Enzyme and Microbial Technology 93 (2016) 122–131

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Evaluation of cysteine ethyl ester as efficient inducer for glutathione overproduction in *Saccharomyces* spp.

Eric Lorenz [a], Maximilian Schmacht [b], Martin Senz [b,*]

[a] Berlin University of Technology, Institute of Biotechnology, Berlin, Germany
[b] Research and Teaching Institute for Brewing in Berlin, Department Bioprocess Engineering and Applied Microbiology, Berlin, Germany

ARTICLE INFO

Article history:
Received 23 May 2016
Received in revised form 13 July 2016
Accepted 5 August 2016
Available online 10 August 2016

Keywords:
Biotransformation
Cysteine
Cysteine ethyl ester (CEE)
Design of experiments (DoE)
Glutathione enriched yeast

ABSTRACT

Economical yeast based glutathione (GSH) production is a process that is influenced by several factors like raw material and production costs, biomass production and efficient biotransformation of adequate precursors into the final product GSH. Nowadays the usage of cysteine for the microbial conversion into GSH is industrial state of practice. In the following study, the potential of different inducers to increase the GSH content was evaluated by means of design of experiments methodology. Investigations were executed in three natural *Saccharomyces* strains, *S. cerevisiae*, *S. bayanus* and *S. boulardii*, in a well suited 50 ml shake tube system. Results of shake tube experiments were confirmed in traditional baffled shake flasks and finally via batch cultivation in lab-scale bioreactors under controlled conditions. Comprehensive studies showed that the usage of cysteine ethyl ester (CEE) for the batch-wise biotransformation into GSH led up to a more than 2.2 times higher yield compared to cysteine as inducer. Additionally, the intracellular GSH content could be significantly increased for all strains in terms of $2.29 \pm 0.29\%$ for cysteine to $3.65 \pm 0.23\%$ for CEE, respectively, in bioreactors. Thus, the usage of CEE provides a highly attractive inducing strategy for the GSH overproduction.

1. Introduction

The worldwide annual turnover of yeast-derived products is supposed to exceed the 9 billon dollar threshold by the year 2019 [1]. Glutathione (GSH) containing yeasts and purified GSH are part of this market.

GSH (γ-glutamyl-cysteinyl-glycine) consists of the three amino acids glutamate, cysteine and glycine. This non-coding tripeptide thiol is found in most pro- and eukaryotic cells with concentrations of 0.2–10 mM and is synthesized via two ATP-dependent enzymatic reactions [2]. Thereby, the first step in GSH biosynthesis is the rate limiting step [3] due to a feedback inhibition of glutamate-cysteine ligase (GSH1, L-glutamate + L-cysteine) by GSH. The second and final reaction is catalyzed by glutathione synthetase (GSH2, γ-glutamyl-L-cysteine + glycine) to glutathione. The redox couple of glutathione ($E_0 = -240$ mV [4]; GSH = reduced; GSSG = oxidized) is located in cytoplasm as well as mitochondria membrane where it maintains the redox status of the organism by e.g. detoxifying reactive oxygen species (ROS) or scavenging free radicals or heavy metals [4,5].

Based on the described properties of GSH, the substance itself but also GSH enriched yeasts can be used as food additive (dough modifier or flavor enhancer) in food industries [6,7]. Other applications are the usage of GSH as skin whitening agent in cosmetics or as supporting medication in cancer therapies [8,9].

The organisms of choice for an efficient production of GSH are yeasts, especially species like *Candida utilis* and *Saccharomyces cerevisiae* [10]. These organisms provide advantages such as the ability to grow fast to high cell densities or in low-price media [8]. In the early past, genetic modified bacteria, e.g. *Escherichia coli*, were also successfully used [11]. However, consumers usually do not accept the use of GMOs particularly in food.

