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Optimization of Expression Conditions for Soluble Protein by Using a Robotic System of Multi-culture Vessels

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Abstract We have developed a robotic system for an automated parallel cell cultivation process that enables screening of induction parameters for the soluble expression of recombinant protein. The system is designed for parallelized and simultaneous cultivation of up to 24 different types of cells or a single type of cell at 24 different conditions. Twenty-four culture vessels of about 200 ml are arranged in four columns×six rows. The system is equipped with four independent thermostated waterbaths, each of which accommodates six culture vessels. A two-channel liquid handler is attached in order to distribute medium from the reservoir to the culture vessels, to transfer seed or other reagents, and to take an aliquot from the growing cells. Cells in each vessel are agitated and aerated by sparging filtered air. We tested the system by growing *Escherichia coli* BL21(DE3) cells harboring a plasmid for a model protein, and used it in optimizing protein expression conditions by varying the induction temperature and the inducer concentration. The results revealed the usefulness of our custom-made cell cultivation robot in screening optimal conditions for the expression of soluble proteins.

Keywords: Parallel culture, high throughput, soluble protein, robotic system

The recent progress of the human and other genome sequencing projects has produced a tremendous amount of genetic information on the entire genomes. Lately, the attention has been turned to take best advantage of such information [16, 22]. Among such effort is to produce many different proteins in throughput mode, so that it can be used to understand the structural properties, functional characteristics, and regulatory behaviors of the proteins that execute the genetic information in the cell. Purified proteins in their naturally folded state are indispensable to address such fundamental questions about proteins. In addition, it is necessary to come up with a high-throughput (HT) mode of production considering the huge number of genes in a single genome [4, 5, 11, 14]. An HT mode is also necessary to screen optimal conditions or gene constructs showing high-yield protein production [17]. Obtaining proteins in high yield is often challenging because proteins sometimes express poorly or fold improperly when produced in heterologous systems [2]. In this condition, success often depends on the time-consuming trial-and-error process of attempting to express different versions of the target protein or to optimize conditions for high efficient expression into soluble and properly folded conformation. The ability to handle many constructs and to try various conditions simultaneously in multiple bioreactors could speed up this screening process considerably [4, 8, 15, 25, 27].

The HT process for efficient cell growth and screening of bacterial expression clones has been developed and used in many areas. Several flasks can be handled manually, but it requires great care and attention. A system that can run 16 parallel flasks with pH control in fed-batch mode has been described [28], but further parallelization of multiple shake flasks has not been reported. Experiments of bacterial cell growth were performed in shaken microwell plates or milliliter-scale bioreactors embedded in a commercial liquid handler [7, 20, 21]. Also reported is a 24-station HT microbioreactor utilizing individually driven impeller agitated units [12]. An HT microliter-scale expression screening protocol for recombinant proteins was described that

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predicted protein behavior expressed in large-scale [18]. A new measurement technique for cell growth has been developed using quasicontinuous scattered light for shaken microtiter plates [23]. Most recently, as part of the Structural Proteomics In Europe project, an automated multifermenter system, GRETA, has been developed [13]. However, these developments are more or less focused on the speed of screening and high yield of cell mass by optimizing dissolved oxygen and pH, and thus far, not much effort has been devoted to the process development for optimizing protein expression conditions, which enables control of important process parameters such as growth temperature and the addition of supplementary materials to the medium.

In this study, we have designed and manufactured a robotic system for HT production of target proteins. The system is equipped with 24 culture vessels arranged in 4 columns×6 rows. Each column, *i.e.*, six culture vessels, is designed to be stationed in an independent thermostated waterbath. The robotic system is specifically tailored to the needs of HT screening of optimal conditions for high-yield heterologous soluble protein production. The system was tested in controlling of cell growth condition, and validated by expressing a model protein. The results revealed the usefulness of our custom-made cell cultivation robot in screening optimal conditions for the expression of soluble proteins.

MATERIALS AND METHODS

Development of a Robotic System for Cell Cultivation

We developed a system that allows simultaneous and parallel cell cultivation. It was manufactured with the help from Robots and Design Co. Ltd. (Technopark E-801, 151 Yatap, Bundang, Seongnam, Gyeonggi 463-760, Korea) (Fig. 1).

