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Nanofiltration concentration of extracellular glutathione produced by engineered *Saccharomyces cerevisiae*

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This study aimed to optimize extracellular glutathione production by a *Saccharomyces cerevisiae* engineered strain and to concentrate the extracellular glutathione by membrane separation processes, including ultrafiltration (UF) and nanofiltration (NF). Synthetic defined (SD) medium containing 20 g L^{-1} glucose was fermented for 48 h; the fermentation liquid was passed through an UF membrane to remove macromolecules. Glutathione in this permeate was concentrated for 48 h to 545.1 ± 33.6 mg L^{-1} using the NF membrane; this was a significantly higher concentration than that obtained with yeast extract peptone dextrose (YPD) medium following 96 h NF concentration (217.9 ± 57.4 mg L^{-1}). This higher glutathione concentration results from lower cellular growth in SD medium (final OD_{600} = 6.9 ± 0.1) than in YPD medium (final OD_{600} = 11.0 ± 0.6) and thus higher production of extracellular glutathione (16.0 ± 1.3 compared to 9.2 ± 2.1 mg L^{-1} in YPD medium, respectively). Similar fermentation and membrane processing of sweet sorghum juice containing 20 g L^{-1} total sugars provided 240.3 ± 60.6 mg L^{-1} glutathione. Increased extracellular production of glutathione by this engineered strain in SD medium and subsequent UF permeation and NF concentration in shortend time may help realize industrial recovery of extracellular glutathione.

[**Key words:** Extracellular glutathione; *Saccharomyces cerevisiae*; Synthetic defined medium; Yeast extract peptone dextrose medium; Nanofiltration]

Glutathione is the most abundant thiol peptide in most organisms, and is predominantly found in eukaryotic cells (1). Glutathione is widely used in medicine, healthcare, and the cosmetics industry (2) because it can act as an antioxidant, immune booster, and detoxify xenobiotics (3–5). Glutathione is also used as a food additive because it provides an umami taste. Therefore, the demand for glutathione is increasing.

In typical intracellular glutathione fermentation processing, yeast cells at the end of the glutathione production stage are concentrated by centrifugation, followed by extraction of the intracellular glutathione (6). Chemical and physical treatments to release glutathione were imposed on cells (7). However, these treatments may be costly (8,9). Only limited research has been conducted on extracellular glutathione fermentation processes (10). Currently, extracellular glutathione is mainly produced using yeast whose intracellular glutathione productivity has been improved by metabolic engineering and which exhibit almost no growth inhibition (2,6). An extracellular glutathione fermentation process has recently been developed by the identification and overexpression of a novel *Saccharomyces cerevisiae* glutathione export ABC protein (Gxa1), thereby avoiding intracellular feedback regulation and inhibition of cell growth (11). However, extracellular glutathione production by engineered yeasts has been hampered by low glutathione production (10,12), necessitating the optimization of glutathione extracellular production using *S. cerevisiae* engineered strain.

Another possible approach is to concentrate the extracellular glutathione from the culture medium in post-fermentation. Membrane separation technologies are used in biorefining processes because they exhibit excellent separation properties, do not require chemicals, and consume little energy (13). Nanofiltration (NF) membranes have a molecular weight cutoff of 150–1000 g mol^{-1} and ultrafiltration (UF) membranes can retain compounds with 1000–100,000 g mol^{-1} (14). Moreover, post-fermentation membrane processes can be compatible with optimization of extracellular glutathione production using engineered strain. However, no NF or UF processes have been investigated to date for concentrating glutathione, although the molecular weight of glutathione (reduced glutathione: 307 Da, oxidized glutathione: 613 Da) is appropriate for concentration by NF and UF membranes.

Glutathione fermentation by yeasts uses sugars as carbon and energy sources (15,16). Sweet sorghum stalks contain high levels of

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fermentable sugars (17) and the juice pressed from the stalks has recently been used in biorefinery processes (18). Therefore, fermentable sugars in sweet sorghum juice were also tested as substrate for glutathione production in this study.

The aim of this research was to optimize the cultivation condition for a *S. cerevisiae* engineered strain to produce extracellular glutathione and to concentrate glutathione using a membrane separation process in post-fermentation. Extracellular glutathione was higher in synthetic defined (SD) medium than in the yeast extract peptone dextrose (YPD) medium. A UF membrane was used to remove macromolecules from the fermentation medium, then NF membranes were examined to concentrate the glutathione (Fig. 1). The combination of increasing extracellular glutathione by a *S. cerevisiae* engineered strain and membrane separation technologies was effective to obtaining high concentrations of extracellular glutathione.

