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RESEARCH ARTICLE

Gas-sensing Array Application for On-line Monitoring in a Heat-responsive Bioprocess of *Streptomyces griseus* HUT 6037

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Abstract The stress-responsive bioprocess concept has been developed into an environmentally friendly biosensor with low energy consumption and high recovery yield. To improve chitosanase production during *Streptomyces griseus* cultivation, heat-stress was applied to the bioprocess with monitoring using an on-line gas sensing system. When exposed to heat-stress with liposomes, the chitosanase productivity was 2.6 times greater than for conventional cultivation. The mixed gas components could be distinguished using a principal component analysis during chitosanase production. The online-monitoring system reflected basic changes in growth conditions and metabolite formation in cells. The array type gas-sensing system was capable of detecting bacterial infection faster than conventional sensor systems. The gas sensor system can play a key role in monitoring and controlling stress levels in a stress-responsive bioprocess.

Keywords: heat-responsive, chitosanase, *Streptomyces griseus*, gas-sensing, principal component analysis

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Introduction

Recently, chito-oligosaccharides have received attention due to water solubility and biological properties of antitumor efficacy (1), antifungal and antibacterial activities (2), and immune-enhancing effects (3,4). Among chito-oligosaccharide production methodologies, enzymatic hydrolysis (chitinase and chitosanase) is preferable for ease of control, mild hydrolysis, and catalytic specificity (5). However, this efficient and environmentally friendly process needs to be developed for hydrolytic enzyme production and separation (6,7).

In a conventional bioprocess for chitosanase production, fed-batch fermentation has commonly been used in industrial operations (8) where the glucose concentration (9,10) or specific growth rate (11) are generally monitored for efficiency. In addition, a target conventional bioprocess production was mainly restricted to control of external effects, such as the dissolved oxygen (DO) concentration, pH, and temperature, which are constantly maintained during a cultivation bioprocess. To overcome these restrictions, a stress-responsive bioprocess has been studied as an environmentally friendly method with low energy-consumption and a high recovery yield (12-15). The most important characteristic of this process is excitation of latent cell functions, which effectively use the stress-response function of bacterial cells, membranes, and enzymes to enhance target production. There are several stressors such as heat, peroxide, metabolic, and oxidative, among others. Heat stress is a sensitive and useful stimulus for biological cells.

A process optimization strategy that uses developed stress sensors was first developed based on previous findings (14) regarding the stress response of proteins, liposomes, and cells. Rapid online detection methods for bioprocess monitoring are uncommon in industry. Routine bioprocess

checks are usually made once a day using time-consuming medium samples incubated in a bioreactor. Conventional standard sensors that detect pH and DO concentrations allow for culture contamination testing only at the end stage and not at the initial or middle stages of cell conditions. Another approach to bioprocess-monitoring is GC-MS (16), which is a complicated sampling and evaluation process with a high set-up cost. In order to improve the bioproduction efficiency, stress-responsive sensors are essential for monitoring a stress-mediated bioreactor. The stress-responsive sensor should not only be capable of monitoring cultivation growth phases, but should also be capable of detecting contamination rapidly in order to control the stress-responsive bioprocess.

The primary aim of this study was an assessment of gas-sensor arrays using conducting polymers for use as an online monitoring system for a heat-responsive bioprocess. Chitosanase production, release, and recovery from *Streptomyces griseus* cell growth were used as a model bioprocess. The study also examined the chitosanase bioproduction efficiency with heat-stress in the presence and absence of liposomes.