Cloning of Model Protein, Microbial Strains, and Media

In order to test and optimize the system, we used yeast YLR301W as a model protein. The gene for YLR301W (GenBank Accession No. AY558217) was PCR amplified from the chromosomal DNA of *Saccharomyces cerevisiae* using the primers, 5'-CAT GCC ATG GTG CCA GCA TTA TTA AAA AGA TTA TTG-3' (sense) and 5'-CCA CTC GAG AGC GTG ATA TTC AAT AAC TTC CC-3' (antisense) containing NcoI and XhoI restriction sites, respectively. The amplified DNA was ligated into the pET28a vector using NcoI/XhoI sites and cloned in *Escherichia coli*

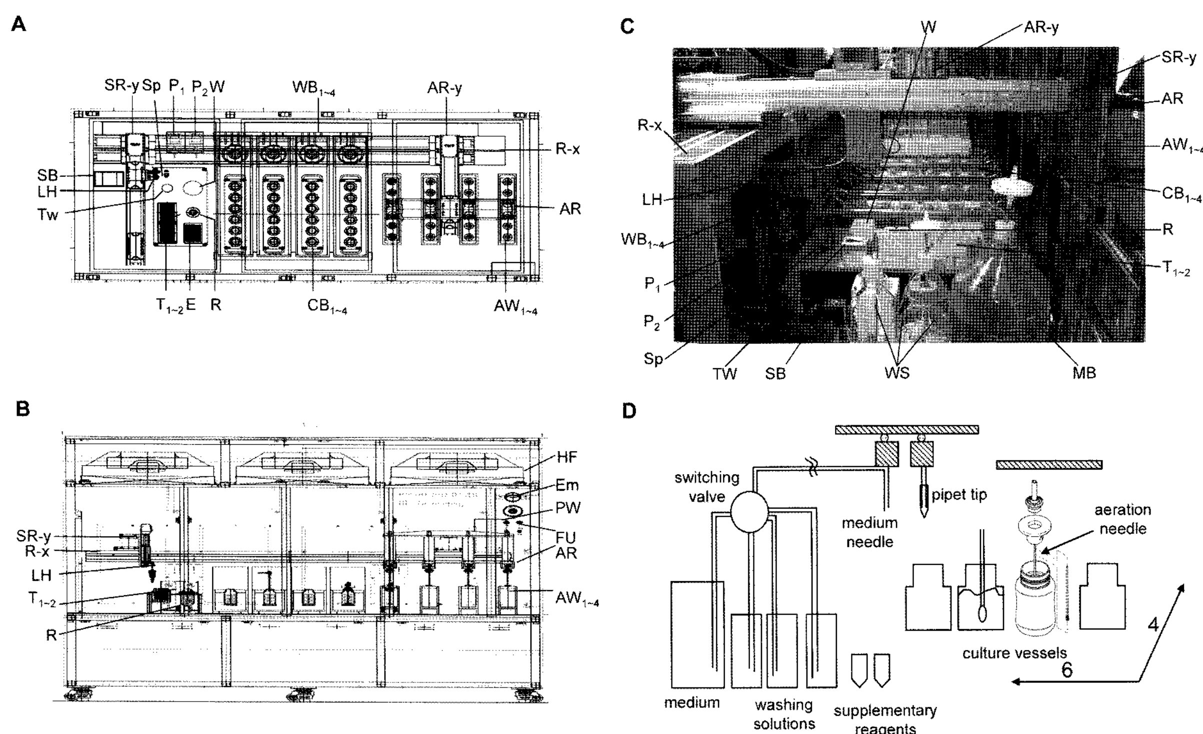

Fig. 1. Layout of the cell cultivation robotic system.

Drawings of the system are presented in plane figure (**A**), in frontal view (**B**), photographic view (**C**), and as a simplified schematic figure (**D**). SR-y: y-axle of pipette head gantry; Sp: spectrophotometer; P_1: syringe pump for movable pipette; P_2: syringe pump for medium and other solutions; W: waste water sink; WB_{1-4}: four thermostated waterbaths; AR-y: y-axle of cover housing gantry; R-x: shared x-axle of the gantries; AR: culture vessel cover housing; AW_{1-4}: parking station for cover housing; CB_{1-4}: 24 culture vessels; R: platform for 50-ml tubes; T_1 and T_2: tip-stacks; Tw: outlet to waste basket for disposable tips; LH: movable pipette head; SB: spectrophotometer control box; E: platform for microwell plate; Em: emergency stop push button; PW: main power on/off switch; HF: HEPA filters; FU: on/off switch for fluorescent and UV lamps; MB: medium reservoir bottle; WS: supplementary bottles for washing solutions.

