Process Biochemistry xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

Process Biochemistry

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

Physiological response of *Kluyveromyces marxianus* during oxidative and osmotic stress

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ARTICLE INFO

Article history:
Received 28 November 2016
Received in revised form 7 February 2017
Available online xxx

Keywords:
Oxidative stress
Osmotic stress
Kluyveromyces marxianus
Lactose
Glutathione

ABSTRACT

In industrial fermentations, yeast cells encounter various stresses that affect the cell growth and productivity, and therefore, cells need to respond immediately to the surrounding environment. The present study helps in understanding the response of fermentative *Kluyveromyces marxianus* toward two stresses found during the fermentation of cheese whey: oxidative stress and osmotic stress. In this article, we demonstrated that *K. marxianus* cells were more resistant to oxidative and osmotic stress than *Saccharomyces cerevisiae* strains. Stationary-phase cells of both yeast strains showed more viability and higher glutathione production than cells in the exponential phase. *K. marxianus* showed high glutathione level (6.8 ± 0.25 μg/mg protein) and high intracellular glycerol (2.2 ± 0.14 g/g CDW) in 150 g/L lactose, which then decreased. In addition, expression analysis was performed, and genes involved in glutathione biosynthesis and glycerol synthesis were upregulated in the presence of oxidative and osmotic stress, indicating the effect of stress protectants at the transcriptional level. We also present preliminary data regarding the use of *TRX* and *GSH* as molecular markers of oxidative and osmotic stress in *K. marxianus*.

1. Introduction

Fermentation has been widely used for thousands of years as a successful and economical resource to conserve the quality and safety of foods. In the group of fermenting microorganisms, yeasts are widely used and play a role in both conventional and modern biotechnological processes, e.g., biofuel production. Yeast cells are vigorously exposed to many stresses such as osmotic, oxidative, thermal, ethanol and starvation stress during industrial applications. These stresses impair the strain's growth and metabolism, which drastically affects ethanol production [1,2]. Therefore, the yeast's ability to produce ethanol is dependent on the tolerance of a strain to ethanol and temperature and suitable physiological characteristics [3].

The yeast *Kluyveromyces marxianus* is favored over other yeasts as it possesses characteristics that are advantageous for biotechnology applications including the capacity to assimilate key sugars, namely lactose and inulin; rapid growth rate, with typical generation times of approximately 70 min; and thermotolerance, with the ability to grow in temperatures up to 52 °C [4]. *K. marxianus,* as a dairy yeast, utilizes lactose as its source of carbon for growth and production of various valuable products such as ethanol, enzymes, and food ingredients [5]. Increase in the concentration of lactose in whey as the substrate will help in increasing the ethanol yield, but higher lactose concentration also presents sugar stress to *K. marxianus.* Some yeast cells acquire an inherent protective mechanism to control the level of reactive oxygen species (ROS), e.g., antioxidative enzymes such as superoxide dismutase, catalase (CAT), and glutathione reductase (GR) and intracellular nonenzyme molecules such as glutathione (GSH) [6]. GSH, a tripeptide (γ-glutamylcysteinylglycine), was first discovered in the ethanolic extracts of yeast. GSH is important because of the redox-active sulfhydryl moiety of its cysteine residue, which also acts as a free radical scavenger. Therefore, GSH (reduced) is involved in the oxidative stress response by reacting with an oxidative agent such as H_2O_2, resulting in glutathione disulfide GSSG (oxidized form). Intracellular GSH cycles include the interconversion of the reduced (GSH) and the oxidized forms (GSSG), generating a redox couple that examine and regulates the redox status of the cell [7]. GSH plays crucial role in the growth of eukaryotic cells. In addition, the levels of GSH are important for the appropriate functioning of cells under stress conditions [8]. GSH also plays an important part in yeast stress response to NaCl in *Saccharomyces cerevisiae* [9].

Fermentations with high sugar concentrations may cause sluggish fermentations or reduction in yeast growth [10]. In hyperosmotic stress response, glycerol is the primary compatible solute produced and accumulates to favor the cell adaptation during osmotic stress. Increased glycerol concentration inside the cells

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http://dx.doi.org/10.1016/j.procbio.2017.03.001
1359-5113/

alters the membrane permeability. In *S. cerevisiae*, glycerol synthesis is encoded by duplicating the genes *GPD* (glycerol-3-P dehydrogenase) and *GPP* (glycerol-3-phosphatase) [11,12]. *GPD1* has an important role in osmoadaptation as cells subjected to high osmolarity show increased GPD1 gene expression, which leads to glycerol accumulation. Tolerances toward high oxidative and osmotic stresses are important characteristics of microorganisms in industrial fermentations.

The mechanism that protects *K. marxianus* cells from ROS during whey fermentation is unknown and therefore is a major part of our study. In the present study, we measured the resistance of the fermentative strain of *K. marxianus* to two different stress conditions. We also analyzed the cell viability under oxidative stress and the correlation of oxidative stress with GSH. The comparative stress protective roles of GSH and glycerol in whey-based media have not been much explored in yeast strains *K. marxianus* and *S. cerevisiae* which we tried to measure. Therefore, we investigated the role of GSH and glycerol in protecting yeast cells against oxidative (H_2O_2) and osmotic stress (concentrated whey). Furthermore, we measured the gene expression levels of various marker genes during oxidative and osmotic stress.