Concerning the development of a production process with high intracellular GSH content, several approaches in the area of biotransformation, genetic-, evolutionary- and bioprocess engineering were done, triggered by high industrial interest. The most common process strategy to produce GSH in an industrial scale is the fed-batch mode. However, production via repeated fed-batch or continuous (chemostat) processes are also content of current research [12,13]. Li et al. and Xiong et al. summarized different successful production processes and control strategies for fed-batch fermentations [10,14]. Besides process strategies, media design and selection of an efficient production strain are key approaches for optimization. Based on the current knowledge, the most frequently

* Corresponding author at: Postal address: Seestraße 13, 13353 Berlin, Germany.
E-mail address: m.senz@vlb-berlin.org (M. Senz).

http://dx.doi.org/10.1016/j.enzmictec.2016.08.004
0141-0229/

used inducer for glutathione overproduction is cysteine, which is often combined with the other two precursor amino acids glutamate and glycine [15]. However, combinations of other inducing amino acids as methionine and serine are also published [16,17]. In Table 7, there is given an overview of current research approaches for an efficient GSH production, wherein also the results of this study were included for comparison.

In the present work, a full factorial screening of known and unknown GSH inducing substances was executed. Subsequently, selected inducers were combined with different concentrations of yeast extract and investigated via response surface model (RSM). Experiments were performed with an affordable screening platform (50 ml shake tubes) adapted from cell culture technique [18]. To underline the reliability of the investigated induction processes, studies were carried out with three different *Saccharomyces* strains (baker's yeast, *S. cerevisiae*; brewer's yeast, *S. bayanus*; probiotic yeast, *S. boulardii*) and finally verified under controlled fermentation conditions in 5-l-stirred tank bioreactors.

2. Material and methods

2.1. Media

For pre-cultures chemically defined WMIX medium (WM = white molasses), a modified version of WMVIII [19], was used. All chemicals were purchased from Sigma-Aldrich, Inc. The composition was as follows (g/l): salt solution (sterilized by autoclaving): NH_4Cl 2.8, *myo*-Inositol 0.075, $MgCl_2{\cdot}6H_2O$ 0.25, $CaCl_2{\cdot}2H_2O$ 0.1, $MgSO_4{\cdot}7H_2O$ 0.55. Moreover 4 ml/l vitamin solution and 1 ml/l trace element solution were aseptically added: vitamin solution (solubility of vitamins can be increased by pH adjustment; vitamins were sterilized by microfiltration) (g/l): biotin 0.625, pantothenic acid calcium salt 12.50, nicotinic acid 2.50, pyridoxine hydrochloride 7.60, thiamine hydrochloride 2.50; trace element solution in 0.01 M EDTA (sterilized by microfiltration) (g/l): $CuSO_4{\cdot}5H_2O$ 0.10, $FeSO_4{\cdot}7H_2O$ 0.50, $MnCl_2{\cdot}4H_2O$ 0.50, $NaMoO_4{\cdot}2H_2O$ 0.10, $ZnSO_4{\cdot}7H_2O$ 0.175. Final concentrations of glucose and glutamic acid monosodium salt monohydrate were (g/l) 50 and 10, respectively. Medium pH was adjusted with 40 ml/l 0.5 M potassium phosphate buffer (pH 6.8) (K_2HPO_4, KH_2PO_4). This medium was also used as batch-medium containing 50 g/l glucose and additionally 10 g/l yeast extract (Leiber GmbH, Bramsche, Germany).

2.2. Strains and pre-cultures

For investigations, three different *Saccharomyces* strains were used. Thereby *S. cerevisiae* Sa-07346 was originally obtained from Organobalance GmbH in Berlin, Germany. The other strains, *S. bayanus* Sa-00645 and *S. boulardii* Sa-07145, were selected from yeast strain collection of the Research and Teaching Institute for Brewing in Berlin (VLB). Pre-culture one was executed by inoculation of 0.1 ml of a cryo-stock into a shaking flask and incubated in WMIX for 24 h at 30 °C and 180 rpm. The second pre-culture was seeded with an optical density (OD_{600nm}) of 0.1 and incubated under the same conditions mentioned above.

