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Preparation of high activity yeast whole cell bioctalysts by optimization of intracellular production of recombinant *Rhizopus oryzae* lipase

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Abstract

Yeast whole cell biocatalysts, which intracellularly overproduced a recombinant lipase with a pro-sequence from *Rhizopus oryzae* IFO4697 (rProROL) were constructed, and the content of active lipase in *Saccharomyces cerevisiae* cells was maximized by optimizing the cultivation procedure. rProROL was overproduced intracellularly under the control of the 5′-upstream region of the isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*) as the inducible system and the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter as the constitutive expression system. Enhancement of expression level of *ProROL* gene at the initial cultivation phase inhibited rProROL accumulation in yeast cells both in GAPDH promoter system by using high glucose concentration at 30 °C and in *UPR-ICL* system by using non-fermentable carbon sources. The highest intracellular lipase activity of 350.6 IU/l was obtained in the inducible *UPR-ICL* system with an initial glucose concentration of 0.5% at 30 °C. To prepare the efficient whole cell biocatalyst by intracellular overproduction of lipase, utilization of inducible *UPR-ICL* and the optimization of cultivation conditions such as temperature, carbon source and its initial concentration are important.

Keywords: Lipase; *Rhizopus oryzae*; *Saccharomyces cerevisiae*; Whole cell biocatalyst; Intracellular overproduction

1. Introduction

Lipases are one of the most commonly used enzymes in many industrial processes because they catalyze the hydrolysis of ester bonds and the reverse reaction, namely ester synthesis and transesterification in nonaqueous systems. For the industrial bioconversion process, the utilization of intracellularly accumulated lipases in the form of whole cell biocatalysts is both cost effective and advantageous. This is because whole cell biocatalysts are prepared simply by cultivation and separated easily. The enzymes trapped inside the cells are regarded as immobilized. Moreover, flocculent microbial cells containing

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enzymes can be spontaneously immobilized within porous support particles during cultivation [1].

Recently, the development of an efficient biodiesel fuel production process using lipase [2,3] is considered of great importance. Biodiesel fuel refers to methylesters (MEs) synthesized from natural triglycerides and methanol (methanolysis reaction) [4–6]. Since biodiesel is a clean fuel [7] and can be produced from waste oil, this is expected to help overcome environmental problems.

In the previous study, we have constructed an yeast whole cell biocatalyst, which intracellularly overproduced recombinant lipase with a pro-sequence from *Rhizopus oryzae* IFO4697 (rProROL), and successfully demonstrated that this whole cell biocatalyst efficiently catalyzed the methanolysis reaction [8] in a solvent-free and water-containing system [9]. This whole cell biocatalyst with high lipase activity may be applicable in many other reaction systems catalyzed by lipases.

In this study, the content of active lipase in the *Saccharomyces cerevisiae* cells was maximized by optimizing the cultivation procedure. Intracellular overproduction of rProROL was carried out under the control of the constitutive glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter [10] and the inducible 5′-upstream region of the isocitrate lyase gene of *Candida tropicalis* (*UPR-ICL*) [11,12]. *UPR-ICL*-mediated transcription is strongly induced by either glucose exhaustion or a non-fermentable carbon source such as ethanol or acetate [11,12]. Effects of cultivation temperature and carbon sources and their concentrations on the intracellular rProROL productivity in these expression systems were investigated.

2. Experimental

2.1. Strains, media and general methods

The *S. cerevisiae* strain used in this work was MT8-1 (*MATa ade his3 leu2 trp1 ura3*) [13]. The *Escherichia coli* strain used for genetic manipulation was Novablue [*endA1 hsdR17* (r_K^- m_K^+) *supE44 thi-1 gyrA96 relA1 lac recA1/F′* {*proAB*$^+$ *lac* I^q *ZΔM15 Tn10* (*tet*^{r})}] (Novagen Inc., Madison, WI, USA). *R. oryzae* IFO4697 was used for cloning the lipase gene.

Yeasts were grown in complete YPD medium (1% yeast extract, 2% peptone, 2% glucose) or selective S medium (0.67% yeast nitrogen base supplemented with appropriate amino acids and nucleotides) with 2% glucose as a solo carbon source (SD medium), unless otherwise noted. To prepare plates, 2% agar was added to these media. *E. coli* was grown in LB medium (1% tryptone, 0.5% yeast extract, 1% sodium chloride) containing 100 mg/ml ampicillin.