2. Materials and methods

2.1. Materials

Ellman's reagent [5,5′-dithiobis-(2-nitrobenzoic acid), DTNB], reduced GSH, GR, KPE buffer (0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt), NADPH, ethylenediaminetetraacetic acid (EDTA), bovine serum albumin, phosphate-buffered saline, phenyl methyl sulfonyl fluoride (PMSF), and all general chemicals suitable for study were purchased from Sigma-Aldrich (St Louis, USA). Hydrogen peroxide (H_2O_2) was obtained from S.D. Fine-Chem. Ltd (Mumbai, India). Cheese whey was collected from Experimental Dairy Plant, NDRI, Karnal, India.

2.2. Yeast strains, media, and growth conditions

K. marxianus MTCC 1389 (ATCC64884) and *S. cerevisiae* MTCC 170 were procured from the Microbial Type Culture Collection (MTCC), Chandigarh, India, and *S. cerevisiae* CEN.PK2 wild-type (MATa; his3D1; leu2-3_112; ura3-52; trp1-289; MAL2-8c; SUC2) strain was kindly provided by Euroscarf (Germany). All the yeast strains were stable in haploid form and cultivated in a YPD medium (yeast extract 10 g/L, bacteriological peptone 20 g/L, and glucose 20 g/L). The cultures were incubated at 30 °C for 24 h, maintained at 4 °C on agar slants, and subcultured fortnightly. The yeast strains were collected and finally added to 50% glycerol solution and maintained at −80 °C.

2.3. Effect of oxidative stress on yeast viability during different growth phases

K. marxianus and *S. cerevisiae* strains were grown in 250-mL Erlenmeyer flasks in a YPD medium (100 mL) up to 72 h at 30 °C and 100 rpm, and the yeast cells were collected at 12, 24, 48, and 72 h of incubation. Cells of OD_{600} 0.8 (corresponding to 1×10^7 cells/mL) were inoculated in a fresh YPD medium containing 0, 5, 10, 20 and 50 mM of H_2O_2. After 2 h of incubation, the yeast cells were collected and colony forming units (CFU/mL) were determined. Plates were prepared in triplicates. Tolerance was calculated as the percentage of viable cells that survived during stress [13].

2.4. Evaluation of the cell resistance to lactose in cheese whey

The deproteinized cheese whey was used as media containing 50 g/L lactose. The lactose was further concentrated up to 200 g/L using reverse osmosis and was used to measure the GSH content in the yeast cells. Standard YPD containing yeast extract (1% w/v), peptone (2% w/v), and dextrose were used as a control media. The Erlenmeyer flasks containing 100 mL of cheese whey for *K. marxianus* and hydrolyzed whey for *S. cerevisiae* were inoculated with cells (approximately 1×10^7 cells/mL) separately. The flasks were incubated at 30 °C and 100 rpm for 2 h; samples were obtained for further analysis.

2.5. Preparation of cell-free extracts

After the treatment of the yeast strains *K. marxianus* MTCC 1389, *S. cerevisiae* MTCC 170, and *S. cerevisiae* CEN.PK2 with H_2O_2 and lactose for 2 h, the treated and nontreated (control) cell suspensions (1.0 mL) were collected. The pellet of each sample was washed three times with ice-cold water by centrifugation at $1000 \times g$ for 3 min at 4 °C. The cell pellets were lysed by adding 0.5 mL of lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 50 mM EDTA, pH 7.2, 50 mMPMSF) and approximately 0.5 g of glass beads (diameter, 425–600 μm; obtained from Sigma) according to the method described by Abegg et al. [14]. The cells were lysed by vortexing for 3 min with 1-min intervals for cooling on ice. The samples were again centrifuged for 10 min at $8000 \times g$ to remove cellular debris and beads. The supernatant was collected for GSH and protein estimation.

2.6. Glutathione content estimation

Total intracellular GSH was determined by the DTNB–GSH disulfide GSSG reductase recycling method. KPE buffer, DTNB, NADPH, and GR reagents were prepared according to Rahman et al. [15]. GSH standard was prepared using 100 μL of standard. A volume of 700 μL of KPE buffer was added to a 1-mL quartz cuvette containing 100 μL of the samples. Equal volumes of DTNB and GR were mixed, and 120 μL of this mixture was added to a cuvette. The mixture was allowed to stand for 30 s for the conversion of GSSG to GSH. Then, 60 μL of NADPH was added to the cuvette. Parafilm was used to invert the cuvette for mixing, and the absorbance was read at 412 nm in a UV spectrophotometer (Shimadzu UV-1800).

2.7. Depletion of glutathione

For GSH depletion, yeast cells were incubated for 1 h in YPD medium containing 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) [16]. Samples were collected for GSH estimation.

2.8. Lipid peroxidation determination by TBARS method

Lipid peroxidation was quantified by the estimation of thiobarbituric acid reactive substances (TBARS). TBARS was determined by the method of Steels [17]. Yeast strains were subjected to different concentrations of H_2O_2. The collected samples were pelleted at $2000 \times g$ for 2 min, and the pellets were washed with distilled water. After washing, the cells were lysed using equal volume of glass beads (0–5 mm diameter) and vortexing for six periods of 20 s. After vortex mixing, the cells were placed on ice. Samples were centrifuged at $2000 \times g$ for 3 min, and 0.1 M EDTA and 0.6 mL of 1% (w/v) thiobarbituric acid in 0.05 M NaOH was added to the supernatant. The reaction mixture was incubated for 15 min in a boiling water bath. After cooling, the absorbance was measured using a Shimadzu UV-1240 spectrophotometer against a reference solution comprising 1 mL of TBA reagent, with the sample replaced by the

Please cite this article in press as: P. Saini, et al., Physiological response of *Kluyveromyces marxianus* during oxidative and osmotic stress, Process Biochem (2017), http://dx.doi.org/10.1016/j.procbio.2017.03.001