2.3. Shake tube and shake flask cultivation

In cell culture, orbital shaking technology is one of the most used techniques for early stage process development especially concerning strain or media screening. This technology has been characterized very well [18,20]. In this study, it was adapted for scheduled screening trials of yeast cultivation. Shake tubes (50 ml) were sealed with AirOtop® Enhanced Seals, pore size 0.22 μm (Thomson Instrument Company, USA) to ensure a suitable aeration and sterility. Cultivations were executed in vertical arrangement at 30 °C with 25 mm offset at 240 rpm. Initial cell concentration was adjusted to approximately $1-2\times10^7$ cells/ml. Working volume was always 10 ml of yeast suspension in WMIX medium. The orbital shaker was equipped with a special rack (Sartorius AG, Göttingen, Germany) for 50 ml reaction tubes.

Concerning shake flask cultivation, cells were cultivated in 250 ml shake flasks (DURAN Group GmbH, Wertheim, Germany) equipped with four baffles on the bottom for increased power input. The working volume, shaking speed and cultivation temperature were always 25 ml WMIX medium, 180 rpm and 30 ± 0.1 °C, respectively. The induction of glutathione overproduction was for both systems (tubes and flasks) executed by spiking of inducer at 24 h. Analysis of biomass and reduced glutathione (see Section 2.5 and 2.7) was done at 32 h of cultivation.

2.4. Batch fermentations

Batch fermentations were carried out in 5 l-bioreactors (Biostat®A_{plus}, Sartorius AG, Göttingen, Germany). 2 l of WMIX medium containing 50 g/l glucose as carbon source was used. The bioreactor was inoculated with 10% of the second pre-culture. During the cultivation, pH was regulated to 5.5 ± 0.1 by 25% H_3PO_4 and 20% ammonia, respectively. Furthermore, temperature was set to 30 ± 1 °C and DO was maintained at 30 ± 5% (via stirrer speed and constant air flow of 0.5 vvm).

2.5. Biomass determination

5 ml cell suspension was centrifuged at 4000 × *g* and washed with 0.9% NaCl solution. The supernatant was taken for additional HPLC analysis (determination of low molecular sugars as described below). Cell pellet was dried for 24 h at 100 °C and cooled in a desiccator. The cell dry weight (CDW) was periodically calculated.

2.6. Determination of low molecular sugars

Sugar analysis was realized by HPLC (KNAUER Wissenschaftliche Geräte GmbH, Berlin, Germany) applying a NUCLEOGEL® ION 300 OA column (Macherey-Nagel GmbH & Co. KG, Düren, Germany). The samples were eluted with 0.005 mol/l H_2SO_4 at a flow rate of 0.4 ml/min.

2.7. Glutathione determination

Determination of GSH was based on the colorimetric method via Ellman's reagent (5,5′-dithio-(bis-2-nitrobenzoic) acid, DTNB, Sigma-Aldrich) based on Tietze [21]. In contrast to Tietze [21], glutathione-disulfide reductase was not used in this assay. That is why only the reduced form (GSH), which shows 30–100 times higher concentrations than GSSG [8,22,23], was detected. Yeast cells were washed with 0.9% NaCl solution, suspended in 0.1 M H_3PO_4 and incubated at 80 °C for 5 min in a water bath. After centrifugation at 4000 × *g* for 10 min, the supernatant was taken for the GSH assay. 100 μl of the sample was pipetted into a 96-well-plate and covered and mixed with 100 μl 0.5 M sodium phosphate buffer, pH 8. Afterwards 5 μl DTNB (4 g/l stock solution) were added and incubated for 10 min. Measurements in triplicates were conducted with a 96-well-plate reader (Molecular Devices GmbH, Biberach, Germany) at 412 nm. Additionally, a GSH standard curve was recorded ranging from concentrations of 12.5–100 mg/l reduced GSH. The intracellular GSH (g/l) content was calculated by using