DH5α [26]. For the cell cultivation test and protein expression, *E. coli* BL21(DE3) was used. The cloned gene is under the control of the T7 promoter, and was indirectly induced by addition of isopropyl-β-D-thiogalactopyranoside (IPTG).

For the growth of *E. coli*, Luria-Bertani (LB) medium (Bacto tryptone 10 g/l, NaCl 10 g/l, and yeast extract 5 g/l) was used. Bacto-tryptone and Bacto-yeast extract were purchased from BD Biosciences Clontech (U.S.A.). The medium was supplemented with 30 mg/ml kanamycin (USB Corp., U.S.A.), just prior to inoculation.

Cell Growth in the Robotic System and Shake Flasks

E. coli BL21(DE3) cells stored in 25% glycerol at −80°C were transferred into 50 ml of LB medium in a 250-ml baffled flask and cultured overnight at 37°C. An aliquot (0.25 ml) of this seed culture was then used to inoculate the fresh LB media (50 ml each) in the culture vessels of the robotic system. Cell growth was monitored by measuring OD_{600} using the built-in spectrometric unit. For shake-flask culture, the same seed was inoculated into 50 ml of LB medium in a 250-ml baffled flask. Cultures were incubated at 37°C on an orbital shaking incubator with an eccentricity of 2.5 cm at 180 rpm. Cell growth was monitored using the Ultrospec 1001 spectrometer (GE Healthcare, U.S.A.).

Expression and Solubility Analysis of Recombinant Model Protein

E. coli BL21(DE3) containing the plasmid for the YLR301W model protein was grown at 37°C in 4×6 culture vessels of the robotic system. Protein expression was induced at an OD_{600} of 0.6 with the addition of 500 μM IPTG to the first vessels in each bath, 200 μM to the second, 50 μM to the third, 20 μM to the fourth, 5 μM to the fifth, and 2 μM to the sixth. Then, the temperature of the second bath was shifted down to 30°C, that of the third to 25°C, and that of the fourth to 18°C, while the first bath was kept at 37°C. Simultaneously, 24 different induction conditions were employed by the combination of four different temperatures and six different IPTG concentrations. After changing the temperature of the waterbaths, the cells were grown for 3 h (37°C), 6 h (30°C), 10 h (25°C), and 18 h (18°C). The cells were incubated at different times for different bath temperatures in order to get similar cell mass throughout the culture. Bacterial cells were harvested and disrupted by using the Ultrasonic processor (Sonics & Materials, U.S.A.). The cell-free supernatant and insoluble protein aggregates were separated by centrifugation at 15,000 ×g for 10 min at 4°C.

SDS-Polyacrylamide Gel Electrophoresis

The separation of proteins was performed by using 12% polyacrylamide gel in a slab gel apparatus (Hoefer Scientific Instrument, U.S.A.) [3]. Samples were prepared in a reducing sample buffer (12.5 mM Tris-HCl, 2% glycerol, 0.4% SDS, 1% β-mercaptoethanol, 0.01% bromophenol blue, pH 6.8) and then boiled for 5 min. Electrophoresis was performed at a constant voltage of 150 V. The gels were stained using Coomassie brilliant blue R-250 (Sigma-Aldrich Inc., U.S.A.).