MATERIALS AND METHODS

Extracellular glutathione fermentation The construction of an extracellular glutathione producing *S. cerevisiae* engineered strain (*GXA1*/*GCS*/*GS*/Δ*CIS2*/Δ*OPT1* strain) was previously reported (11). Reagent-grade YPD medium (10 g L^{-1} yeast extract, 20 g L^{-1} bacto-peptone, and 20 g L^{-1} glucose) was used for extracellular glutathione fermentation and cultivation was carried out as described previously (11). Extracellular glutathione fermentation from a reagent-grade SD medium (6.7 g L^{-1} yeast nitrogen base without amino acids and 20 g L^{-1} glucose) was also conducted as described previously (11). Aureobasidin A (0.5 μg mL^{-1}; Takara, Kyoto, Japan) was added to the YPD, SD, and following sweet sorghum juice media. Supernatants in the YPD and SD fermentation media were sampled after 0, 3, 6, 9, 12, 15, 18, 21, 24, 27, 30, and 48 h of cultivation.

Japanese sweet sorghum cv. SIL-05 was grown at the Togo Field Science and Education Center of Nagoya University (Aichi, Japan) in 2013. The juice was extracted using a juice extractor (Ohuhara Tekko, Okinawa, Japan) and stored at −20°C until use. Prior to fermentation by the engineered *S. cerevisiae*, the juice was filtered through a RS50 membrane (Nitto Denko Corporation, Osaka, Japan) as described below. Sweet sorghum juice medium contained 20 g L^{-1} sugars (11.3 g L^{-1} sucrose, 5.0 g L^{-1} glucose, and 3.7 g L^{-1} fructose) and 6.7 g L^{-1} yeast nitrogen base.

Membrane separation Sulfonated polyethersulfone NF membrane NTR-7450 and polyvinylidene fluoride UF membrane RS50 were obtained from Nitto Denko Corporation. The membranes were cut into circles (diameter: 7.5 cm; effective area: 32 cm^2). RS50 membrane was soaked in 50% ($v \cdot v^{-1}$) ethanol solution for 15 min, then in deionized water for 15 min, and soaked in fresh deionized water overnight before use.

Membrane separation experiments were carried out at 25°C using a flat membrane test cell (diameter: 104 mm, height: 147 mm, working volume: 380 mL; model C40-B; Nitto Denko Corporation) as described previously (18). The feed solution in the cell was stirred at 400 rpm under pressurized nitrogen gas controlled by a pressure valve. The process steps were: (i) extracellular glutathione fermentation; (ii) UF permeation; and (iii) NF concentration (Fig. 1). Three cultures of *S. cerevisiae* engineered strain were grown for 48 h, then the medium was separated from the cells by centrifugation at 3000 ×*g* for 10 min at 4°C. Each supernatant was passed through a UF membrane to remove macromolecules; 0.5–2.0 h was required to pass 350 mL of supernatant. Then, each permeate was passed through an NF membrane to concentrate the glutathione. The pH of the supernatant was not regulated during membrane separation. In addition, cultures of the *S. cerevisiae* engineered strain after 48 h growth were directly passed (without centrifugation) through a UF membrane and the results (with and without removal of the cells) were compared.

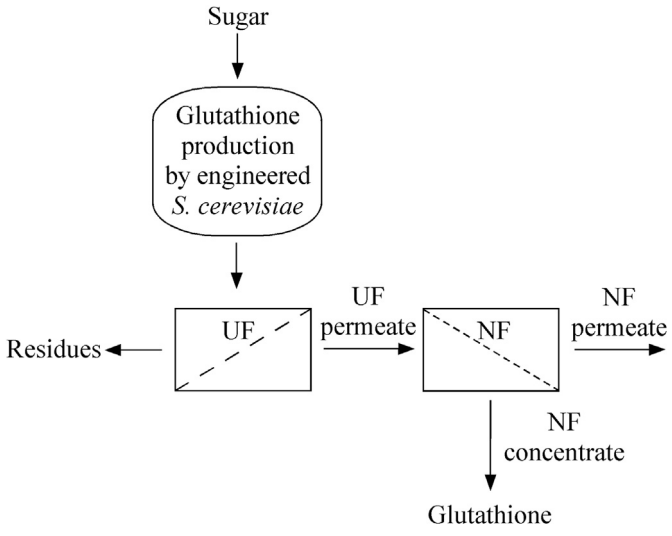

FIG. 1. Scheme showing extracellular glutathione production by engineered *Saccharomyces cerevisiae* and the proposed membrane separation process. Ultrafiltration (UF) permeation to remove residues. Nanofiltration (NF) concentration to increase glutathione. UF permeate, NF permeate, and NF concentrate were sampled.

