Cloning and functional expression of a human lysozyme gene (*hly*) from human leukocytes in *Pichia pastoris*

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Abstract. *hly* is a cDNA gene derived from human leukocytes that encodes a mature human lysozyme (abbreviated to hLY). The aim of the present study was to determine the effect of cloned *hly* on recombinant hLY (r-hLY) activity under optimized conditions. *hly* was amplified by RT-PCR and ligated into the pPIC9K plasmid. The cloned cDNA (*hly*) was 393 bp in length, encoding a 130 amino acid hLY with a calculated molecular mass of 14,698 Da. The recombinant expression plasmid, designated as pPIC9K-*hly*, was linearized with *Sac*I and transformed into *Pichia pastoris* GS115 (*his4*, Mut^+) by electroporation. The integration of *hly* into the *P. pastoris* genome was confirmed by PCR analysis using 5'-*AOX1* and 3'-*AOX1* primers. Yeast extract peptone dextrose (YPD) plates containing different concentrations of geneticin (G418) were used for the screening of *P. pastoris* transformants (His^+, Mut^+) with multiple *hly* copies. One transformant resistant to 4.0 mg/ml of G418, designated as *P. pastoris* GShLY4-6, expressing the highest r-hLY activity was selected by the shake-flask test, and used for the optimization of expression conditions. When the *P. pastoris* GShLY4-6 was induced under optimized conditions, the expressed r-hLY activity was up to 533 U/ml, which was 1.52 times as high as that (351 U/ml) expressed using the standard protocol. SDS-PAGE assay demonstrated that the r-hLY with an apparent molecular mass of approximately 14.7 kDa was extracellularly expressed in *P. pastoris*. In conclusion, r-hLY increased following the cloning of *hly* and the optimized conditions as compared to standard protocol.

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Introduction

Human lysozyme (EC 3.2.1.17) is a bacteriolytic enzyme that is widely distributed in a variety of tissues and body fluids (1). It hydrolyzes preferentially the β-1,4 glycosidic linkages between the N-acetylmuramic acid and N-acetylglucosamine residues that occur in the peptidoglycan cell wall structures of certain microorganisms, particularly those of Gram-positive bacteria, and therefore appears to have a role in host defense (2). The hLY comprises 130 amino acid residues and belongs to the group of lysozymes called c-type lysozymes (3). Due to its bacteriolytic activity, it is considered to be useful for medical and food uses. There is a reemergence of uses of lysozyme in various clinical trials. The addition of lysozyme to baby formulas was proposed (4), and lysozyme had also been shown to inhibit the growth of HIV-1 *in vitro*. Of note, the anti-HIV activity of lysozyme is independent of its muramidase activity (5). In addition, findings of previous studies have shown that it is effective in inactivating spores of *Bacillus cereus* in cheese by causing high hydrostatic pressure following the addition of lysozyme (6).

Currently, hLY is mainly extracted from human milk and placenta, which is restricted by many factors, such as the lack of raw materials and high cost of purification. Therefore, the hen egg-white lysozyme is the most readily available form of commercial lysozyme, since it is relatively inexpensive compared to human lysozyme (3). However, the hLY is the body's own protein and has a natural compatibility; therefore, it may exhibit greater safety than other lysozymes in clinical trials (7). Furthermore, the hLY has two and a half times higher antimicrobial activity than the hen egg-white lysozyme (8). Therefore, hLY is likely to be more suitable for medical and food uses than hen egg-white lysozyme. Due to the advances in recombinant DNA technology, hLY has been expressed in various organisms, including rice (9), goats (10), bacteria (11) and yeast (3). However, the yield and activity of the r-hLY remains extremely low, making it difficult for r-hLY to be widely applied. In this work, a cDNA gene (*hly*) encoding the hLY was cloned and extracellularly expressed in *P. pastoris* G115. Most significantly, expression conditions of the transformant were optimized by using a 'one-factor-at-a-time' method. The expressed r-hLY activity under the optimized conditions was much higher than that expressed using the standard protocol.

