

Full Length Research Paper

A novel protein expression system-PichiaPink™- and a protocol for fast and efficient recombinant protein expression

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Pichia pastoris is a eukaryote and has many of the advantages of higher eukaryotic expression systems, such as protein processing, protein folding, and the availability of posttranslational modifications. It is as easy to manipulate as *Escherichia coli* or *Saccharomyces cerevisiae*. However, some serious and unavoidable problems occur with the *Pichia pastoris* system that is difficult to overcome, such as low transforming efficiency and degradation of protein. Recently, a new protein expression system named PichiaPink™ was introduced. We have used the pichia-based system to express some proteins successfully, such as human serum albumin, *Ganoderma lucidum* immunoregulatory protein (Lz-8), and found that the system can improve the transformation of the *Pichia* stain, the low yields and the degradation of the protein of interest. In the example of human serum albumin (HSA), the results indicated two facts: (1) the protease knockout PichiaPink™ strains are quite helpful in decreasing the degradation of HSA without affecting the yield of HSA or the strain growth, and the high-copy expression vector worked better than the low-copy expression vector in terms of the HSA yield. The example of rLz-8 showed that compared with the traditional *Pichia* strain Gs115, the new system provided an easier selection method for screening correct and higher level of expression transformants.

Key words: *Pichia pastoris*, protease knockout strain, ADE.

INTRODUCTION

Over the last several decades, researchers have learned how to manipulate DNA to identify, move and place genes into a variety of organisms that are quite different genetically from the source organism (Miyamoto et al., 1985). A major use for many of these recombinant organisms is to produce proteins. As a result many proteins have immense commercial value, numerous studies have focused on finding ways to produce them inexpensively, easily and in a fully functional form (Carbonell et al., 1985). The production of a functional protein is related to the cellular machinery of the organism producing the protein. *Escherichia coli* has been the "factory" of choice for the expression of many

proteins, but it is a prokaryote and lacks intracellular organelles, such as the endoplasmic reticulum and the golgi apparatus, that are present in eukaryotes. These structures are responsible for modifying the proteins being produced. Many eukaryotic proteins can be produced in *E. coli*, but they are produced in a non-functional form because glycosylation or posttranslational modifications do not occur. Therefore, researchers have recently turned to eukaryotic yeast and mammalian expression systems for protein production (Miyamoto et al., 1985; Carbonell et al., 1985).

Compared with other eukaryotic expression systems, *Pichia pastoris*, offers many advantages because it does not have the endotoxin problem associated with bacteria or the viral contamination problem of proteins produced in animal cell culture. Furthermore, *P. pastoris* can utilize methanol as a carbon source in the absence of glucose. The *P. pastoris* expression system uses the methanol-

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induced alcohol oxidase (AOX1) promoter, which controls the gene that codes for the expression of alcohol oxidase, the enzyme that catalyzes the first step in the metabolism of methanol (Vassileva et al., 2001; Vassileva et al., 2001).

However, after over 20 years of use, some limitations and disadvantages of the *P. pastoris* system have been gradually uncovered. These are summarized as follows: (1) The expression vectors that are designed to integrate into the Pichia chromosome enable stable expression (Cregg and Russell, 1998), but the transformation efficiencies are very low (Brzobohaty and Kovac, 1986; Gietz and Woods, 2001; Kobayashi et al., 2000; Suga and Hatakeyama, 2001; Thompson et al., 1998). Unlike other yeast systems, the vector of *P. pastoris* must both enter the cell and integrate into specific locations in the host chromosome, an inherently inefficient process (Vassileva et al., 2001). (2) Proteases are known to be secreted into the medium during Pichia fermentation, which can result in the degradation of the desired protein product (Kobayashi et al., 2000). (3) After transformation of the *P. pastoris* strain Gs115 by the gene of interest, most of the transformants that were picked from the G418-resistant plate had lower levels of expression than 20 mg/L. Hence, it is very difficult to screen engineering strain for the production of protein (especially as some new proteins have no corresponding antibody). (4) AOX1 and AOX2 are two genes in *P. pastoris* that code for alcohol oxidase. The majority of the alcohol oxidase activity in the cell is attributable to the AOX1 gene, but when researchers pick colonies, some transformants with AOX2 may be chosen. These transformants are hard to effectively induce with methanol (Ohi et al., 1994).