Materials and Methods

Chemicals Poly (ethylene glycol) (PEG) 1540, 4, and 6 k (average Mw 1.5, 3, and 7.5 k, respectively), dithiothreitol (DTT), N-hydroxysuccinimide (NHS), dioxane, and potassium hexacyano-ferrate (II) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Dextran T-500 was obtained from Amersham Biosciences (Uppsala, Sweden). Chitosanase from *S. griseus*, 16-mercapto-hexadecanoic acid, Triton X-405 (nonionic surfactant), tetra-*n*-butylammonium perchlorate (Bu_4NClO_4) and tetra-ethylammonium tetrafluoroborate (Et_4NBF_4) (TCI) (used as doping agents), acetonitrile and propylene carbonate (TCI) (used as solvents), polypyrrole (PPy) and poly-thiophene (pTh) derivatives, and 2,2-bithiophene (used as a conducting polymer for preparation of sensor array devices) were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). 3-*n*-dodecylthiophene, also used as a conducting polymer, was obtained from TCI (Tokyo, Japan). 1-Palmitoyl-2-oleoyl-sn-glycero-phosphocholine (POPC) used as a phospholipid for preparation of liposomes was obtained from Avanti Polar Lipid (Alabaster, AL, USA). 1-Ethyl-3-(3-dimethlaminopropyl)-carbodiimide hydrochloride (WSC) was purchased from Dojindo Laboratories (Kumamoto, Japan). All other analytical grade chemicals were purchased from Wako Pure Chemical Industries and used without purification.

Preparation of sensor array devices and conductive films Sensor array devices were prepared following the method of a previous report with slight modification (17). Electrochemical polymerization was carried out using a potentiostat HA-501G and a potentiosweeper HB-105 (Hokuto Denko Corp., Osaka, Japan). Indium tin oxide (ITO) was used as a working electrode for conducting polymers. Doped pTh, poly (3-*n*-dodecylthiophene) (pD), and thioxyrenol modified PPy (Thiox/PPy) films were prepared based on application of 1.8, 2.0, and 2.2 V (vs. Ag/AgCl), respectively. These films were polymerized through the gap to form a resistor element between separated electrodes. After polymerization, films on the ITO electrode were rinsed several times with acetonitrile and propylene carbonate as solvents then finally dried at 150°C for 2 h.

Cultivation and stress exposure of *S. griseus* HUT 6037 cells The bacterial strain used in this study was Gram-positive *S. griseus* HUT 6037, which is constitutive for chitosanase as a secondary metabolite. Cells were maintained on an agar slant containing 1.0% manitol, 0.2% peptone, 0.1% meat extract, 0.1% yeast extract, and 0.05% $MgSO_4 \cdot 7H_2O$. Cells were transferred to 20 mL of a pH 7.0 seed culture medium with 1.0% manitol, 0.2% peptone, 0.1% meat extract, and 0.1% yeast extract, then grown at 37°C for 36 h. The seed culture (2 mL) was inoculated into a 300 mL Erlenmeyer flask containing 100 mL of pH 7.0 enzyme production medium with 0.05% KCl, 0.1% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4$, and 0.2% soluble chitosan as a carbon source. Cultivation was performed at 37°C on a circular shaker.

When *S. griseus* cells reached the late exponential growth phase, they were heated to temperatures from 37 to 45°C for 30 min in the presence and absence of 0.013 mM POPC liposomes in the cultivation medium. The intracellular and extracellular chitosanase activities were measured using a protocol described previously (8,18). Other gas stress sensing methods characterized cell responses against heat stresses.

Chitosanase assay The chitosanase (EC 3.2.1.132) activity was measured using glycol chitosan as a substrate (19). A reaction mixture containing 0.5 mL of 2% (w/v) glycol chitosan in a 0.1 M phosphate buffer (pH 7.5) and 0.5 mL of a crude enzyme solution was incubated (Sanyo MIR-S100C; SANYO Electric Co. Ltd., Osaka, Japan) at 37°C for 10 min. The enzymatic reaction was stopped by heat-inactivation via boiling for 4 min. After cooling, 1 mL of acetyl acetone and 1 mL of distilled water were added to the reactant, followed by boiling again for 20 min.