Materials and methods

Strains and plasmids. *Micrococcus lysodeikticus* WX-2008, used for lysozyme activity assay, was preserved in our laboratory. *E. coli* JM109 and pUCm-T plasmid (Sangon, Shanghai, China) were used as a host-plasmid system for gene cloning and DNA sequencing. *E. coli* DH5α and pPIC9K plasmid (Invitrogen, Grand Island, NY, USA) were used for construction of the recombinant expression plasmid. A cDNA gene (*hly*) encoding the hLY was heterologously expressed in *P. pastoris* GS115 (Invitrogen).

Reagents, media and sample. A DNA marker and a EZ-10 Spin Column DNA Gel Extraction kit were purchased from Sangon. X-Gal, IPTG, T4 DNA ligase, *Taq* DNA polymerase, restriction enzymes, protein marker and the RNA PCR kit were purchased from Takara (Dalian, China). The Yeast Genomic DNA Extraction kit was purchased from Tianwei (Beijing, China). The BCA-200 Protein Assay kit was purchased from Pierce (Rockford, IL, USA). The 10-kDa cut-off membrane was purchased from Millipore (Billerica, MA, USA). Coomassie Brilliant Blue R-250 was purchased from Sigma (St. Louis, MO, USA). All other chemicals were of analytical grade. The preparation of LB, yeast extract peptone dextrose (YPD), MD, buffered minimum glycerol medium (BMGY) and buffered minimum methanol medium (BMMY) was performed according to the Multi-Copy Pichia Expression kit (Invitrogen). Human peripheral blood was provided by Wuxi Red Cross Blood Center (Jiangsu, China).

Total RNA extraction. Fresh human peripheral blood was separated to obtain leukocytes through centrifugation. The leukocytes were washed two times with 0.9% normal saline (pH 7.4). Total RNA was extracted by using an RNA Extraction kit (Sangon) according to the method reported previously (12).

Cloning of a cDNA gene encoding the hLY. Based on the complete DNA sequence of the hLY gene (GenBank accession: NM_000239), a pair of PCR primers was designed. Forward and reverse primers, synthesized by Sangon (China), were *hly*-F: 5'-CGGAATTCAAGGTCTTTGAAAGGTGTG AGTT-3' with an *Eco*RI site (underlined) and *hly*-R: 5'-ATT TGCGGCCGCTTACACTCCACAACCTTGAACAT-3' with a *Not*I site (underlined), respectively.

An Oligo dT-Adaptor primer provided by the RNA PCR Kit (Takara), 5'-GTTTTCCCAGTCACGAC(dT_{18})-3', was used for reverse transcription of the first-strand cDNA from the human leukocyte total RNA, using the following conditions: one cycle at 50˚C for 30 min, 99˚C for 5 min, and 5˚C for 10 min. Using the resulting first-strand cDNA as a template, the first-round PCR amplification was carried out using the primers *hly*-F and M13 Primer M4 (identical to Oligo dT-Adaptor Primer but not Oligo dT alone) under the following conditions: an initial denaturation at 94˚C for 2 min; annealing of 30 cycles at 94˚C for 30 sec, 53˚C for 30 sec, 72˚C for 90 sec; an extra elongation at 72˚C for 10 min. The first-round PCR product was then subject to the second-round PCR amplification using the primers *hly*-F and *hly*-R. The target gene of RT-PCR amplification was purified with the EZ-10 Spin Column DNA Gel Extraction kit and ligated into pUCm-T plasmid. The ligated solution was transformed into *E. coli* JM109 competent cells, followed by blue-white screening, PCR confirmation and DNA sequencing. The recombinant plasmid containing the *hly* gene was designated as pUCm-T-*hly*.