In order to overcome the disadvantages of existing *P. pastoris*-based protein expression systems, a new *P. pastoris* system called PichiaPink™ is reported. It supplies a new and efficient way to screen high-expression transformants. The strains in the PichiaPink™ system are ADE2 auxotrophs that are unable to grow in the absence of adenine because of full deletion of the ADE2 gene and part of its promoter. The ADE2 gene encodes phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step in the de novo biosynthesis of purine nucleotides (Jones and Fink, 1982). The expression plasmids included in the kit contain the ADE2 gene (under its own promoter) as the selection marker (Cregg and Russell, 1998). Transformation of the PichiaPink™ strains with the expression plasmids enable the strain to grow on medium lacking adenine (ADE dropout medium or minimal medium). The pink colonies express very little ADE2 gene product, while the white colonies express higher amounts of the ADE2 gene product, suggesting that those colonies have more copies of the integrated construct. Also, to help reduce the impact of proteases and the need for heavy protease inhibitor use, the PichiaPink™ system offers three protease knockout strains, including *pep4* knockout, which prevents it from synthesizing protease A, *prb1* knockout,

which prevents it from synthesizing protease B, and double knockout. Furthermore, to avoid adverse influence of AOX2 promoter, in the expression plasmids of this system, only AOX1 promoter is used to drive expression of the gene of interest encoding the desired heterologous protein.

Here, we intended to express Human serum albumin (HSA, MW: 66 kDa) and Ganoderma lucidum immune regulatory protein using *P. pastoris*-based PichiaPink™ system, and observed transformation efficiencies, yield of recombinant proteins and anti-degradation ability. As a well-known protein, HSA has been expressed in *P. pastoris* Gs115 by many research groups over time. It can most easily and clearly illustrate some of the details of and usage tips for the PichiaPink™ system.

LZ-8, another example in this work, comes from a medicinal fungus (*Ganoderma lucidum*). Zhao et al. (2008) and our team (Liang et al., 2009) both use the traditional *P. pastoris* system (Gs115 strain) to complete the recombinant expression of LZ-8, but after transformation in the first round of selecting high-expressing strains, the yields of recombinant LZ-8 (rLZ-8) are not more than 20 mg/L without optimizing the parameters of induction. Low yield of recombinant protein is a problem that many researchers confront using the *P. pastoris* Gs115 strain. In most cases, it is not possible to find a commercial antibody for recombinant protein, so the SDS-PAGE assay is a frequently used method to determine and screen for the correct transformants, but a low concentration of the protein of interest and the natural protein from yeast both make identifying a positive-expressing yeast strain difficult.

Recombinant expression was conducted of the same rLZ-8 gene sequence using the PichiaPink™ system. The yields of all transformants of rLZ-8 were greatly improved, and screening for the correct transformants was easier than ever before.

MATERIALS AND METHODS

Reagent, strains, plasmid and gene of interest

DNA restriction and T4 DNA ligase enzymes were purchased from the TaKaRa Biotech Corporation. The PichiaPink™ system that was purchased from the Invitrogen Corporation included PichiaPink™ strains (1 to 4), pPink-LC vector, pPink- α -HC vector, pPink-HC vector, and signal sequence. The plasmid extraction kit and the PCR production and yeast genome extraction kit were all from Promega. The sorbitol and D-glucose were from Sigma Aldrich Corporation. All other reagents were made in China and were of analytical grade. The HSA ELISA kit was produced by Cygnus Technologies, Inc.

The total DNA of *G. lucidum* was extracted as described by Al-Samarrai and Schmid (Al-Samarrai and Schmid, 2000). The LZ-8 gene was amplified from the total DNA sample by PCR, and Stu I and Kpn I restriction sites were designed for flanking the PCR product at the 5'- and 3'-terminus, respectively, using Primer 1 (TATAGGCCTTCCGACACTGC) and Primer (CGGGGTACCTTACTAGTCCACT) 2. The HSA gene was obtained from the total RNA by RT-PCR amplification (Brzobohaty and Kovac 1986;

Table 1. The details of the ligation reaction in a 0.5 ml tube.

| Component | Amount |
|---|-------------------------|
| 5× ligase buffer | 2 µL |
| T4 DNA ligase | 0.5 µL |
| pPink-HC or pPink-LC (approximately 4 fmol) | 1 µL (at 10 – 25 ng/µL) |
| HSA or LZ-8 sequence (approximately 10-20 fmol) | 1 µL (at 10 – 25 ng/µL) |
| Secretion signal sequence (1× 0.1 pmol) | 1 µL (at 10 – 25 ng/µL) |
| Sterile water | To 10 µL |

Table 2. The details of a ligation reaction in a 0.5 mL tube.

| Component | Amount |
|---|-------------------------|
| 5× ligase buffer | 2 µL |
| T4 DNA ligase | 0.5 µL |
| pPink-HC or pPink-LC (approximately 4 fmol) | 1 µL (at 10 – 25 ng/µL) |
| HSA sequence (approximately 10 – 20 fmol) | 1 µL (at 10 – 25 ng/µL) |
| Sterile water | To 10 µL |

Kobayashi et al., 2000; Ohi et al., 1994). Mly I and Kpn I restriction were also added to the 5'- and 3'-terminus using Primer 3 (GTATTGAGTCCTGACATGAAGTGGGT) and Primer 4 (CGGGGT ACCTTATTATAAGCCTAAG).

