Journal of Trace Elements in Medicine and Biology 27 (2013) 7–11

Contents lists available at SciVerse ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.de/jtemb

Applied methodology

Selenium-enriched *Candida utilis*: Efficient preparation with L-methionine and antioxidant capacity in rats

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ARTICLE INFO

Article history:
Received 15 December 2011
Accepted 17 June 2012

Keywords:
Candida utilis
Glutathione
L-Methionine
Selenium-enriched yeast
Antioxidant capacity

ABSTRACT

Selenium-enriched *Candida utilis* has attracted much attention due to its expanding application in food and feed additives. The objective of this study was to efficiently prepare selenium-enriched *C. utilis* and to investigate the effects of the prepared yeast on antioxidant capacity in rats. A batch culture of selenium-enriched *C. utilis* was first carried out, and the addition of sodium selenite (Na_2SeO_3) after all glucose had been consumed was found to favor higher intracellular glutathione and organic selenium content. Moreover, L-methionine boosted yeast cell growth and glutathione biosynthesis, and prevented glutathione from leaking to the extracellular space that can be caused by Na_2SeO_3. We therefore developed a two-stage culture strategy involving supplementation with L-methionine and Na_2SeO_3 at separate culture phases to improve the performance of selenized *C. utilis*. Using this two-stage culture strategy, intracellular glutathione content reached 18.6 mg/g and 15.5 mg/g, respectively, in batch and fed-batch systems, and organic selenium content reached 905.2 μg/g and 984.7 μg/g, respectively. The effects of selenium-enriched *C. utilis* on the activities of antioxidant related enzymes in rats were investigated, and the prepared selenium-enriched *C. utilis* was shown to be an optimal dietary supplement for enhancing antioxidant capacity in rats.

Introduction

Selenium (Se) is an essential micronutrient in humans and animals, and dietary deficiencies are linked to chronic diseases including cancer and heart disease [1,2]. Dietary supplementation with selenized *Saccharomyces cerevisiae* is a popular approach to prevent Se deficiency, which in turn improves immunity, as well as antioxidant and detoxification capacities of higher eukaryotic organisms [3–5]. Recently, many researches have focused on Se-enriched *Candida utilis*, which can accumulate high concentrations of inorganic Se and transform it mainly into selenomethionine. It also has a high protein content and valuable molecules including glutathione (GSH) and *S*-adenosylmethionine (SAM) [6]. To date, no research has investigated the role of GSH on the performance of selenized yeast.

In our previous study, a strain of *C. utilis* capable of accumulating GSH during Se enrichment was screened and efforts were made to improve intracellular GSH and organic Se levels [6]. During selenized *C. utilis* preparation, the absorption of inorganic selenite (Na_2SeO_3) elevated glutathione peroxidase (GSH-Px) activity [7]; moreover, GSH leaked into the medium [8]. Both of these events decreased intracellular GSH level in the selenized yeast. In the present study, we explored strategies to boost GSH biosynthesis and maintain intracellular GSH levels.

L-Cysteine has been shown to be the most effective amino acid for the promotion of GSH biosynthesis [9]. However, its inhibitory effects on yeast cell growth and its reaction with Na_2SeO_3 inhibited L-cysteine utilization during Se assimilation [6,10]. Recently, it was reported that L-methionine, the precursor of SAM, can be subsequently transformed to L-cysteine in yeast cells [11]. Therefore, we used L-methionine as a substitute of L-cysteine. For the first time L-methionine was evaluated as a means of promoting high-performance (as defined as high levels of GSH and organic Se) selenized *C. utilis* through a two-stage culture strategy under batch and fed-batch cultivations. In addition, we also evaluated the effects of the selenized *C. utilis* on the growth performance and antioxidant capacity in rats.

Materials and methods

Yeast strain and media

C. utilis SZU 07-01 was grown in a seed medium consisting of 20 g/L of glucose, 20 g/L of peptone, and 10 g/L of yeast extract with an initial pH of 6.0 at 30 °C for 20 h on a reciprocal shaker

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http://dx.doi.org/10.1016/j.jtemb.2012.06.001

at 200 r/min. The seeds were then inoculated at 10% (v/v) in a 5 L stirred fermentor (Minifors, INFORS HT, Basel, Switzerland) with a working volume of 3 L. The fermentation medium contained 30 g/L of glucose, 8 g/L of $(NH_4)_2SO_4$, 3 g/L of KH_2PO_4 and 0.25 g/L of $MgSO_4$. The media were sterilized by autoclaving at 121 °C for 15 min. Before supplementation, L-methionine and Na_2SeO_3 were separately microfiltered using a Sartorious® membrane (Sartorius Stedim Biotech SA, Aubagne, France) with a pore size of 0.20 μm.

Culture conditions

Batch fermentation was conducted in a fermentor at 27 °C with an agitation rate of 350 r/min and an aeration rate of 3 L/min for 30 h. pH was automatically controlled at 6.0 ± 0.02 by the addition of 3 mol/L H_2SO_4 or 3 mol/L NaOH. The fed-batch culture was initiated in the same manner as the batch culture, and the concentrated medium was fed beginning at 15 h using the polynomial glucose feeding strategy with a total concentration of 150 g/L of glucose [12]. The level of dissolved oxygen was maintained at over 35% relative to saturation by controlling the agitation rate. Supplementation with 15 mg/L of Na_2SeO_3 and 10 mmol/L of L-methionine were performed according to the experimental scheme.