RESULTS

Layout of the Automatic Cell Cultivation System

A robotic system that allows parallel and simultaneous cultivation of bacterial cells in flask level has been designed and developed. This system can handle up to 24 different culture conditions in four different temperature settings. Fig. 1 represents the overall layout of the system. The culturing unit is located in the center and 24 culture vessels are arranged in 4 columns×6 rows. A set of six vessels, each holding up to 200 ml, are placed in a thermostated waterbath. Supply of water to the waterbath and its drainage as well as setting of the bath temperatures are all controlled by a Windows-based software. The system contains two x-y-z gantries sharing the x-axle rail in common: one for transferring reagents and taking samples from the culture vessels, and the other for handling a cover housing of the culture vessels. Attached to the first gantry are a pipetting head that is equipped with a removable 2-ml pipetting tip, and a stainless needle through which medium is supplied. Attached to the second gantry is the cover housing in which the lids of all 24 culture vessels are arranged, with an aeration needle protruding downward from the center of each lid. The cover housing was designed to close all the vessels. A central air inlet contains a filter and a pressure reducing valve and splits into four channels to each of which a pneumatic mass flow controller is attached. Sterile air flows from the central inlet through aeration needles and sparges into the culture fluids. The aeration needle is 9 cm length of stainless steel (3 mm OD; 2 mm ID). The culture fluids are agitated by air-driven turbulence. A ventilation port is also installed in the lid featured with a foam-suppressing device (Yu *et al.*, 2006. Korean patent 10-611685). Samples are taken, if directed, only after the cover housing is lifted and shifted sideway to a parking station. The system is provided with an electronic measurement for cell density in culture fluid: a spectrometric unit is incorporated, which is equipped with a photodiode laser and a flow cell.

A platform for two tip-stacks, one standard microwell plate (128×86 mm) and six 50-ml plastic tubes (ϕ30×115 mm), was prepared next to the culture vessels. Fifteen-ml tubes (ϕ17×120 mm) can also be placed in the 50-ml tube holder by using adaptors. Another platform was prepared for mounting four different solution bottles: one is exclusively used as a reservoir for fresh medium, the others for distilled water or other kinds of solutions. One 50-ml syringe to draw and dispense any of the solutions

including the medium and one 2.5-ml syringe to manipulate the movable pipette are mounted.

The whole system is enclosed in a single space of a HEPA-filtered hood to maintain a clean environment inside and reduce unwanted contamination during the cell cultivation. The interplay of the different robotic parts and the job process given by a user are directed by a software implemented on a PC using Windows-based Visual C++.

Simultaneous Measurement of Cell Growth at Four Different Temperatures

As the robotic system is equipped with four independent thermostated waterbaths, one type of cells can be grown simultaneously at four different temperatures. To test this type of versatility, fresh LB media (50 ml each) were transferred to the first culture vessels of each waterbath and then inoculated with 1/200 volume of *E. coli* BL21(DE3) seed culture, which had been grown overnight in a shake flask. The temperatures were set to 37°C, 30°C, 25°C, and 18°C, respectively. When cell growth was monitored (Fig. 2A), results indicated that our robotic system seemed to control growth temperature quite accurately.

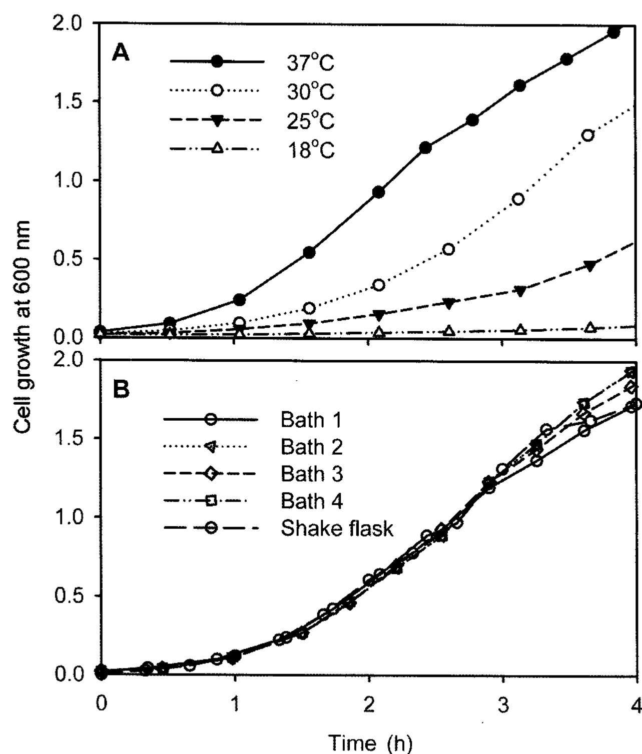

Fig. 2. Cell growth at the robotic system.
A. *E. coli* BL21(DE3) cells were grown in the culture vessels of the robotic system at four different temperatures of 37°C, 30°C, 25°C, and 18°C. Cell growth was monitored by measuring OD_{600} with the built-in spectrometer according to the pre-programmed time schedule as indicated by the markers. **B.** Cells were grown in the first representative culture vessel of each thermostated waterbath at 37°C in the robotic system. Cells were also grown separately in a baffled flask at 37°C with shaking at 180 rpm.