Construction of the recombinant expression plasmid. After the pUCm-T-*hly* was digested with the restriction enzymes *Eco*RI and *Not*I, the released *hly* was agarose gel-purified, and then inserted into pPIC9K plasmid digested with the same enzymes, followed by transformation into *E. coli* DH5α competent cells. The recombinant plasmid, designated as pPIC9K-*hly*, was confirmed by restriction enzyme analysis and DNA sequencing.

Transformation and r-hLY expression. The pPIC9K-*hly* was linearized with *Sac*I, and transformed into *P. pastoris* GS115 by eletroporation using a Gene Pulser apparatus (Bio-Rad, Hercules, CA, USA) according to the manufacturer's instructions. The screening of geneticin G418-resistant *P. pastoris* transformants was first carried out on an MD plate, and then on YPD plates containing geneticin G418 at increasing concentrations of 1.0, 2.0 and 4.0 mg/ml. The integration of *hly* into the genome of *P. pastoris* GS115 was confirmed by PCR analysis using primers 5'-*AOX1* and 3'-*AOX1*, and yeast genomic DNA as a template extracted by the Yeast Genomic DNA Extraction kit. Expression of *hly* in *P. pastoris* GS115 was performed according to the instructions of the Multi-Copy Pichia Expression kit (Invitrogen). Each single colony of transformants was inoculated in 30 ml of BMGY medium in a 250 ml conical flask, and grown at 30˚C on a rotary incubator (220 rpm) until the OD_{600} reached 2-4. The cells were then harvested by centrifugation at 3,000 rpm and suspended in 30 ml of BMMY medium in a 250 ml conical flask. The r-hLY expression was then induced at 30˚C for 96 h by adding methanol to a final concentration of 0.5% at 24 h intervals. One transformant, termed *P. pastoris* GShLY4-6, expressing the highest r-hLY activity was selected for the subsequent studies.

r-hLY activity assay. The r-hLY assay was carried out using a method adapted from Morsky (13). An enzyme solution of 300 μl was mixed with 2.7 ml *Micrococcus lysodeikticus* WX-2008 cell suspension (OD_{450} approximately 0.7) in 50 mM phosphate buffer, pH 6.2. A decrease in absorbance at 450 nm of the mixture caused by the lysis of the bacterial cells was monitored at room temperature. One unit (U) was defined as the activity reducing absorbance by 0.001 OD_{450} per minute.

Protein assay. Protein concentration was determined by using the BCA-200 Protein Assay kit, with bovine serum albumin as the standard. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed on a 12.5% gel according to a standard protocol (14), and isolated proteins were visualized by staining with Coomassie Brilliant Blue R-250.

Optimization of conditions for r-hLY expression. In this study, various parameters, such as initial pH value, methanol addition, and induction temperature and period for r-hLY expression were optimized, respectively, by maintaining all

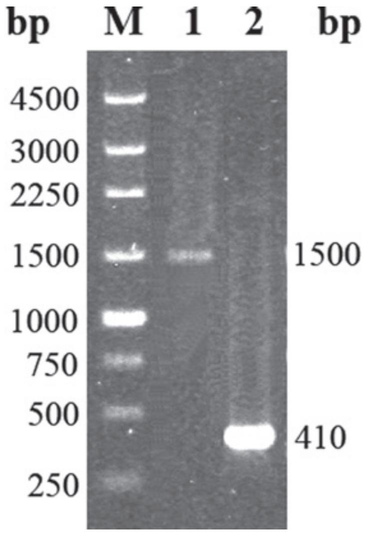

Figure 1. Cloning of a cDNA gene (*hly*) encoding the hLY from human leukocytes. Lane M, DNA marker; lane 1, the first-round PCR amplification products using primers *hly*-F and M13 Primer M4; lane 2, the second-round PCR amplification product using primers *hly*-F and *hly*-R.

Table I. Screening of multi-copies of integration of the gene *hly* into the *P. pastoris* genome and its expression in *P. pastoris* GS115.