Subsequently, 5 mL of ethanol and 1 mL of the enriched reagent *p*-dimethylamino-benzaldehyde (DMAB) were added, followed by incubation (Sanyo MIR-S100C; SANYO Electric Co. Ltd.) at 65°C for 10 min. After centrifugation (Beckman Coulter, Inc., Brea, CA, USA) at 5,100×*g* for 5 min for measurement of the extracellular activity in an unreacted substrate precipitate (glycol chitosan), the supernatant absorbance was measured at 530 nm wavelength using a UV-Vis spectrophotometer (UV-1600A; Shimadzu, Kyoto, Japan). One unit of chitosanase activity was defined as the amount of enzyme needed to release 1 μmol of glucosamine per min under the assay conditions.

An enzyme solution was prepared for determination of the intracellular activity. Cells harvested from the cultivation were resuspended in a 0.1 M phosphate buffer (pH 7.5) and disrupted using an ultrasonic generator at 20 kHz (UD-200; Tomy Seiko Co. Ltd., Tokyo, Japan) with a horn-type probe (TP-040; Tomy Seiko Co. Ltd.). The resulting lysate was centrifuged (Beckman Coulter, Inc.) at 3,900×*g* for 10 min to remove cell debris. The supernatant was obtained for a subsequent chitosanase assay.

Measurement of gas components A gas sensor array equipped with pTh, pD, and Thiox/PPy gas sensor films was used to monitor the heat-stress bioprocess for chitosanase production by *S. griseus*. The independent array device electrodes were operated at temperatures of 25, 40, and 60°C. The gas sensor arrays were connected to the bioreactor off-gas line outside the sterile barrier in the bioreactor. Sampling from the bioreactor headspace was performed continuously every 1 min during the entire bioprocess. Nine signal parameters were collected from each gas sensor, and the measurement system consisted of a data acquisition/switch unit (34970A; Hewlett-Packard, Palo Alto, CA, USA) and a personal computer for data acquisition.

Results and Discussion

Preparation of conducting films and sensor array devices The gas-sensing capability of the conductive polymer sensor was confirmed, then each conducting polymer response was acquired and investigated against different gases and temperatures. The sensing films for the gas array were prepared on a patterned electrode, which was prepared using a photolithography method on the ITO substrate (17). The isolation gap between the electrodes was 50 μm. Electrodes for gas component analysis with pTh, pD, and Thiox/PPy applied sensors were prepared. Electrochemical polymerization was performed to fabricate conductive films on electrodes. Other conditions were the same as described in previous studies (20).

The growth state of the bacterial cell culture was monitored based on exhaust gas from the cultivation process using the array-typed gas sensor (17). Online monitoring and control are essential for improvement of product quality and bacterial cell culture yield, which both require a better understanding of environmental effects on cell physiology. The conducting film response varied depending on the temperature, gas type, and other conditions. Therefore, gas responses under different conditions were discriminated using a pattern recognition technique. The principle gas sensor array used the principal component analysis (PCA) technique (20), which is a type of multivariate analysis that is used to reduce the amount of data without losing information. PCA was used to analyze the exhaust gases from cultures using gas sensor arrays sensitive to different conducting polymers. A schematic illustration of chitosanase preparation using *S. griseus* with a heat-stress bioreactor, a gas sensor array, and a heat controller are shown in Fig. 1.

Online monitoring of cultivation using sensor array devices An optical density (OD) time series and sensor film electric resistance for produced gases from an *S. griseus* culture, measured using a Thiox/PPy gas sensor film at room temperature, are shown in Fig. 2A. Based on the OD value, *S. griseus* cells grew in the medium in an exponential manner for approximately a day before reaching the stationary growth phase. The electrical response to 3 kinds of gas sensor films controlled at 3 independent temperatures was monitored online during a 36 h cultivation. Thiox/PPy film resistance in response to exhaust gas varied dynamically during the cultivation period. In particular, the Thiox/PPy gas sensor film demonstrated high sensitivity to NH_3, indicating detection of the gas. The behavior of the Thiox/PPy sensor correlated with cell growth states and with metabolite formation.