Choice secretion signal sequences for the gene of interest

The serum albumin signal sequence from *Homo sapiens* and *S. cerevisiae* α -mating factor pre-sequence were selected for secreted expression of HSA and rLZ-8. In addition, in order to compare the expression efficiency of different signal sequences for the same protein, we used the two signal sequences mentioned above in the expression of HSA.

Construction of the expression vector

Construction of pPink-HC-HSA and pPink-LC-HSA

Cloning of the HSA sequence and its secretion signal sequences into pPink-HC vector is a three-way ligation. First, pPink-HC was digested with EcoR I and Kpn I, which created compatible ends to the 5'EcoR I end of the signal sequence and the 3'end of the insert containing the HSA gene. In this procedure, it is very important to add two units of calf intestinal alkaline phosphatase (CIAP) to the reaction mix and to incubate for 1 h at 37°C. CIAP can prevent self-ligation of the vector. If this is not done, a large number of colonies with self-ligation will appear on the plate used for the transformation. Any colonies of interest will not be identifiable. This is the key procedure for the construction of a vector.

Then serum albumin signal sequence from *H. sapiens* was digested with EcoR I and the HSA gene sequence were digested with Mly I and Kpn I. The AxyPrep™ PCR Cleanup Kit was used to purify and condense the sequences.

The details of the ligation reaction in a 0.5 ml microcentrifuge tube are described in Table 1. To conduct the reaction, it was mix gently, centrifuge briefly, and incubate at 25°C for 1 to 2 h or at 16°C overnight.

The ligated product was used to transform the *E. coli* DH5 α

competent cells, and the transformed cells were plated on LB media with ampicillin. There was no blue/white screening available to detect the presence of the insert with PichiaPick™ vectors. The correct transformants were checked by restriction endonuclease digestion and agarose gel electrophoresis. Finally, the recombinant vector was obtained, which was later confirmed by sequencing with 5' AOX primers.

Construction of pPink α -HC-HSA, pPink α -HC-LZ-8

First, the pPink α -HC vector was digested with Stu I and Kpn I. Adding CIAP to the ligation reaction is also important. The other details of the ligation system are given in Table 2.

Transformation of *P. pastoris* and induced expression of recombinant proteins

After extraction, the recombinant vector pPink was linearized by PME I. It was found that for optimum transformation efficiency, > 1 µg of purified linearized DNA fragments (containing HSA, LZ-8 gene) were used that were transformed into PichiaPink™ strains. In this step, how the yeast cells are cultured in order to generate electrocompetent PichiaPink™ strains will determine whether transformation is successful. An easier method was provided for the step, as following: Day 1, 8:00 am: Streak each strain from the working glycerol stock with 10 µl to 10 ml of YPD media in a 100 to 125 ml flask. Incubate with shaking at 300 rpm for 5 h. This is the starter culture. Day 1, 1:00 pm: Use the starter culture to inoculate 100 ml of YPD media in a sterile 1 l flask and shake it at 300 rpm for 22 h. Day 2, 11:00 to 12:00 a.m.: The OD₆₀₀ of the culture will reach 1.3 to 1.5 (log-phase growth). It is very important that the intensity of the yeast cell reaches the optimum range. If your strain's growth speed seriously deviates from the above schedule, repeat the steps again.

Competent cells were selected by electroporation using a gene pulsar JY2000-1B (Ningbo, China) at 1.5 kV cm⁻¹, a capacitance of 25 IF and a resistance of 200 X. PAD selection media was used to check the yeast transformants. Spread 300 µL of the cell mixture on

PAD selection plates, and incubate at 28°C for 3 to 8 days, until distinct colonies are formed. Pick the large white colonies from each plate and restreak onto fresh PAD selection plates. The pink colonies cannot grow any larger. After spreading for 4 to 5 days, they will stop growing totally. Then, the genomic DNA of the yeast transformants is extracted, and PCR with primers for the gene of interest (HSA, LZ-8) is conducted to examine the correct transformants.

Analyzing protein expression by SDS-PAGE and ELISA assay

The correct recombinant yeasts were cultured in BMGY (10 ml/100 ml flask) and expression was induced in BMMY (50 ml/500 ml flask) with methanol (1%, v/v) for 4 days. After centrifugation, the supernatant was carefully collected and later dialyzed against dH₂O (pH 8.0). Ten microliters of the induced supernatant of recombinant LZ-8 was applied to SDS-PAGE for testing. The ELISA assay for detecting HSA was performed according to the manufacturer's (Cygnum Technologies, Inc.) instructions.