G418 (mg/ml)	Representative strains	r-hLY activity (U/ml)[a]
1.0	GShLY1-2	236±5.1
	GShLY1-5	258±6.5
	GShLY1-7	218±4.8
2.0	GshLY2-1	266±5.2
	GshLY2-3	280±4.9
	GShLY2-6	275±5.5
4.0	GShLY4-3	330±6.9
	GShLY4-5	325±7.4
	GShLY4-6	351±6.2

[a]Data are the means ± SD from three independent experiments.

other parameters at constant levels, with the exception of the one being studied. For example, to obtain the highest r-hLY activity towards initial pH, the *P. pastoris* transformant of GShLY4-6 was cultured in BMMY media with different initial pH values.

Purification of the r-hLY. After the GShLY4-6 strain was induced under the optimized conditions, the cultured broth was centrifuged at 10,000 rpm for 10 min to remove yeast cells. The resulting supernatant (30 ml) was brought to 65% saturation by adding solid ammonium sulfate and left overnight. The resulting precipitate was collected by centrifugation at 10,000 rpm, dissolved in 3.0 ml of 20 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7.4), and then dialyzed against the same buffer overnight. The dialyzed solution was concentrated to 1.0 ml by ultrafiltration at 8,000 rpm using a 10-kDa cut-off membrane, applied onto a Sephadex G-50 column (ϕ1.6x80 cm), and eluted with the same buffer at a flow rate of 0.4 ml/min. Aliquots of 2.0-ml eluent containing the r-hLY activity were pooled and concentrated by ultrafiltration. All purification procedures were performed at 4˚C.

Statistical analysis. Data of the enzyme activity of the r-hLY were shown as the means ± standard deviation (SD) from three independent experiments or parallel measurements. Statistical comparison was made using Student's t-test. The level of statistical significance was defined as $P<0.05$ or $P<0.01$.

Results

Cloning of cDNA encoding the hLY. Analytical results of the total RNA isolated from the human leukocyte showed that the ratio of OD_{260} to OD_{280} was 1.95, and that the 18S rRNA and 28S rRNA bands, characterized as eukaryotes, on formaldehyde denatured agarose gel electrophoresis were specific (data not shown), indicating that the total RNA has high purity and is not decomposed.

The reversely transcribed first-strand of cDNA was used as a template for a first round of PCR using the primers *hly*-F and M13 Primer M4. As a result, an approximate 1.5-kb cDNA fragment was amplified by PCR (Fig. 1, lane 1). Based on the principle of the nested PCR, the resulting PCR product was subjected to a second round of PCR with the primers *hly*-F and *hly*-R. A 410-bp cDNA fragment (containing *Eco*RI and *Not*I sites) was therefore amplified by PCR (Fig. 1, lane 2). DNA sequencing results verified that the cDNA fragment inserted either in the pUCm-T-*hly* or in the pPIC9K-*hly* was identical to the one reported previously (GenBank accession: NM_000239).

Screening and expression of the transformants. *P. pastoris* transformants that are capable of resisting a higher concentration of geneticin G418 may have multi-copies of integration of the heterologous gene into the *P. pastoris* GS115 genome, which could lead to a higher expression level of the heterologous protein (15). However, the expression level was not directly proportional to the concentration of geneticin G418 or the copy number of heterologous gene integration, as explained in the manual of the Multi-Copy Pichia Expression kit (Invitrogen). Therefore, we selected 10 transformants resistant to 1.0, 2.0 and 4.0 mg/ml of geneticin G418, designated as *P. pastoris* GShLY1-1 to GShLY1-10, GShLY2-1 to GShLY2-10 and GShLY4-1 to GShLY4-10, for shake flask tests using the standard protocol (Invitrogen). *P. pastoris* GS115 transformed with pPIC9K plasmid was used as a negative control (designated as *P. pastoris* GSC). Following 96 h of induction, the cultured supernatants of the transformants were collected by centrifugation and used for the r-hLY activity assay (Table I). From the transformants tested, one transformant expressing the highest r-hLY activity (351 U/ml), termed *P. pastoris* GShLY4-6, was selected and used for subsequent studies. No r-hLY activity was detected in the cultured supernatant of the negative control under the same expression conditions.