In addition, gas component identification from the mixture was enhanced by use of PCA, which is a useful statistical tool for differentiation of off-gases produced by the *S. griseus* cell culture during chitosanase production. Data obtained from 3 sensor films at different temperatures were analyzed using PCA as a function of cultivation time. The first principal component (PC1) was plotted against the second principal component (PC2) (Fig. 2B). A 9 dimensional space made from 3 film types at 3 different operating temperatures was reduced to a 2 dimensional plot. The PCA pattern was classified into 3 distinctive groups of lag, exponential, and stationary phases of *S. griseus* cultivation.

The PCA pattern of cultivation data was compared with one of the 5 sample gases reported previously (20). For NH_3, the PC1 value was greater than for any other sample gas. The PC1 value clearly increased in the exponential growth phase (7-25 h) and a cluster was formed on the

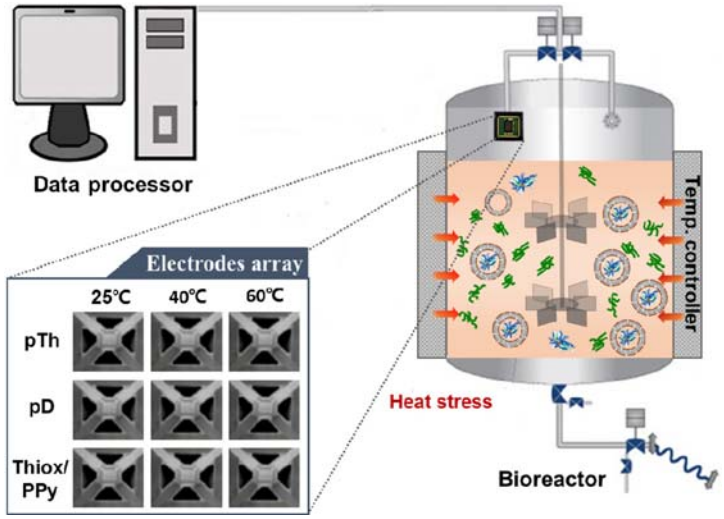

Fig. 1. A schematic illustration of chitosanase preparation using *S. griseus* with a heat-stress bioreactor, a gas sensor array, and a heat controller system with a digital multimeter to measure resistance, and a computer for data acquisition.

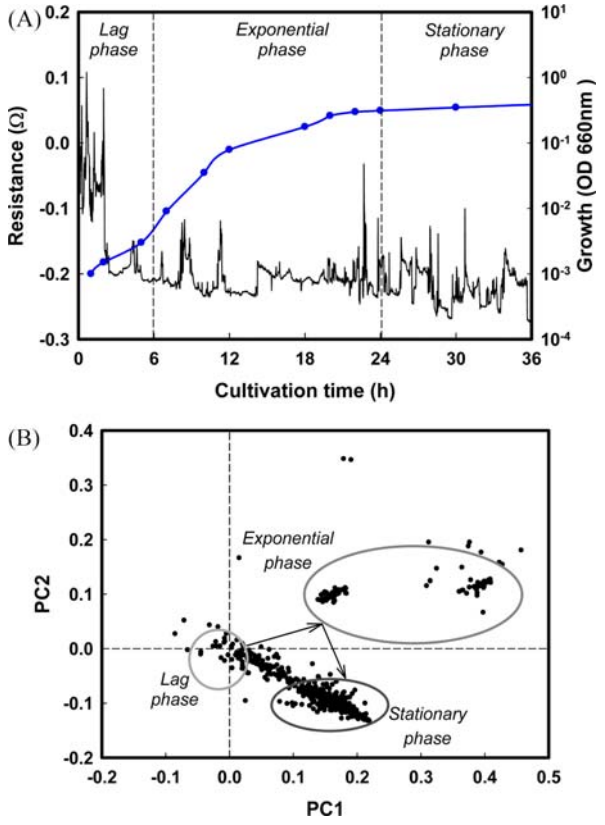

Fig. 2. (A) Time course of *S. griseus* cell culture growth based on optical density (OD) and the electric resistance value of Thiox/PPy sensor films at 25°C. (B) Results of PCA applied to response patterns for *S. griseus* cultivation plotted as a scatter diagram made using 3 types of gas sensor films (pTh, pD, and Thiox/PPy) at 3 different operating temperatures (25, 40, and 60°C).