Preparation of monoantibody

For the preparation of mAbs, 6 weeks old BALB/c mice were immunized with the purified 3.5 mg rLz-8 mixed with Freund incomplete adjuvant (Sigma, St. Louis, USA) intraperitoneally and given booster injections (3.5 mg) after 3 weeks. Four days after a second intravenous booster dose, the spleen was removed and the cells were fused with Sp2 mouse myeloma cells. The culture supernatant from the growing cells was examined for the presence of antibodies to rLz-8. The colonies producing anti-rLz-8 antibodies were cloned twice at a density of 0.3 per well by limiting dilution. The cloned cells were injected into the mouse belly cavity and ascetic fluids with anti-rLz-8 mAbs were generated. The culture supernatant from the cloned cells was used to characterize the mAbs. The mAbs were purified using protein A sepharose as usual.

The titers of mAbs were examined using an indirect enzyme-linked immunosorbent assay (ELISA). The wells of polystyrene microtiter plates were coated with purified rLz-8 protein. After incubation overnight at 4°C, the wells were washed three times with PBST buffer (0.1% Tween-20 in PBS). The coated wells were blocked with 200 ml of 1% BSA for 1 h at 37°C and then washed as previously described.

RESULTS AND DISCUSSION

Construction of pPink™ expression vector

The HSA gene (1755 bps) and the rLz-8 gene (330 bps) were amplified by PCR. Their PCR product was then introduced into a pPink™ expression vector through an enzyme site and the recombinant vector was later confirmed by sequencing with 5'AOX1 primers. The sequencing result showed that the nucleic acid sequence was the same as HSA and LZ-8. The schematic diagrams of the recombinant expression vector pPink™ are shown in Figure 1.

Transforming and screening

In the transformation of expressing recombinant Pichia-Pink™ strains, cell mixtures are spread on PAD selection plates. After 3 to 8 days, approximately 100 distinct colonies are formed. The color of the colonies indicates

the relative expression levels of the protein of interest. The pink colonies express very little ADE2 gene product, while the white colonies express higher amounts of the ADE2 gene product, suggesting that those colonies have more copies of the integrated construct.

The pink colonies could not grow larger than 2 mm in diameter, while the white ones did not stop growing as the pink ones did. After transformation, approximately 8 to 10 days, their diameters become greater than 4 mm. The strains needed are large, white colonies. As shown in Figures 2A and B, we displayed 2 PAD plates on which pink and white colonies grew after transforming for 7 days.

The result of rLz-8 expression indicate an important fact that easy selection of expression clones using ADE2 complementation rather than antibiotic resistance. One of the main hurdles associated with recombinant expression of protein in traditional Pichia system is always the hard-to-obtain correct transformants. Direct selection of G418 resistance in yeast does not work well because newly transformed cells need time to express sufficient amounts of the resistance factor. Since yeast grow much more slowly than bacteria, significant numbers of recombinant yeast are killed before they accumulate enough of the resistance factor to survive direct plating on antibiotic.

Apparently, pichia based expression system need a more efficient and workable selection of expression clones. The ADE2 gene encodes phosphoribosylaminoimidazole carboxylase, which catalyzes the sixth step in the de novo biosynthesis of purine nucleotides. Expression can also be slightly enhanced under general amino-acid starvation conditions. Although not experimentally shown for *P. pastoris*, ADE2 expression is thought to be regulated in a similar manner. In *S. cerevisiae*, *P. pastoris* and other yeast strains, mutations in ADE2 lead to the accumulation of purine precursors in the vacuole, which causes the colony to be red in color. The pigmentation phenotype can be used as a tool for selection and screening. In addition, *ade2* mutants are adenine auxotrophs that are unable to grow on medium lacking adenine and have a slow growth phenotype on rich medium.

The ADE2 auxotrophs strains are unable to grow in the absence of adenine due to the full deletion of the ADE2 gene and part of its promoter. The expression plasmids included in the kit contain the ADE2 gene (under its own promoter) as the selection marker. Transformation of the *ade2* auxotrophs strains with the expression plasmids enable the strain to grow again on medium lacking adenine. Further, the color of the transformant colonies indirectly indicates the relative expression levels of your protein of interest.

Expression of rHSA in PichiaPink™ strains 1, 2, 3 and 4 (concentration of HSA)