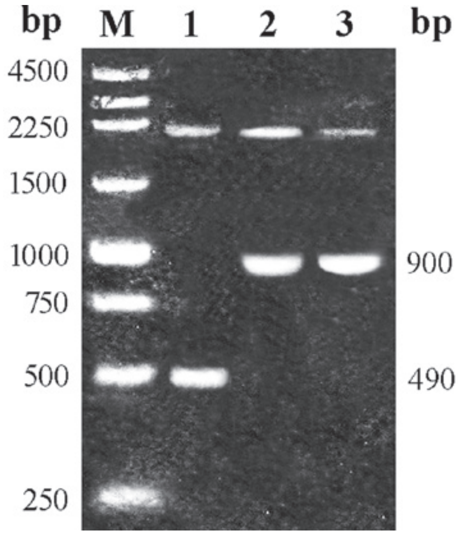

Figure 2. Verification of three representative *P. pastoris* transformants, GSC, GShLY1-2 and GShLY4-6, by PCR analysis using the primers 5'-*AOX1* and 3'-*AOX1*. Lane M, DNA marker; lane 1, PCR products of the GSC genome; lane 2-3, PCR products of the GShLY1-2 and GShLY4-6 genomes, respectively.

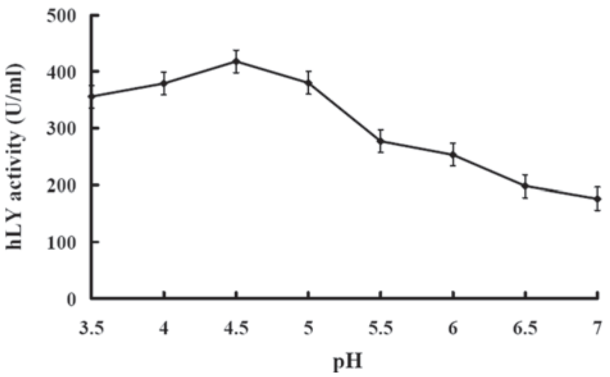

Figure 3. Effect of the initial pH value on the r-hLY expression. The GShLY4-6 was induced in BMMY media with different pH values (3.5-7.0), respectively, at 30˚C for 96 h by adding methanol to a final concentration of 0.5% at 24 h intervals. The maximum r-hLY activity reached 418 U/ml at pH 4.5, and decreased gradually on both sides of this point.

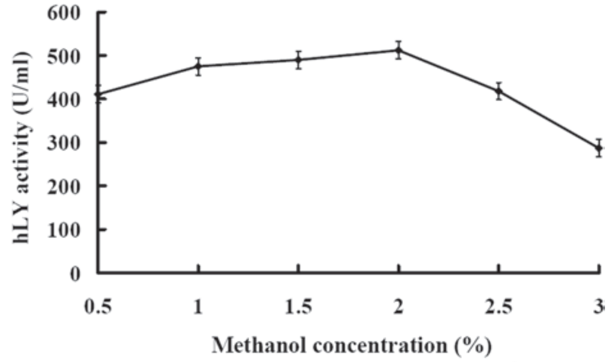

Figure 4. Effect of methanol concentration on the r-hLY expression. In BMMY medium with initial pH 4.5, the GShLY4-6 was induced at 30˚C for 96 h by adding different concentrations of methanol at 24 h intervals. The highest r-hLY activity reached 512 U/ml with 2.0% methanol.