Plasmids were transformed into *S. cerevisiae* cells using Yeast Maker^{TM} (Clontech Laboratories Inc., Palo Alto, CA, USA), and the transformants were selected on SD-medium plates.

2.2. Construction of two intracellular overexpression systems of ProROL

For intracellular overproduction of rProROL in yeast cells, plasmids pWI3ProROL and pWGP3ProROL constructed in our previous study [8] were used for the constitutive and inducible expression, respectively, of the *ProROL* gene encoding ROL together with the pro-sequence (Fig. 1). *ProROL* gene used in this study was amplified from *R. oryzae* IFO4697 chromosomal DNA by using following two oligonucleotides as primers: ICs (5′–CTCCGGATCCATGGTTCCTGTTTCTGGTAAATCTGGATCT–3′) and ROLrvSalI (5′–CGATGTCGACTTACAAACAGCTTCC–3′). This fragment was digested with *Bam*HI and *Sal*I and inserted into multicopy plasmids pWI3 [12] digested with *Bgl*II and *Sal*I or pWGP3 [10] digested with *Bam*HI and *Sal*I. The resulting plasmids

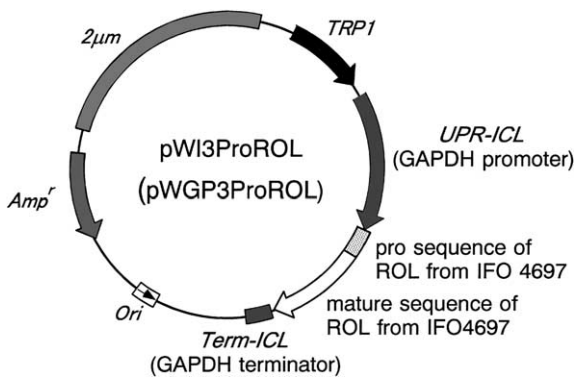

Fig. 1. Construction of plasmids pWI3ProROL and pWGP3ProROL for expression of *ProROL* gene from IFO4697 under the control of *UPR-ICL* and GAPDH promoter, respectively.

were named pWI3ProROL and pWGP3ProROL, respectively.

2.3. Cultivation

Transformants harboring the plasmid for intracellular overproduction of rProROL were precultivated in SD medium at 30 °C for 30 h (OD_{600} > 1.5) and were used as starters to inoculate 150 ml of SDC medium (SD medium containing 2% casamino acids) in 500 ml shaking flasks to give an initial OD_{600} of 0.03. The initial glucose concentration was varied between 0.25 and 2.0%. To enhance the transcription level of *UPR-ICL*, SEC medium (S medium with 1.5 % ethanol and 2% casamino acids) and SAC medium (S medium with 2.67% sodium acetate and 2% casamino acids) were used.

2.4. Measurement of intracellular lipase activity

The hydrolytic activity of lipase in yeast cells was measured with Lipase Kit S (Dainippon Pharmaceutical Co., Osaka, Japan) according to the protocol specified by the supplier and indicated by international units (IU). One IU of lipase activity was defined as the amount of enzyme catalyzing the formation of 1 μmol of 2,3-dimercaptopropan-1-ol from 2,3-dimercaptopropan-1-ol tributyl ester per min. To measure the intracellular lipase activity, intracellular soluble fractions were extracted by the following procedure. Harvested yeast cells were washed twice with 5 mM ethylenediaminetetraacetic acid disodium salt (EDTA) in 50 mM Tris–HCl buffer (pH 8.0) and resuspended in the same buffer with 1 mM phenylmethylsulfonyl fluoride (PMSF), 3 mg/l leupeptine and 3 mg/l pepstatin A to inhibit the activity of certain proteases. This mixture was agitated for 30 s using a vortex mixer at maximum speed with a half volume of glass beads, and then cooled on ice. After 10 cycles of agitation and cooling, the intracellular soluble fraction was obtained as the supernatant by centrifugation at 12,000 rpm at 4 °C for 10 min.