Three methods of secreted expression were employed in

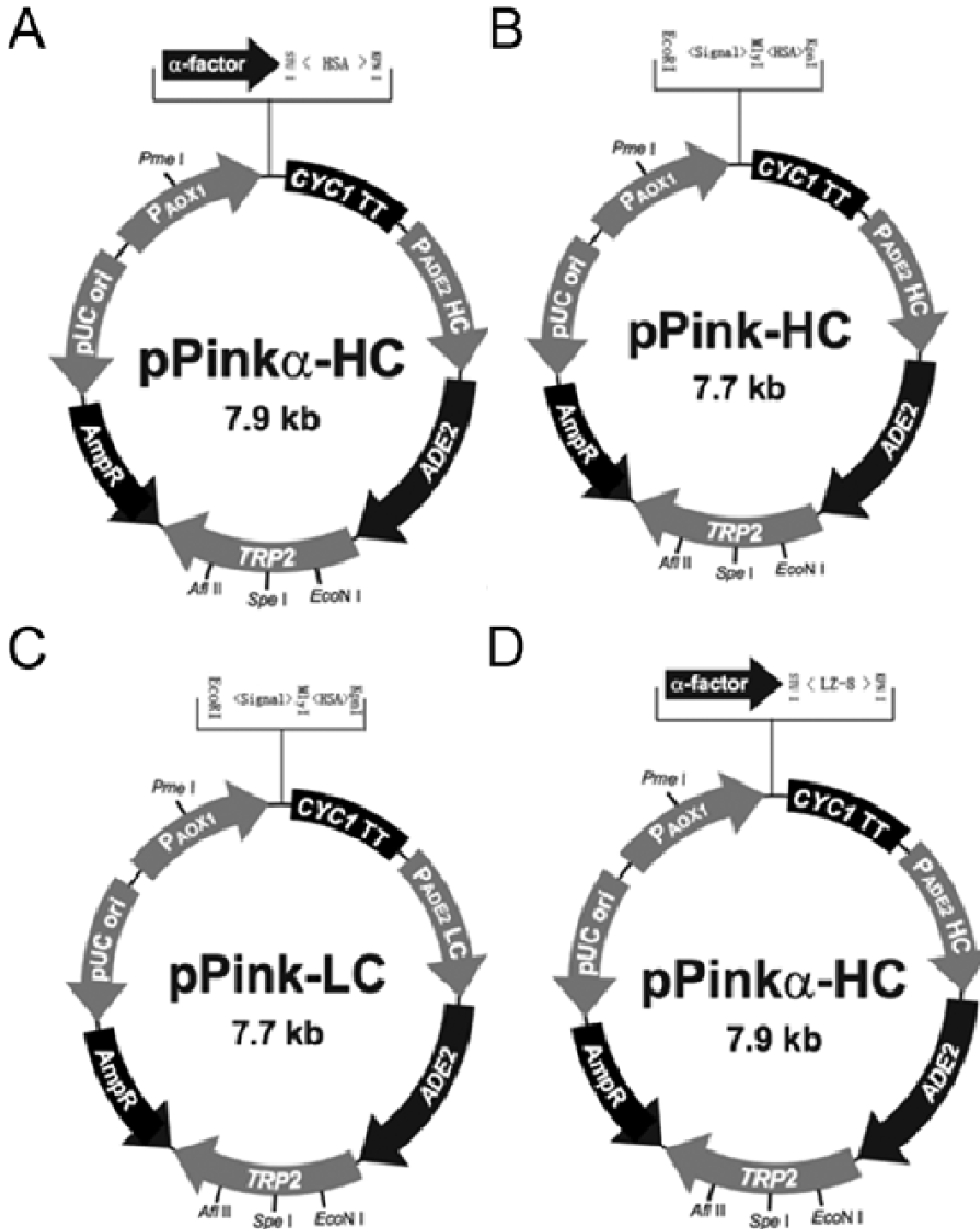


Figure 1. A schematic diagram of the constructed recombinant vector pPink α -HC-HSA (A), pPink-HC-HSA (B), pPink-LC-HSA (C) and pPink α -HC-LZ-8 (D) PAOX1: 5'AOX1 promoter region α -factor: α -mating factor secretion signal Signal: Serum albumin signal sequence CYC1 TT: CCY1 transcription termination region, PADE2 HC: High-copy ADE2 promoter region, PADE2 LC: Low-copy ADE2 promoter region, ADE2: ADE2 ORF, TRP2: TRP2 gene, pUC ori: Oriental promoter of pUC, AmpR: Ampicillin (bla) resistance gene.

the rHSA pilot expression:

(A) Expression Vector: pPink α -HC, Signal Sequence: α -

factor signal sequence from *S. cerevisiae*.

(B) Expression Vector: pPink HC, Signal Sequence: HSA signal sequence from *H. sapiens*.