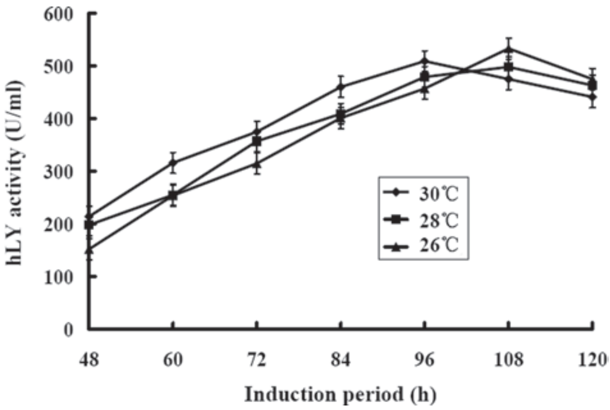

Figure 5. Effects of the induction temperature and period on the r-hLY expression. GShLY4-6 was induced in BMMY with initial pH 4.5 and by adding methanol to a final concentration of 2.0% at 24 h intervals for 120 h at three temperatures of 26, 28 and 30˚C, respectively. The maximum r-hLY activity increased to 533 U/ml at 26˚C for 108 h.

Verification of P. pastoris transformants. To verify whether the *hly* was integrated into the *P. pastoris* GS115 genome, the genomic DNAs of three representative *P. pastoris* transformants, GSC, GShLY1-2 and GShLY4-6, were extracted as templates and then analyzed by PCR amplification, respectively, using the primers 5'-*AOX1* and 3'-*AOX1*. A 2.2-kb complete alcohol oxidase 1 (AOX1) gene in *P. pastoris* GS115 and a 490-bp AOX1 gene fragment on pPIC9K plasmid were amplified by PCR from *P. pastoris* GSC (Fig. 2, lane 1). However, a 2.2-kb complete AOX1 gene and a 900-bp DNA fragment consisting of a 490-bp AOX1 gene fragment and a 410-bp cDNA gene (*hly*) were amplified by PCR from *P. pastoris* GShLY1-2 or GShLY4-6 (Fig. 2, lane 2 or 3). These results demonstrated that in positive transformants the *hly* encoding the hLY was successfully integrated into the *P. pastoris* GS115 genome.

Optimization of conditions for the r-hLY expression. The possibility of increasing the protein expression level of the *P. pastoris* transformant has been explored (16). Optimization of induction conditions is one of the methods that is capable of enhancing the expression levels of recombinant proteins (17,18).

The effect of the initial pH value on r-hLY expression was assessed by cultivating the *P. pastoris* GShLY4-6 in BMMY media with different pH values (3.5-7.0), respectively, induced at 30˚C for 96 h (220 rpm) by adding methanol to a final concentration of 0.5% at 24 h intervals. Maximum r-hLY activity increased to 418 U/ml at pH 4.5, and decreased gradually on both sides of this point (Fig. 3).

In BMMY with an initial pH of 4.5, the GShLY4-6 strain was induced at 30˚C for 96 h (220 rpm) by adding different concentrations of methanol. The highest r-hLY activity achieved with 2.0% methanol was 512 U/ml (Fig. 4). During the induction expression of the transformants, the added methanol concentrations ranged generally from 0.5 to 3.0%, and a high methanol concentration was toxic to yeast, whereas, extremely low concentrations failed to induce protein expression effectively (19).

The GShLY4-6 strain was induced in BMMY with an initial pH of 4.5 and by adding methanol to a final concentra-