2.5. Western blotting analysis of rProROL

rProROL expressed in yeast cells was analyzed by Western blotting. Intracellular soluble fraction was obtained as described above. Proteins from culture supernatants and cell extracts were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) using a 12.5% gel. The proteins separated on the gel were electroblotted on polyvinylidene difluoride (PVDF) membrane (Millipore Co., Boston, MA, USA) and reacted with primary rabbit anti-ROL IgG antibodies [13] and secondary goat anti-rabbit IgG alkaline phosphatase (AP)-conjugated antibodies (Promega Co., Madison, WI, USA). Then the membrane was stained with nitro-blue tetrazolium chloride (NBT, Promega Co.) and 5-bromo-4-chloro-3-indolylphosphate toluidine salt (BCIP, Promega Co.).

3. Results and discussion

3.1. Construction of two intracellular overexpression systems of rProROL

Based on the finding of Takahashi et al. that rProROL has high hydrolysis activity and higher stability than r28ROL, the recombinant mature lipase with 28 amino acids of the prosequence [13,14], we developed two intracellular overproduction systems of active lipase shown in Fig. 1 to obtain whole cell biocatalysts with high lipase activity [8]. In the plasmids pWI3ProROL and pWGP3ProROL, *ProROL* gene is expressed under the control of *UPR-ICL* and GAPDH promoter, respectively.

3.2. Effects of initial glucose concentration on the intracellular lipase activity

The effect of the initial glucose concentration on rProROL productivity of two intracellular expression systems was investigated. The time courses of intracellular lipase activity and cell density of the *UPR-ICL* system and the GAPDH promoter system in flask cultivation are shown in Figs. 2 and 3, respectively. In all systems and conditions, no lipase activity was detected in the culture supernatant.

In both expression systems, the initial glucose concentration significantly affected the intercellular production of active rProROL. In the inducible expression system with *UPR-ICL*, the intracellular lipase activity of yeast strain MT8-1 harboring pWI3ProROL (MT8-1/pWI3ProROL) increased rapidly as soon as glucose was exhausted (Fig. 2A).

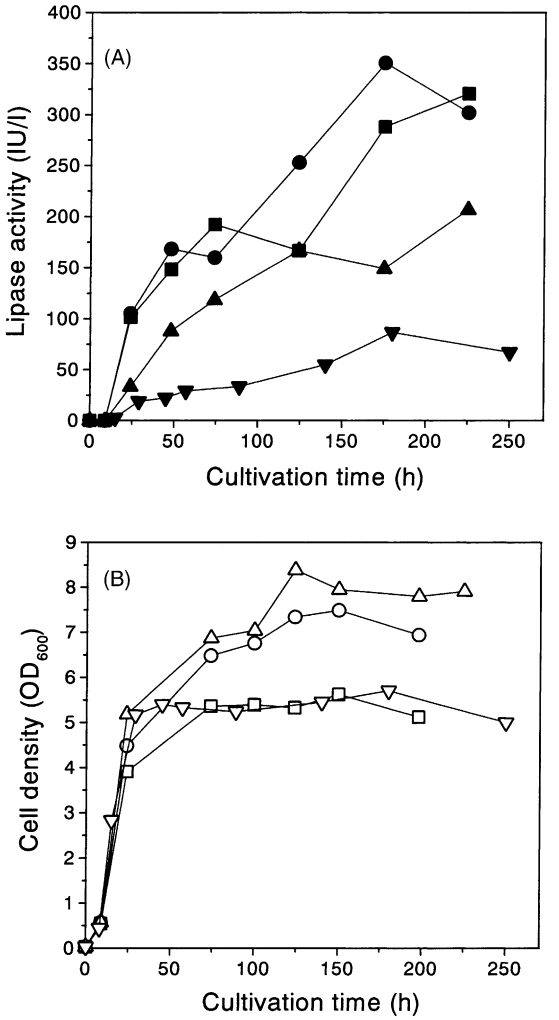

Fig. 2. Effects of initial glucose concentration on intracellular lipase activity (A, filled symbols) and cell density (B, open symbols) of MT8-1/pWI3ProROL. Cultivation was carried out in SDC medium at 30 °C. Symbols: (■ , □) 25% glucose; (●, ○) 0.5% glucose; (▲„ △) 1.0% glucose; (▼ , ▽) 2.0% glucose.