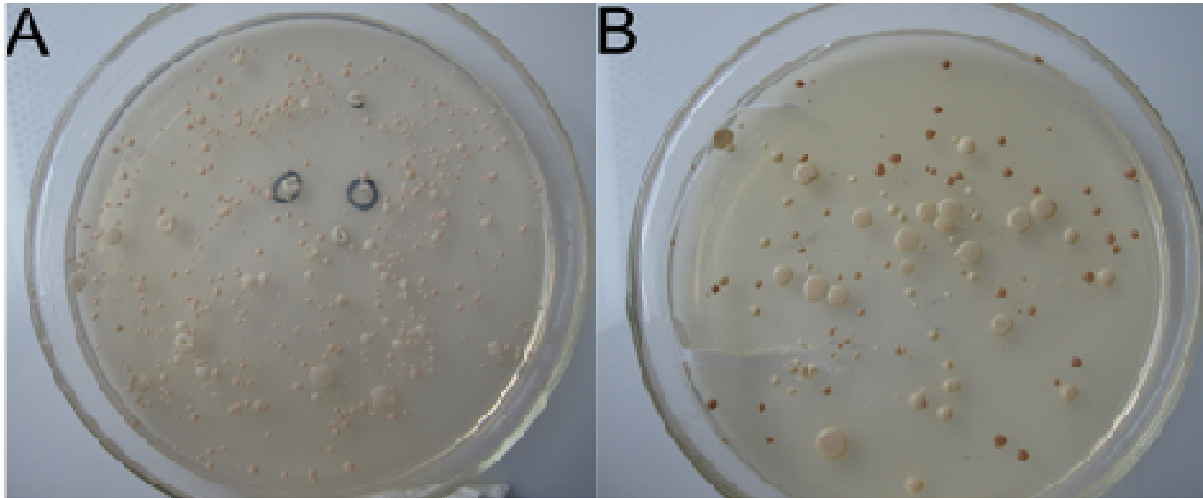


Figure 2. After transformation, spread the cell mixtures on PAD selection plates, and incubate at 30°C for 4 days (A) and 8 days (B) until white and pink colonies are formed.

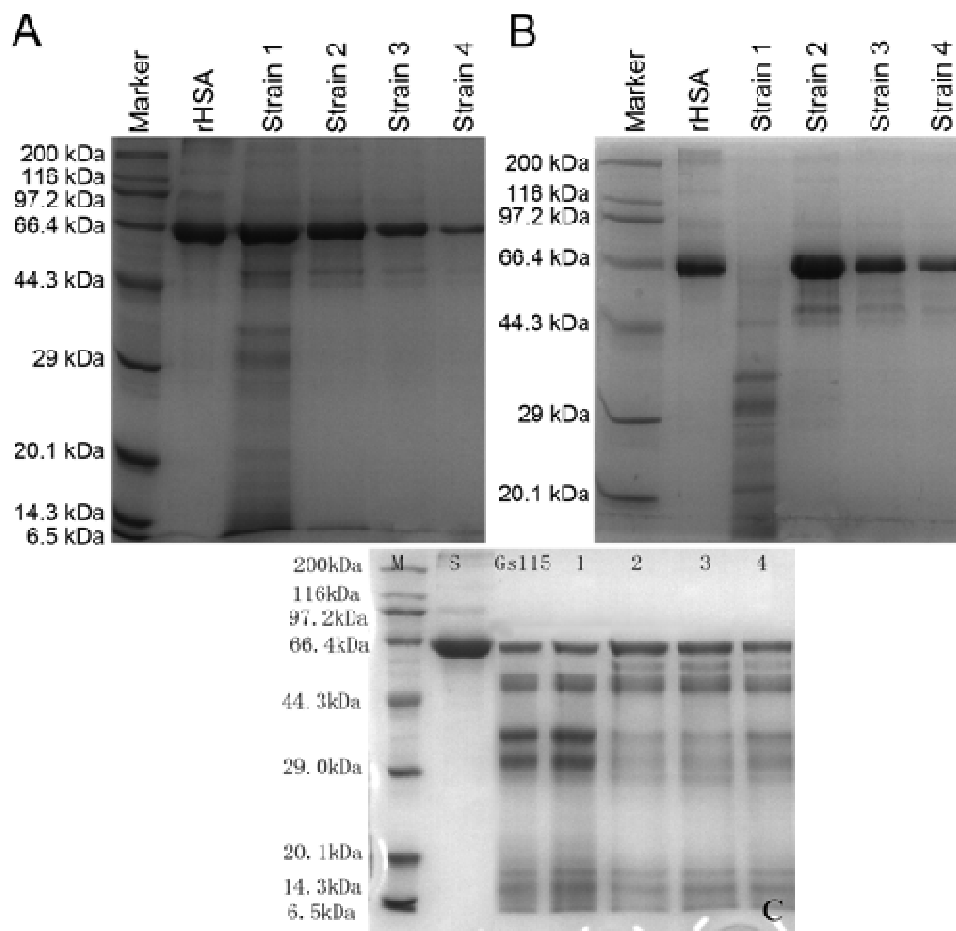


Figure 3. SDS-PAGE results for the supernatants of four induced PichiaPink™ transformants. **A:** induced with methanol for 72 h, **B:** induced with methanol for 144h, **C:** induced with methanol for 144h and loaded the same amount of HSA). M: Protein molecular weight marker, S: Standard of rHSA, line 1: The supernatant of induced PichiaPink™ strain 1, line 2: The supernatant of induced PichiaPink™ strain 2, line 3: The supernatant of induced PichiaPink™ strain 3, line 4: The supernatant of induced PichiaPink™ strain 4.