PCA plot, which suggests that NH_3 was dominantly produced during the exponential growth phase. There have been several studies on NH_3 generation during chitosan hydrolysis (21) and studies of different gases produced, including NH_3, during cell cultivation (22). Based on PCA results, the NH_3 component was a key parameter for the cell stress response function due to chitosanase secretion and chitosan hydrolysis under cultivation.

The gas sensor array was investigated with emission gases and tested for early detection of cell culture infection during the bioprocess. The response signal of a gas sensor array device that detected bacterial infection using *E. coil* is shown in Fig. 3. Three sensor film (pTh, pD, and Thiox/PPy) signals were observed during the entire 36 h cultivation. After 20 h of cultivation, 5 mL of the *E. coil* suspended solution was added to the bioreactor to simulate bacterial infection of the culture. The pD sensor film signal (Fig. 3B) immediately responded to the contamination while the pTh (Fig. 3A) and Thiox/PPy (Fig. 3C) sensor films barely showed noticeable changes in the signal. Results of this study were compared with results obtained previously using sensor films for each of the gases (17). In this case, generated gases were composed of hydrophobic and alcohol gases, except for NH_3. Currently, the compositions of gasses generated when the bioreactor was contaminated with bacteria cannot be explained. The gas sensor array is capable of detecting bacterial infection faster than conventional sensor systems (pH, DO electrode).

Monitoring the heat stress response of *S. griseus* cells in the presence of liposomes The effects of POPC liposome addition to the medium on the array-type gas sensor signals were investigated under heat-stress conditions. Variations in the gas sensor array response signals when cells were cultivated in the presence of 0.013 mM POPC liposomes are shown in Fig. 4. The response signal of the gas sensor was similar to the response of cultivation without heat-

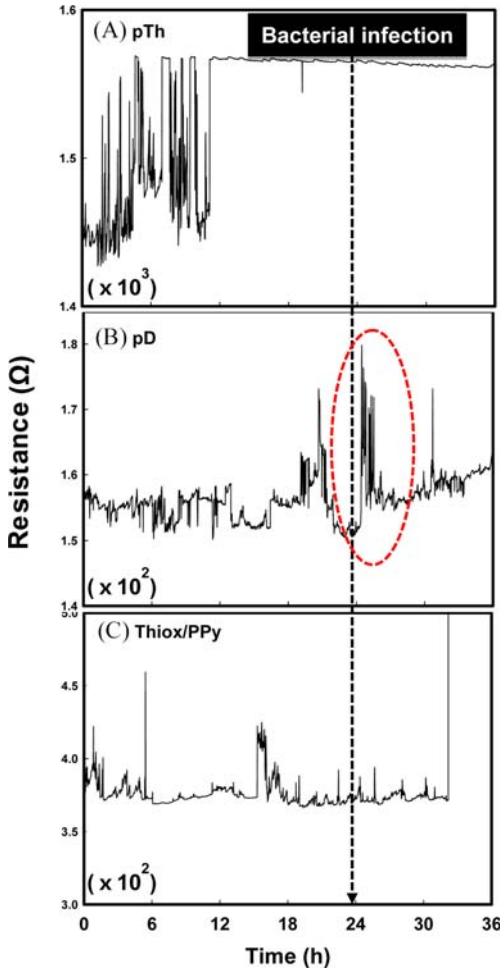

Fig. 3. Electric resistance values of (A) pTh, (B) pD, and (C) Thiox/PPy sensor films at 25°C based on a cell culture bioprocess and intentional bacterial infection with *E. coli* after 24 h of cultivation.