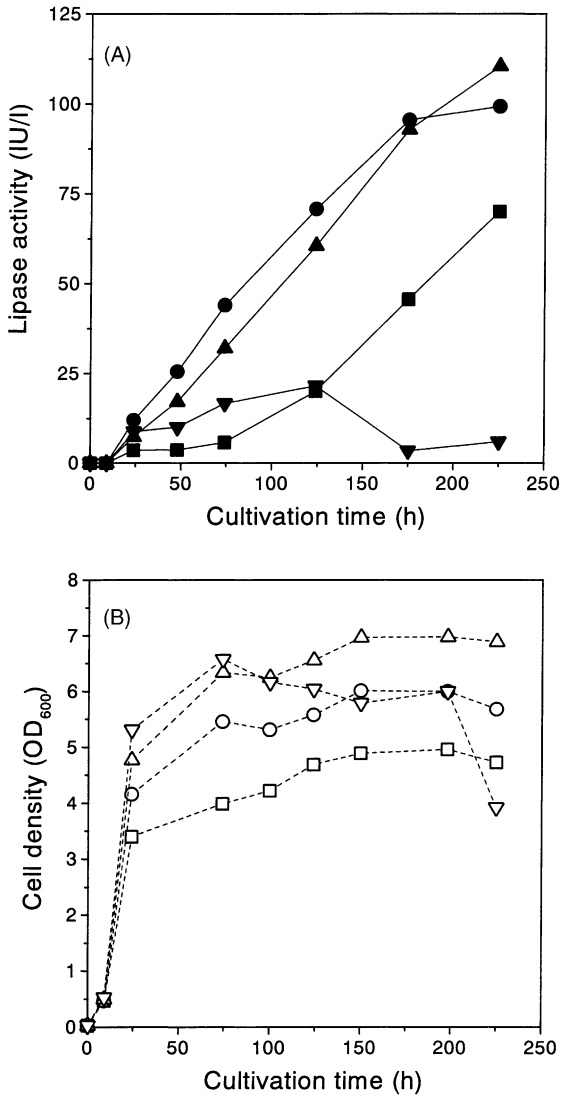

Fig. 3. Effects of initial glucose concentration on intracellular lipase activity (A, filled symbols) and cell density (B, open symbols) of MT8-1/pWGP3ProROL. Cultivation was carried out in SDC medium at 30 °C. The symbols are the same as those in Fig. 2.

At an initial glucose concentration of 0.5%, intracellular lipase activity reached 350.6 IU/l at 175 h. At initial glucose concentrations above 0.5%, however, intracellular lipase activity decreased with increasing initial glucose concentration. Furthermore, growth inhibition was observed at an initial glucose concentration of 2.0% (Fig. 2B). Therefore, the optimum initial glucose concentration was 0.5%. A similar tendency was observed in yeast strain MT8-1 harboring pWGP3ProROL (MT8-1/pWGP3ProROL) (Fig. 3A and B). The optimum initial glucose concentration was 1.0%. However, intracellular lipase activity reached only 110.5 IU/l at 225 h. At an initial glucose concentration of 2.0%, strong inhibition in intracellular lipase production was observed.

On the other hand, in the secretory overproduction of r28ROL under the control of GAPDH promoter,

higher lipase activity (880 IU/l) was obtained in the culture supernatant at 72 h cultivation by using 2.0% glucose [unpublished results]. This difference between intracellular and secretory expression systems might be attributable to the folding problem of rProROL in the intracellular environment. In the intracellular expression system, too high production rate lead to the accumulation of incompletely folded or misfolded rProROL, which is toxic to the cells and easy to degrade by intracellular protease systems.