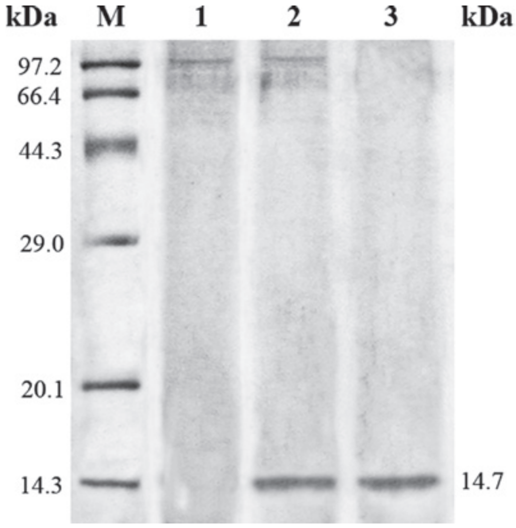

Figure 6. SDS-PAGE analysis of the cultured supernatants of the *P. pastoris* transformants (GSC and GShLY4-6) and the purified r-hLY. Lane M, protein marker; lane 1, cultured supernatant of the GSC; lane 2, cultured supernatant of the GShLY4-6; lane 3, the r-hLY purified by a combination of ammonium sulfate fraction, ultrafiltration and Sephadex G-50 gel filtration.

tion of 2.0% at 24 h intervals for 120 h (220 rpm) at three temperatures (Fig. 5). The results indicated that the highest r-hLY activity achieved at 26˚C for 108 h was 533 U/ml. The temperature for the protein expression in *P. pastoris* was generally at the range of 28-30˚C, whereas our study showed that the highest r-hLY expression level in the *P. pastoris* GShLY4-6 was at 26˚C.

Purification and SDS-PAGE assay of the r-hLY. In this study, the purity of the expressed r-hLY in the cultured supernatant of *P. pastoris* GShLY4-6 was >85% (Fig. 6, lane 2), which greatly facilitated the purification of the r-hLY and decreased the industrial production and application costs. Therefore, the expressed r-hLY may be purified to homogeneity only by a simple combination of ammonium sulfate fraction, ultrafiltration and Sephadex G-50 gel filtration. The specific enzyme activity of the purified r-hLY was 3118 U/mg. SDS-PAGE analysis of the purified r-hLY showed a single protein band with an apparent molecular mass of approximately 14.7 kDa (Fig. 6, lane 3), which was similar to the calculated molecular mass (14,698 Da) of the hLY.

Discussion

Lysozyme is one of the best characterized enzymes and has been used as an anti-inflammatory agent and a bactericide (20); however, its applications are restricted due to the lack of sufficient resources. Production of hLY has been attempted in various organisms, such as *Saccharomyces cerevisiae* (21), *Aspergillus oryzae* (22) and *Kluyveromyces lactis* (3). However, it remains difficult to meet these application needs.

In pharmaceutical research, *P. pastoris* has been used to successfully express human serum albumin (23), human interferon α2b (24) and hepatitis B virus surface antigens (25) on a large scale. Currently, heterologous expression is the main tool for the production of protein drugs, and *P. pastoris* is one of the attractive expression hosts due to the numerous advantages over other hosts. In this study, a cDNA gene (*hly*) encoding the hLY was cloned and extracellularly expressed in *P. pastoris* G115. Most significantly, the expression conditions were optimized by using a 'one-factor-at-a-time' method. The expressed r-hLY activity under the optimized conditions increased to 533 U/ml, which was higher than the activity expressed with a standard protocol.

Yeast is a useful host for the extracellular production of recombinant gene products, and *P. pastoris* could express heterologous protein efficiently at a broad pH range of 3.0-8.0 (26) and by adding methanol to a final concentration of 0.5-3% at 24 h intervals (27). The potential for enhancing the protein expression level of the *P. pastoris* transformant (16) by controlling the physical and chemical parameters, such as pH value, methanol addition, induced temperature and time period remained to be determined. Therefore, in this study, the production of r-hLY was investigated using shake flasks under different culture conditions, which demonstrated that acidic conditions were able to enhance r-hLY production. Findings of this study may provide more insights to investigators regarding the manner in which various cultivation conditions affect r-hLY production in *P. pastoris* GS115.

In conclusion, in this study, the cDNA gene (*hly*) was cloned and the expression conditions of *P. pastoris* GShLY4-6 were optimized to increase r-hLY activity. Moreover, purification of the expressed r-hLY was also performed. Future studies should therefore determine how to best realize an industrial scale production of r-hLY with high enzyme activity in a cost-effective manner.

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