Analytical methods for Se-yeast

A fermentation broth of 25 mL was centrifuged at 8000 × *g* for 10 min and then washed twice with distilled water. The wet cells were dried at 70 °C to a constant weight to determine dry cell weight (DCW). GSH was extracted from the wet cells by 40% (v/v) ethanol at 30 °C for 2 h and centrifuged at 8000 × *g* for 10 min. The supernatant was then used for a GSH assay described by Tietze [13]. Glutathione reductase, 5,5′-dithio-bis(2-nitrobenzoic acid) (DTNB), and nicotinamide adenine dinucleotide phosphate (NADPH) were purchased from Sigma–Aldrich Co. (St Louis, MO USA). Glucose concentration was determined by the 3, 5-dinitrosalicylic acid (DNS) method [14]. Organic Se and inorganic Se were analyzed as described previously [6].

Intracellular GSH content (IGC), intracellular organic Se content (ISeC), and Se biotransformation rate (SeBTR) were defined as follows:

$$\mathrm{IGC\,(mg/g)} = \frac{\text{intracellular GSH (mg/L)}}{\text{DCW (g/L)}} \quad (1)$$

$$\mathrm{ISeC\,(\mu g/g)} = \frac{\text{organic Se (mg/L)}}{\text{DCW (g/L)}} \times 1000 \quad (2)$$

$$\mathrm{SeBTR\,(\%)} = \frac{\text{organic Se (mg/L)}}{\text{Se added (mg/L)}} \quad (3)$$

Animals and experiment design

Forty Sprague-Dawley (SD) rats with a mean initial body-weight of 150 ± 15 g were purchased from the Experimental Animal Center of Soochow University, Suzhou, China. The rats were randomly divided into 4 groups of 10 rats each (the male and female ratio was 50:50), and fed for 6 weeks with the following experimental diets: group 1 – control of Se-free diet, group 2 – diet with Na_2SeO_3, group 3 – diet with Se-yeast, and group 4 – diet with Se-free yeast. The Se dose in the diets was controlled at a level of 3.0 mg/kg. All rats were housed in cages and maintained at a temperature of 25 ± 1 °C and humidity of 50 ± 5%, and allowed free access to food and distilled water. At the end of experiment, the rats were feed-deprived for 24 h, anesthetized with bioketan and blood was collected by heart puncture into heparinized tubes. Plasma was separated by centrifugation at 3000 r/min for 20 min. Liver samples were collected after the cervical vertebrae were dislocated, washed in a cold saline (0.85% NaCl, w/v), weighed and stored at −20 °C until analyzed. Before determination, liver samples were homogenized in three-fold volume of buffer solution (50 mmol/L Tris, 5 mmol/L EDTA, pH 8.0). The supernatant was obtained by centrifugation of the homogenate at 4 °C and 10,000 r/min for 20 min and used for Se, GSH and enzyme assay.

Antioxidant capacity assay

The activities of glutathione peroxidase (GSH-Px), catalase (CAT), superoxide dismutate (SOD), total antioxidant capability (T-AOC), and malondialdehyde content (MDA) in serum and liver were assayed with commercial assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) and a spectrophotometer (UV2550, Shimadzu, Tokyo, Japan) according to the manufacturer's instructions [15]. The enzyme activity, expressed in relative units, was calculated as activity per mL serum or per mg liver.

Statistical analysis

The data were reported as means ± standard deviation (SD) of triplicate or ten determinations. Statistical calculations were carried out using SPSS version 16.0 software (SPSS Inc., Chicago, IL, USA), and a p-value <0.05 was considered statistically significant.

Results

Effects of sodium selenite on batch culture of C. utilis

The effect of Na_2SeO_3 addition at different growth phases on batch culture of *C. utilis* SZU 07-01 is shown in Fig. 1 and Table 1. Na_2SeO_3 supplementation at 0 h inhibited glucose consumption and retarded yeast cell growth. In contrast, no significant negative effect was observed when Na_2SeO_3 was added at 15 h (after glucose had been consumed). In addition, compared to Na_2SeO_3 addition at 15 h, Na_2SeO_3 addition at 0 h resulted in only 67.8% of GSH biosynthesis, of which 43.9% was excreted to the outer broth. Taken together, our results demonstrated that Na_2SeO_3 addition after glucose consumption dramatically increased and maintained intracellular GSH levels in *C. utilis*.

Effects of L-methionine on Se-enriched C. utilis preparation

L-methionine supplementation was accompanied by the addition of Na_2SeO_3 at 0 h for Se-enriched *C. utilis* preparation (Fig. 2 and Table 1). Cell growth, GSH biosynthesis, and intracellular GSH and Se levels increased by 34.8%, 61.9%, 92.0%, and 22.1%, respectively, relative to culture systems without L-methionine. Less than 11.1% of GSH leaked out of the cells in the presence of L-methionine, and there was an increase in Se biotransformation rate of over 26.1%. Our data clearly showed that L-methionine not only enhanced GSH biosynthesis and maintained intracellular GSH, but also improved Na_2SeO_3 assimilation and conversion to organic Se.

Two-stage culture strategy for the preparation of Se-enriched C. utilis

Based on the above results, a two-stage culture strategy involving L-methionine and Na_2SeO_3 supplementation at 0 h and 15 h, respectively, was developed. Details are given in Fig. 2 and Table 1. Relative to cultures without L-methionine, DCW and total GSH level increased by 10.7% and 41.0%, respectively. Of the latter, 93.4% of the GSH remained inside the cells. The biotransformation rate of Na_2SeO_3 to organic Se was considerably improved in this two-stage culture system, and ISeC reached a high level of 905.2 μg/g. With the high performance (as defined by high levels of intracellular