intracellular GSH was determined according to Rollini and Manzoni [12]. Samples at different incubation time were centrifuged (10,600 × *g*, 6 min), and obtained cells were washed twice with H_2O, then thermally treated at 100 °C for 12 min. After cooling in ice bath, samples were centrifuged (15,300 × *g*, 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 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_2PO_4, pH 3.5, at 0.3 ml/min. Standard GSH (reduced form) and GSSG (oxidized form) were purchased by Sigma.

2.4. Transmission electron microscopy (TEM)

Samples (2 ml) obtained at different biotransformation reaction times were centrifuged (5200 × *g* for 10 min) and the obtained cells were prepared for transmission electron microscopy as previously reported [12]. 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

3.1. Intracellular GSH production

In the preliminary part of the research, a set of biotransformation trials were performed in order to evaluate GSH physiological accumulation by *S. cerevisiae* in a post-fermentative procedure, employing either a collection strain and commercial baker's yeasts. 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 [13,16]. Table 1 reports intracellular, extracellular and total GSH levels (g/l) obtained at 24 and 48 h incubation. No GSSG production was observed, and GSH present in samples resulted stable in the reaction conditions as well as during the permeabilization procedure and subsequent analysis. Samples pH was always found in the range between 4.5 and 5.5, without any correlation with GSH accumulation. To evaluate the equilibrium between intra- and extracellular GSH forms, intracellular 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.

Table 1
GSH production levels (g/l) either as total, intra- and extracellular forms, obtained at 24 and 48 h incubation time (data reported as mean of four different trials ± standard deviation).

Sample	Time (h)	GSH (g/l)		
		Total	Intracellular	Extracellular
NCYC 2959	24	0.7 ± 0.1	0.7 ± 0.2	0
	48	0.8 ± 0.2	0.8 ± 0.1	0
GB Italy	24	1.6 ± 0.3	1.4 ± 0.2	0.2 ± 0.1
	48	1.7 ± 0.3	1.5 ± 0.1	0.2 ± 0.1
Lievitalia	24	1.3 ± 0.2	1.2 ± 0.2	0.1 ± 0.1
	48	1.1 ± 0.1	1.1 ± 0.2	0
Zeus	24	1.5 ± 0.1	1.3 ± 0.3	0.2 ± 0.1
	48	1.8 ± 0.2	1.7 ± 0.2	0.1 ± 0.1

In these trials GSH, as expected, was mainly found as intracellular metabolite at interesting levels in all yeast samples. Trials carried out by suspending cells in a solution not containing the precursor aminoacids (cysteine and glycine) evidenced that intracellular GSH remained at stable low levels during incubation (0.2–0.3 g/l, data not shown). Extracellular GSH was always found in the range 0–0.2 g/l, thus confirming its intracellular physiological nature. 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, 0.06 g/l h productivity), and at 48 h with Zeus (1.7 g/l, 3.4% dw, 0.02 g/l h productivity). For the reference collection *S. cerevisiae* strain, yields of 0.7–0.8 g GSH/l with the highest values for intracellular form, 0.03–0.06 g/l h at 24 h, to decrease to 0.004–0.02 g/l h from 24 to 48 h.

Results obtained in this phase were considered as reference data, and used to evaluate GSH release from cells.

3.2. Extracellular GSH production

The addition of surface-active agents to the reaction solution can be considered a strategy to alter cells properties, particularly the transport mechanisms across cell surrounding structures, and to obtain metabolites release from yeast [17]. GSH release was investigated by adding different substances to the reaction solution. 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 membrane-modulating agent [18]. Octyl-glucoside, Mega 10 and Triton-X 100 are mild non-ionic detergents used for solubilization of cytoplasmatic membrane proteins. Digitonin, non-ionic detergent, permeabilizes plasma membranes of eukaryotic cells by forming complexes with membrane cholesterol and other hydroxysterols conjugates. Lauroyl sarcosine, a cationic detergent, does not precipitate and is commonly used instead of SDS for proteins solubilization [19–22].

GSH release was 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