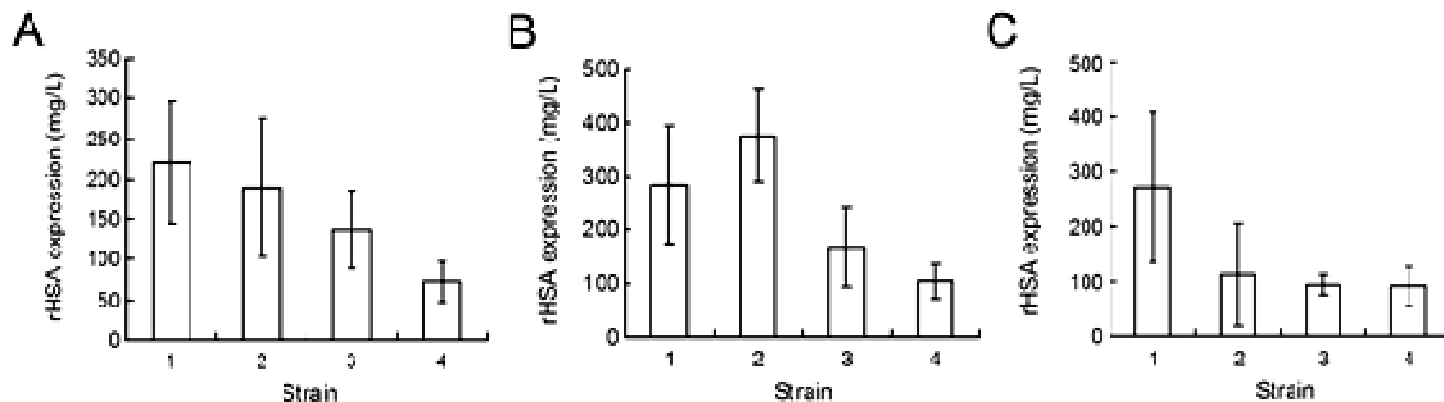


Figure 4. The colonies that were selected to analyze the rHSA expression from a transformed PAD plate by an ELISA assay.

(C) Expression Vector: pPink LC, Signal Sequence: HSA signal sequence from *H. sapiens*.

Twenty colonies were selected to analyze rHSA expression from transformed PAD plates by an ELISA assay. The result showed that all of the colonies that were picked were the correct transformants.

As shown in Figure 4A, in Part (A) of the pilot expression examination, the single colonies from Strain 1 produced more rHSA than the other 3 strains. The peak level of expression is 334 mg/L and the averaging is 219 mg/L. The colonies from Strain 2 produced slightly more than those of Strain 3, averaging 189 and 137 mg/L respectively. Although, the transformants from Strain 4 had the lowest expressing ability, their average still reached into the 73 mg/L range.

The results (Figure 4B) from Part (B) of the pilot expression examination indicate that all kinds of strains showed a more powerful expression capacity than the ones in Part (A). It should be noted that the transformants from Strain 2, being protease knockout yeast, produced more rHSA than the ones from Strain 1 and the other strains, averaging 376 mg/L. In Part (C) of the pilot expression examination (Figure 4C), the capacities of all of the transformants from the 4 strains were lower than the ones in Parts (A) and (B). The expression levels of 3 protease knockout strains were less than 100 mg/L. It is easy to discover from these data that the expression levels of transformants from Strains 1 and 2 are much higher than those of the other 2 strains. Strain 4, being a protease double knockout yeast, is not stronger than the other 3 strains, but it still reached 100 mg/L, which may be better than the traditional *Pichia* Gs115 strain.

Expression and degradation of rHSA in *PichiaPink* strains 1, 2, 3 and 4

Many studies have shown that *P. pastoris* is indeed an excellent host strain for high-level expression of heterologous proteins. However, the presence of high protease activity in the culture broth would have an