3.3. Effect of temperature on the intracellular lipase activity

Since expression level has found to significantly affect intracellular productivity of rProROL, cultivation temperature was optimized for the *UPR-ICL* and the GAPDH promoter systems using 0.5 and 1.0% glucose, respectively. Fig. 4A shows the effect of cultivation temperature on intracellular productivity of rProROL in the inducible *UPR-ICL* system. The highest intracellular lipase activity was obtained at 30 °C. On the other hand, as shown in Fig. 4B, the highest intracellular lipase activity of MT8-1/pWGP3ProROL (300.8 IU/l, 142 h) was obtained at 25 °C and was approximately three times higher than that cultivated at 30 °C. Growth inhibition was not observed either in the inducible *UPR-ICL* system or in the constitutive GAPDH promoter system, and the cell density reached up to the same level as that in the control strain MT8-1 harboring pWI3 or pWGP3 (data not shown).Higher cultivation temperature generally increases transcriptional level and hence enhances intracellular productivity of rProROL in the inducible *UPR-ICL* system (Fig. 4A). On the other hand, a high transcription level at a high cultivation temperature inhibited intracellular production of rProROL in the constitutive GAPDH promoter system. Therefore, strong induction in the initial growth phase of yeast cells probably inhibited intracellular production of rProROL.

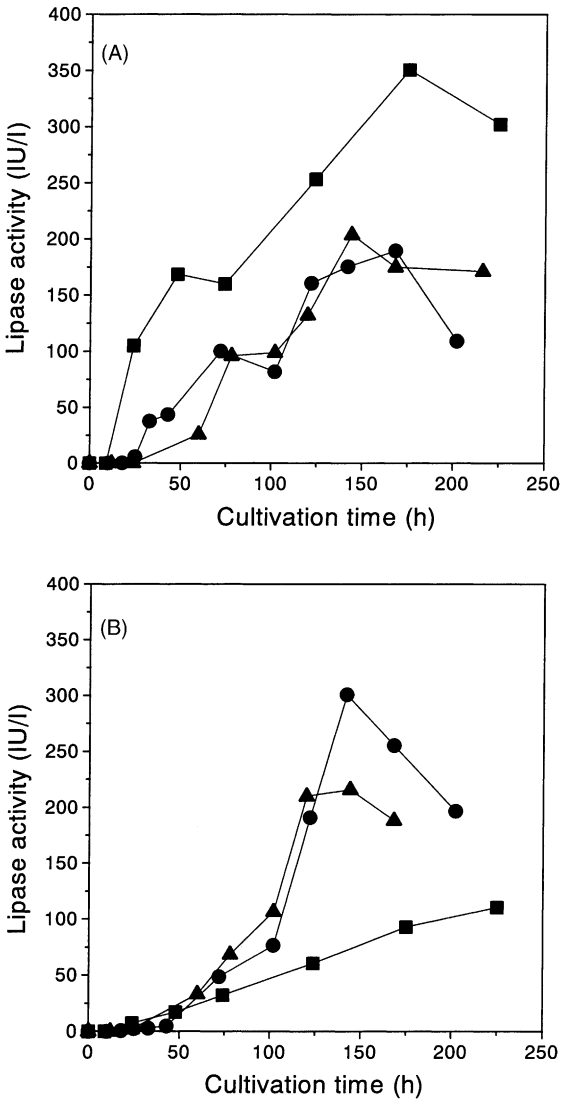

Fig. 4. Effects of cultivation temperature on intracellular lipase activity of MT8-1/pWI3ProROL (A) and MT8-1/pWGP3ProROL (B). The initial glucose concentrations were 0.5% (A) and 1.0% (B), respectively. Symbols: (▲) 20 °C; (●) 25 °C; (■) 30 °C.

3.4. Effect of enhancement of transcription level of UPR-ICL by non-fermentable carbon sources on the intracellular lipase activity

To confirm the consideration described above, induction at initial cultivation phase in the inducible *UPR-ICL* system was carried out using either ethanol or sodium acetate as a carbon source. Presence of these non-fermentable carbon sources and absence of glucose enhances the transcription level under the control of *UPR-ICL* [11,12]. Fig. 5 shows the time courses of intracellular lipase activity of MT8-1/pWI3ProROL. In case of the cultivation using SEC medium with 1.5% ethanol and SAC medium with 2.67% acetate,