The observed rejection (*R*) of glutathione by the NF membrane, NTR-7450, was calculated from the following equation,

$$R = \left(1 - \frac{C_p}{C_f}\right) \times 100\% \quad (1)$$

where C_f and C_p are the concentrations of glutathione in the feed medium without *S. cerevisiae* cells and in the membrane permeate, respectively. Glutathione (10 g L^{-1}) (Wako Pure Chemicals Osaka, Japan) was dissolved in SD medium without *S. cerevisiae*. The concentrations before and after membrane separation were averaged for the calculation of C_f. For calculation of R, the medium was concentrated 5.6–5.9 times with 0.5 MPa nitrogen pressure.

Analytical methods Extracellular glutathione concentration (reduced glutathione plus oxidized glutathione) was measured using the 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) method as described previously (19) using reduced glutathione as a standard. The concentrations of glucose, fructose, and sucrose in the sweet sorghum juice were determined by applying the supernatant to a high performance liquid chromatography (HPLC) (Shimadzu, Kyoto, Japan) system equipped with a Shim-pack SPR-Pb column (Shimadzu) and an RID-10A refractive index detector (Shimadzu), as described previously (20). Ethanol was measured on the HPLC system equipped with an Aminex HPX-87H column (Bio-Rad Laboratories, Hercules, CA, USA) operated at 65°C and using 5 mM H_2SO_4 as the mobile phase at a flow rate of 0.6 mL min^{-1}. The optical density at 600 nm (OD_{600}) was measured using a UV-VIS spectrophotometer (UVmini-1240, Shimadzu). The specific growth rate was calculated from the slope of OD_{600} versus time as follows: $\Delta \ln(OD_{600})/\Delta t$, where *t* is the cultivation time (h).

Glycine concentration was determined as described previously (21). In short, dried samples were derivatized at 30°C for 90 min with 100 μL of 20 mg mL^{-1} of methoxyamine hydrochloride in pyridine, followed by incubation at 37°C for 30 min after the addition of 50 μL *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (GL Science, Tokyo, Japan) (22). The derivatives (1 μL) were used for GC–Q–MS analysis (GCMSQP-2010 system, Shimadzu) equipped with CP-Sil 8 CB-MS column (Varian Inc., Palo Alto, CA, USA).

RESULTS AND DISCUSSION

Testing the NF membrane NTR-7450 was selected as the NF membrane for concentrating glutathione because it was reported to reject 82.6% and 54.9 % of sucrose (molecular weight: 342 Da) and glucose (molecular weight: 180 Da) in sugar beet molasses (23), respectively, indicating its utility for removing compounds with lower molecular weight than glutathione. The rejection of glutathione dissolved in SD medium without *S. cerevisiae* was evaluated at 0.5 MPa pressure and showed that NTR-7450 rejected 92.0 ± 0.4% of the glutathione, which is adequate for efficient concentration. Therefore, the molecular weight cutoff of NTR-7450 was lower than the molecular weight of glutathione. As expected, NTR-7450 rejected only 12.2% of glycine, one of precursor amino acids of glutathione (6).

NF concentration of extracellular glutathione cultured in reagent-grade media Extracellular glutathione production was investigated in amino acid-rich YPD medium containing 20 g L^{-1} glucose (pH: 5.3) to grow *S. cerevisiae* engineered strain. The specific growth rate was 0.23 ± 0.01 h^{-1}, as calculated from 0 h to 9 h (Fig. 2A). Final OD_{600} was 11.0 ± 0.6 at stationary phase after 48 h of cultivation and the cells produced 9.2 ± 2.1 mg L^{-1} of extracellular glutathione, similar to that previously reported (11.4 mg L^{-1}) (11). Next, the supernatant was permeated through a RS50 UF membrane under 0.5 MPa pressure. The glutathione permeated completely, judging from the similar glutathione concentration in the original supernatant (9.2 mg L^{-1}) and the