To compare the cell growth in the robotic system with the shake flask culture, 50 ml of LB medium in a 250-ml baffled flask was inoculated with the same seed and by the same fold of dilution in parallel with the robotic system and was incubated at 37°C with shaking at 180 rpm. As shown in Fig. 2B, all of the four culture vessels in the robotic system yielded the same growth and they showed almost identical growth curves to the shake-flask culture (Fig. 2B). The results suggest that cell cultivation using the robotic system has little difference with the shake-flask culture in cell growth rate and is analogous to a situation in which cells are grown in 24 baffled flasks simultaneously, each six of them being placed in an independent shaking incubator respectively.

Control of the Temperature Change

Since the growth temperature has significant effect on the expression of heterologous protein, especially on the folding of the protein [9, 24], the time span required for shifting the culture temperature after initiation of protein induction to a target one is critical. To test the efficiency of temperature change, we first ran all the baths at 37°C and then changed the bath temperature settings to 25°C, 20°C, 18°C, and 15 °C, respectively. After the change of temperature settings, the temperatures of culture fluids along with the readout of the bath temperatures were recorded at one-min intervals (Fig. 3). It took less than 20 min for the bath temperature to reach the target value. The time to reach the final temperature depended largely on the time required to change the water in the bath (data not shown). Although the culture fluid required more time to reach the designated target temperature than it was required for the bath water, temperature change, even to 18°C, was completed within 40 min (Fig. 3). The results suggest that our

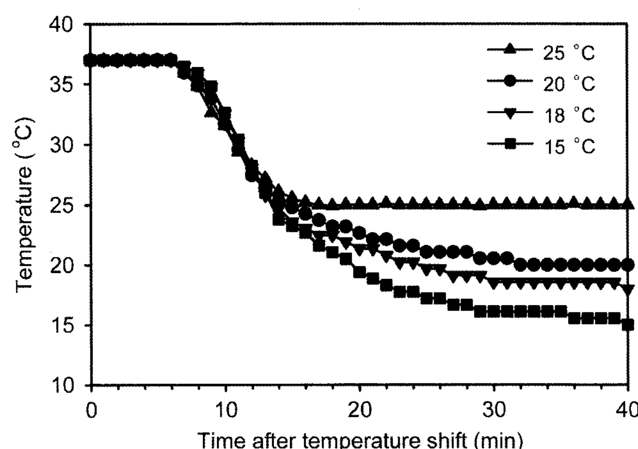

Fig. 3. Control of the temperature change by the robotic system.
All waterbaths were set to 37°C initially. After thermal equilibrium was attained, the temperature set values were changed to 30°C, 25°C, 18°C, and 15°C. The temperatures of culture fluids were recorded at each designated time point.

robotic system can control the temperature of bioreactors and shift it to a different temperature very efficiently.

Optimization of Temperature and Inducer Concentration for Soluble Protein Expression

We tested our robotic system in a specific task to optimize the expression condition for improved soluble expression with high production yield of a model recombinant protein. *E. coli* BL21(DE3) containing the plasmid pET28a-YLR301W was grown at 37°C until OD_{600} reached 0.6. Protein expression was induced at 24 different conditions in 24 culture vessels. The conditions were a combination of four different temperatures (18°C, 25°C, 30°C, and 37°C) and six different IPTG concentrations (500 μM, 200 μM, 50 μM, 20 μM, 5 μM, and 2 μM). Whole-cell extract, cell-free supernatant, and insoluble protein aggregate were analyzed by SDS-PAGE to determine the effects of the temperature and inducer concentration on the productivity and the solubility of the protein. As shown in Fig. 4, expression efficiency was notably high in the range of 200–500 μM IPTG at 30°C, 20–500 μM at 25°C, and 50–500 μM at 18°C. Although a band for the recombinant protein was also observed in the samples of 37°C, productivity was lower than the samples obtained from different temperatures. As judged by the expression efficiency, 25°C was the best temperature for the expression of YLR301W. Furthermore, more than 60% of the expressed protein was recovered in the soluble supernatant in the range of 20–200 μM IPTG, indicating that the optimal condition for a high yield of properly folded YLR301W is 25°C and 20–200 μM IPTG. The result suggests that the robotic system developed in this study is adequate in screening optimal cultivation conditions to induce heterologous expression of soluble proteins.