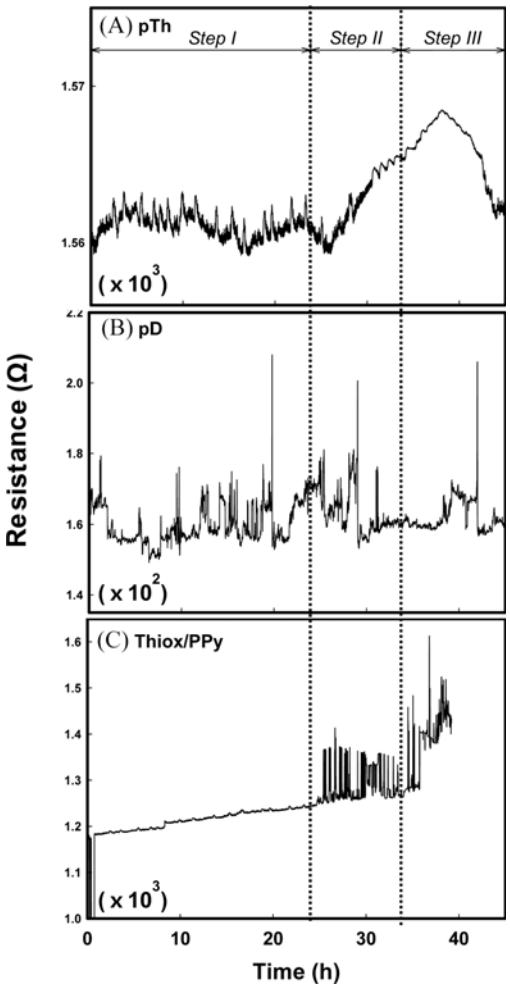

Fig. 4. Electric resistance values of (A) pTh, (B) pD, and (C) Thiox/PPy sensor films at 25°C based on a cell culture bioprocess under heat-stress. Heat was applied 2 times at 45°C for 30 min after 24 h and 33 h of cultivation (step II).

stress for 36 h after the beginning of cultivation (step I). When the growth curve reached the stationary phase, cells in the bioreactor exposed to heat-stress at 45°C (step II) changed to the conventional condition (step III). The sensor signal from the pTh film gradually increased after heat stress exposure (Fig. 4A), while the Thiox/PPy film signal fluctuated drastically (Fig. 4C). No noticeable signal changes; however, were observed from the pD film (Fig. 4B). A pattern analysis of sensor signals showed that it is plausible that NH_3 was also produced during the stationary phase, depending on the heat-stress. The presence of liposomes might reactivate the cell culture to produce chitosanase even in the stationary phase.

The first principal component (PC1) was plotted against the second principal component (PC2) (Fig. 5). The gas pattern was also verified and classified into 3 major groups of lag, exponential, and stationary *S. griseus* cultivation. When cells were exposed to heat stress during the stationary phase, the PC1 and PC2 values formed a cluster in the plot around NH_3 generation. POPC liposome addition further enhanced cell growth under heat stress conditions, indicating that liposome addition to the medium enhanced production and/or release of chitosanase from *S. griseus* cells. On the other hand, the heat stress treatment enhanced secretion of chitosanase from *S. griseus* cells based on enzyme activity and dielectric dispersion analysis (23). Liposomes could act as a positive modulator of proteins or biological membranes for regulation of *S. griseus* cell growth. Thus, *S. griseus* cells continuously produced chitosanase after heat treatment via chitosanase binding or translocation to the liposome membrane, although NH_3 was exhausted in the cell culture at the stationary phase. Online PCA-based monitoring of exhaust gases from a stress-responsive bioprocess can spontaneously represent the culture phase or conditions of *S. griseus* cells under heat-stress with POPC treatments.