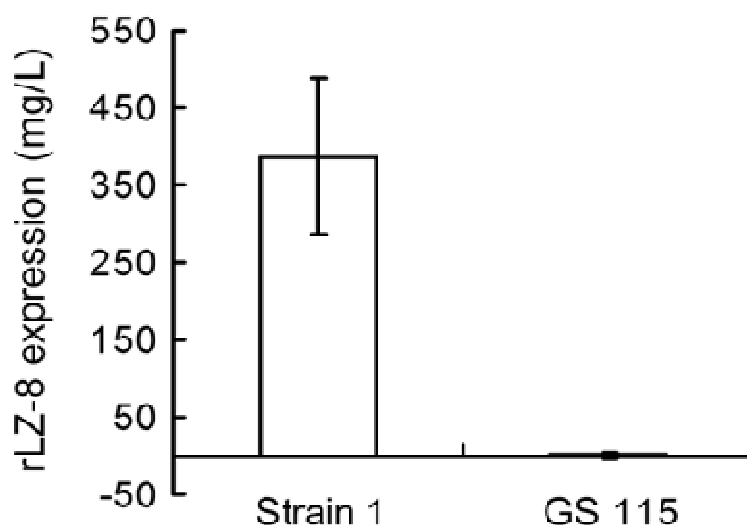
adverse effect on the final protein levels and the subsequent purification of the product. There are 4 *Pichia* strains in the *PichiaPink*TM system. *PichiaPink*TM strain 1 is the parental strain from which the rest of the *PichiaPink*TM strains are derived. The others are all protease knockout *PichiaPink*TM strains. Strain 2 is a *pep4* knockout, which prevents it from synthesizing proteinase A, a vacuolar aspartyl protease capable of self-activation. Due to the fact that proteinase A also plays a role in the subsequent activation of additional vacuolar proteases, *pep4* knockout strains have a diminished proteinase B activity and lack carboxy-peptidase Y activity altogether. Strain 3 is a *prb1* knockout, which prevents it from synthesizing proteinase B, a vacuolar serine protease of the subtilisin family. Strain 4 is double knockout for both proteinases A and B (i.e., *pep4* and *prb1*); therefore, it has the lowest protease activity among the *PichiaPink*TM strains.

It was reported that the rHSA secreted into the medium was a mixture of the rHSA monomers (67 kDa) and degraded fragments (around 43 kDa), as detected by western blot analysis using anti-HSA antibody. As shown in Figure 3A, compared with the standard of rHSA from *P. pastoris* (Line 2) (Sigma Co., Ltd.), an apparent extra protein band of approximately 66.7 kDa was observed in the supernatant of the four induced *PichiaPink*TM transformants. In addition, the rHSA expression increased along with the induction time, and the yield reached its highest level at 72 h. Accordingly, the degraded fragments (around 43 kDa) of HSA from Strain 1 had some distinct and dark bands, while in the lines of Strain 2, 3 and 4, no significant degraded fragments of rHSA could be found, suggesting that protease knockout is helpful in preventing the degradation of secreted rHSA. Among these strains, recombinant strain 2 can express rHSA in very much the same way as Strain 1 and in much higher amounts than Strains 3 and 4. Much lower levels of rHSA degradation have been detected in the supernatant of induced Strain 2 transformants. In particular, the supernatant of induced strain 1, 2, 3 and 4 transformants, which were stored at room temperature for

Table 3. The colonies from Strain 1 and Gs115 that were selected to analyze the rLZ-8 expression from a transformed PAD plate by an ELISA assay (mg/L).

| Strain | *1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 |
|----------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Strain 1 | 251 | 280 | 289 | 301 | 320 | 320 | 343 | 350 | 380 | 420 | 430 | 440 | 440 | 480 | 520 | 630 |
| Gs115 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 15 | 0 |

* Number

**Figure 5.** The colonies from Strain 1 and Gs115 that were selected to analyze the rLZ-8 expression from a transformed PAD plate by an ELISA assay.

3 days, were examined by SDS-PAGE. The results indicate that the rHSA in the supernatant of induced strain 1 all was degraded. Surprisingly, similar behavior was not observed in the supernatant of the other three protease knockout strains, especially when the same amount of HSA was loaded (Figures 3B). It can be concluded from the above results that strain 2 is the most likely to be developed into a high-expression, low-degradation engineering strain that can be used to produce recombinant HSA.

Expression of rLZ-8 in PichiaPink Strain 1 and GS115 (Content of LZ-8)

17 colonies were picked from a transformed plate of Pichia Gs115 and examined for the level of expression. It was only 15 mg/L by ELISA. To compare the expression ability between PichiaPink™ strains and traditional Pichia Gs115, 17 colonies were also picked from a transformed PAD selection plate. All of them were correct transformants. The average of the expression level was 387 mg/L, and 2 transformants produced more than 500 mg/L of rLZ-8 (Table 3 and Figure 5). It is very clear that the transforming efficiency and the expression capacity of

the PichiaPink™ strains are better than those of traditional Pichia Gs115.

Conclusions

Series of protocols was developed and tips for a new Pichia-based PichiaPink™ expressing system, which improves the transformation of the Pichia strain, low yields and degradation of the protein of interest. In the example of HSA, the results indicated two facts: (1) the protease knockout PichiaPink™ strains are quite helpful in decreasing the degradation of HSA without affecting the yield of HSA or the strain growth, and the high-copy expression vector worked better than the low one in terms of the yield of HSA. The example of RLZ-8 showed that compared with the traditional Pichia strain Gs115, the new system provided an easier selection method, more correct transformants and a higher level of expression without any optimization.

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