DISCUSSION

We have developed a robotic system for parallelized automatic cell cultivation in flask level, which was utilized to optimize conditions for high efficient expression of soluble proteins.

Heterologous expression of foreign proteins in *E. coli* frequently appears in the form of inclusion body [2, 9]. Besides aspects of the protein molecule itself and the host strain used, cultivation conditions affect the form in which foreign proteins appear [9]. Temperature and special medium components exert direct influences on the protein folding. Coexpression of chaperones, the concentration of IPTG used for induction, the duration of induction, and the cell growth stage at which the protein is induced have also been identified to influence the *in vivo* folding of foreign proteins [6, 10, 19, 30]. One lesson from all these previous

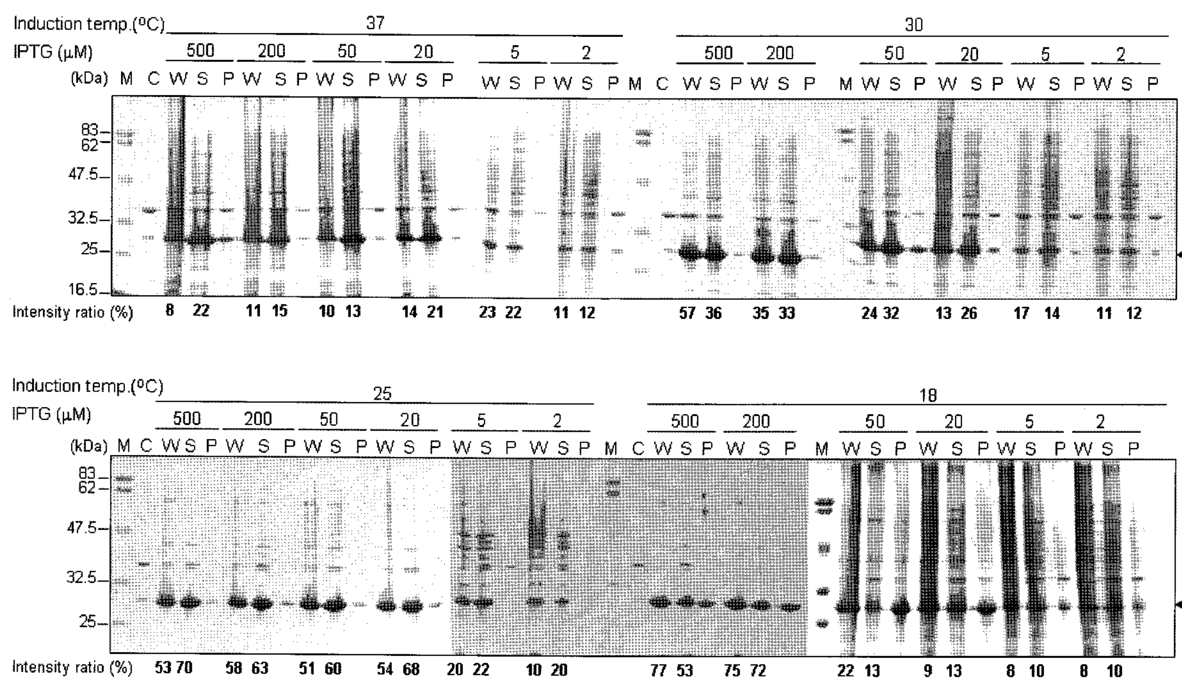

Fig. 4. Optimization of induction condition for highly efficient expression of soluble protein.
E. coli BL21(DE3) containing pET28a-YLR301W was grown at 37°C. When OD_{600} reached 0.6, protein expression was induced at 24 different conditions as indicated above the gel images. Cells were grown for additional 3 h (37°C), 6 h (30°C), 10 h (25°C), and 18 h (18°C), harvested, disrupted by sonication, and analyzed by SDS-PAGE. M, protein molecular weight marker; C, control sample served as a milestone to locate the YLR301W protein band; W, whole-cell extract; S, cell-free supernatant; P, insoluble aggregates. Arrows indicate the YLR301W protein band. Intensity ratios representing the intensity value of the YLR301W band relative to the total protein are indicated below the gel images.