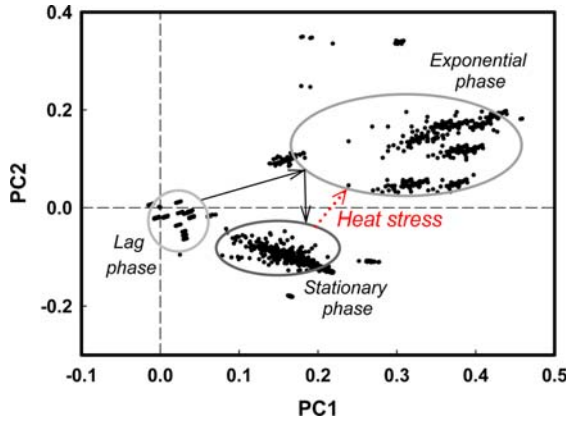

Fig. 5. Electric resistance values of 3 types of gas sensor film for generation of gases during *S. griseus* cultivation under heat-stress conditions plotted as a scatter diagram obtained using PCA.

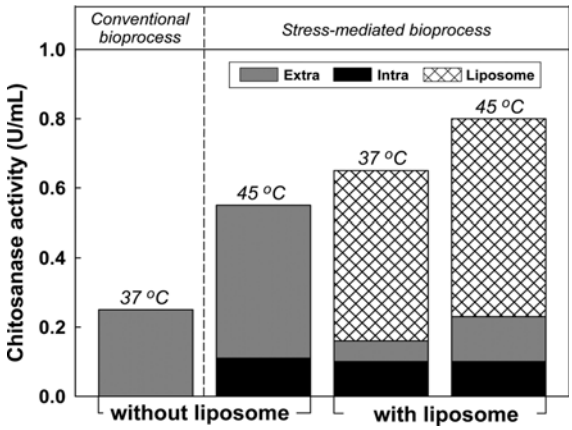

Fig. 6. The intracellular and extracellular chitosanase activities of *S. griseus* compared with conventional and heat-stress cultivation. Extra: extracellular activity, Intra: intracellular activity, Liposome: extracellular activity of POPC liposomes after surfactant treatment.

Comparison of chitosanase production efficiencies

Chitosanase production efficiencies, combining the intracellular and extracellular chitosanase activities between conventional and heat stress-responsive bioprocesses, are shown in Fig. 6. Chitosanase was collected after 4 days of *S. griseus* cell cultivation for a conventional bioprocess at 37°C. The chitosanase activity of the conventional bioprocess was approximately 0.25 U/mL. The stress-responsive bioprocess required only 1 day for chitosanase collection and the amount of obtained enzyme was more than double the amount for conventional bioprocess. The heat stress-responsive bioprocess performed better, improved the bioprocess production, and also saved cultivation time.

The heat stress-responsive bioprocess in the presence of POPC liposomes appeared to improve the chitosanase activity because the released chitosanase from *S. griseus* cells bound to the liposome membrane and translocated to the inner aqueous phase of the liposome, leading to chitosanase production in cells. Liposomes have a chaperone function in protein refolding. Moreover, heat activates molecular chaperones in bacteria, which are important in protein refolding and translocation (24,25).

For these reasons, liposomes can be a useful biomaterial to increase the yield of bio-production via molecular chaperoning and cell protection under excessive stress conditions. Use of this novel strategy based on a stress-responsive bioprocess presented in this study can optimize stress conditions for cell fermentation. However, difficulties in the stress responsive bioprocess with liposomes due to liposome separation from chitosanase occur because of liposome binding or entrapment. This problem can be solved using immobilized liposome chromatography or gel chromatography technologies, which would require further study.

In conclusion, to improve chitosanase production during *S. griseus* cultivation, heat-stress was applied to a bioprocess that was monitored using an online gas sensing system. When cultivations were exposed to heat-stress in the presence of liposomes, the chitosanase productivity was 2.6× greater than for the conventional cultivation method. Furthermore, the gas sensor array system can detect bacterial infection faster than conventional sensor systems (pH, DO electrode). Cell growth phase conditions can be observed using PCA for generated gas compositions. Therefore, a gas sensor system can play a key role in monitoring and controlling stress levels in stress-responsive bioprocesses.

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Disclosure The authors declare no conflict of interest.

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