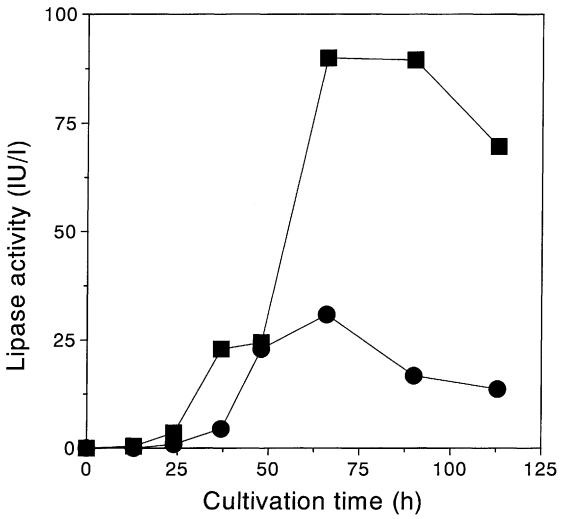

Fig. 5. Effect of non-fermentable carbon sources on intracellular lipase activity of MT8-1/pWI3ProROL. Cultivation was carried out in SEC medium (■) and SAC medium (●) at 30 °C.

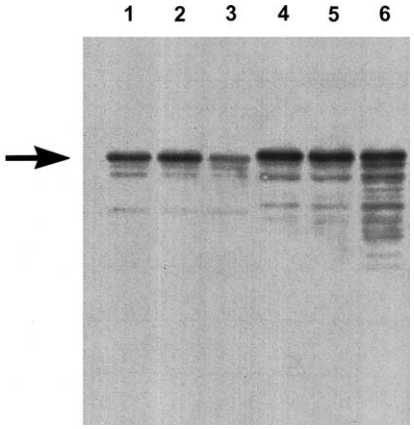

Fig. 6. Comparison of intracellularly produced rProROL by immunoblotting. Lanes 1–3, MT8-1/pWGP3ProROL cultivated with an initial glucose concentration of 1.0% at 20 °C (144 h cultivation), 25 °C (142 h cultivation), and 30 °C (175 h cultivation), respectively. Lanes 4–6, MT8-1/pWI3ProROL7 cultivated with an initial glucose concentration of 0.5% at 20 °C (144 h cultivation), 25 °C (168 h cultivation), and 30 °C (175 h cultivation), respectively. Twenty micrograms protein of soluble cell homogenate prepared from yeast cells cultivated in each condition was applied on each lane. The arrowhead indicates the band corresponding to rProROL.

the intracellularly produced lipase activity was approximately 25% and less than 10% of that produced using SDC medium with 0.5% glucose, respectively. In contrast, it was previously reported that secretory production of β-galactosidase was enhanced over 300-fold by acetate [12]. These results are also consistent with the above observation. That is, in case of rProROL, which is difficult to fold and toxic to cells, strong induction at the initial cultivation phase inhibits intracellular production of rProROL.

3.5. Western blotting analysis of intracellular rProROL

Since the intracellular activity of rProROL was found to depend highly on the systems and cultivation conditions, molecular states and production level of rProROL were analyzed by Western blotting. Fig. 6 shows an immunoblot analysis of proteins prepared from soluble cell homogenates of MT8-1/pWGP3ProROL and MT8-1/pWI3ProROL cultivated in various temperatures at an initial glucose concentration of 1.0 and 0.5%, respectively. Intracellular rProROL with a predicted molecular weight of approximately 46 kDa was found in all soluble cell homogenate fractions. The amounts of whole proteins cross-reacting with anti-ROL antibody was measured with NIH image (Version 1.62) (data not shown). The expression amount of rProROL in the GAPDH promoter system at 20 and 25 °C was larger compared with that at 30 °C (lanes 1–3). On the other hand, the expression amount of proteins cross-reacting with anti-ROL antibody in the *UPR-ICL* system increased with cultivation temperature (lanes 4–6), although the larger amounts of degradation bands of rProROL were observed when cells were cultivated at 30 °C. These results were consistent with the results described in Fig. 4. Since the amounts of whole proteins cross-reacting with anti-ROL antibody correlated well with the lipase activity of the homogenate, degraded rProROL proteins cross-reacted with anti-ROL antibody probably possessed lipase activity.

In conclusion, to prepare the efficient whole cell biocatalyst, both the selection of promoter system and the optimization of cultivation conditions such as temperature, carbon source and its initial concentration are important. In case of rProROL, which is difficult to fold and toxic to cells, expression by the inducible *UPR-ICL* system under the optimum initial glucose

concentration and temperature is effective for intracellular production of rProROL.

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