studies is that each individual protein behaves differently in the same expression condition. Therefore, in order to get a high yield in soluble form, it is necessary to find the optimal expression condition in which various environmental factors are optimized for improved soluble expression with high production rate. Although our system is equipped with several pneumatic air flow controllers, the rate of oxygen supply to the culture medium and the pH of the culture media are not measured to give a feedback control. Oxygen and pH are considered as very important process parameters in bacterial growth because the extent and the speed of cell growth is often limited by the concentration of dissolved oxygen and pH of the growth medium [7, 12, 20, 21, 28]. However, it is unlikely that the two parameters are also critical in making recombinant proteins soluble during expression inside the bacterial host [1, 2, 10, 30]. It is well known that growth temperature has significant effects on the protein production and the folding of the protein [9, 24]. Fast growth often exerts an adverse effect on the solubilization of recombinant protein; on the contrary, recombinant protein expression at reduced growth temperatures increases the solubility of recombinant proteins [1]. Therefore, we developed the system to focus more on the control of the growth temperature and the addition of supplementary material to the culture medium, for the system to be specifically tailored to the needs of HT screening of optimal conditions for high-yield soluble protein production. We adopted four independent waterbaths, which made possible to explore different induction temperatures.

The robotic system developed in this study was designed to handle 24 culture vessels simultaneously. In parallel cultures using multiple bioreactors, it is critical to maintain the robotic system in the determined conditions throughout. When the system was tested by growing *E. coli* BL21(DE3) cells at 37°C, there was very little fluctuation in the OD_{600}, suggesting cell growth is quite reproducible in any of the culture vessels. The optical densities were monitored until they reached above 1.5. Considering that protein expression is induced around 0.6, it will be enough to measure the OD_{600} at any one bioreactor out of 24 in determining the proper induction time for all bioreactors, because all cultures will be at the same growth stage. The GRETA multifermenter system developed by Hedren *et al.* [13] is similar to ours, in that six culture vessels are arranged in a single unit and four units are integrated into one system. However, GRETA is more likely that multiple independent fermenters are arranged in a row and controlled by a single operating system, and does not seem suitable for screening of expression parameters because each fermenter chamber has a one-liter working volume. In contrast, the maximum volume of our culture vessel is 200 ml, allowing cell culture in the range of 20–150 ml. Furthermore, our system is quite versatile in controlling the addition of various supplementary materials at any time during culture.

One special feature that our robotic system is equipped with is four independent thermostated waterbaths. As noted, temperature is one of the factors that exert significant effect on protein folding. The usefulness of our robotic system in optimization of temperature as well as inducer concentration was demonstrated by using a yeast model protein, YLR301W. The protein was identified by yeast two-hybrid as an *in vivo* interacting partner of Sec72, one component of the SEC complex, which is responsible for posttranslational translocation of secretory proteins in yeast [29]. Expression of YLR301W in its properly folded form would help to understand its structural property and functional characteristic in relation to posttranslational protein translocation. The optimized temperature for highest expression and more soluble form was 25°C for the model protein (Fig. 4). Not only the steady-state temperature, but the way to change the temperature is also important. Our system was very efficient in controlling the temperature change of the waterbath (Fig. 3). A water cooler can drop the bath temperature. However, in order to get a higher speed in changing the temperature, the system was developed to have an option that it replaces the existing warm water with cool tap water. Because of this, the bath temperature drops down to 15–25°C from 37°C within 20 min (data not shown), and the temperature of the culture fluid reaches the target temperature within 40 min (Fig. 3).

Our robotic system was utilized to optimize specific growth conditions for the soluble protein expression. Although temperature and inducer concentration were the parameters we optimized, apart from those, various other factors can also be optimized. Moreover, OD_{600} at which induction starts can be adjusted according to the user's need. Furthermore, the system can be used for screening 24 different cell types that contain distinct plasmid constructs. We have demonstrated that the system developed here can reduce not only human interventions, but also the time and expense needed to optimize the conditions for protein production. It can be used for both initial screening and production in a large quantity of protein. Our HT production technology applied to proteins of commercial value, especially where obtaining soluble forms is critical (*e.g.*, crystallization for structure determination, or antibody production for immunohistochemistry), would contribute greatly to the development of biotechnology.

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