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Codon modification for the DNA sequence of a single-chain Fv antibody against clenbuterol and expression in *Pichia pastoris*

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Abstract The expression efficiency was improved for the recombinant single-chain variable fragment (scFv) against clenbuterol (CBL) obtained from mouse and expressed in the methylotrophic yeast *Pichia pastoris* GS115, by redesigning and synthesizing the DNA sequence encoding for CBL-scFv based on the codon bias of *P. pastoris*. The codons enco4ding 124 amino acids were optimized, in which a total of 156 nucleotides were changed, and the G+C ratio was simultaneously decreased from 53 to 47.2 %. Under the optimized expression conditions, the yield of the recombinant CBL-scFv (41 kDa) antibodies was 0.223 g L^{-1} in shake culture. Compared to the non-optimized control, the expression level of the optimized recombinant CBL-scFv based on preferred codons in *P. pastoris* demonstrated a 2.35-fold higher yield. Furthermore, the recombinant CBL-scFv was purified by Ni-NTA column chromatography, and the purity was 95 %. The purified CBL-scFv showed good CBL recognition by a competitive indirect enzyme-linked immunoassay. The average concentration required for 50 % inhibition of binding and the limit of detection for the assay were 5.82 and 0.77 ng mL^{-1}, respectively.

Keywords Single-chain variable fragment · Clenbuterol · Codon optimization · Expression · *Pichia pastoris*

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Introduction

Clenbuterol (CBL) is a β-agonist bronchodilator drug, which is prohibited as an additive in animal feed because it constitutes a severe risk for animal welfare and exposes consumers to a pharmacological active drug (Sporano et al. 1998). However, for pursuing economic benefits, CBL has been illegally abused to improve meat production (Sporano et al. 1998), and CBL residues have caused acute food poisoning to consumers (Barbosa et al. 2005).

At present, many methods have been used to analyze for CBL residues (Damasceno et al. 2002; Pan et al. 2006; Melwanki et al. 2007). Among them, the immunoassay is widely used as a screening technology because of its high

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sensitivity, rapid detection, and moderate price (Mörtl et al. 2013), and production of high-quality antibodies is important for development of an immunoassay method. Currently, recombinant single-chain variable fragment (scFv) antibodies with good specificity and sensitivity to the determined antigens have become a new focus of immunoassay applications (Chan et al. 2008). A scFv can be produced by fungal fermentation over a short period of time and at a low cost. These advantages benefit the large-scale production of a scFv (Sommaruga et al. 2011).

In previous work, a mouse scFv antibody against CBL was expressed in *Escherichia coli* BL21 (DE3) as inclusion bodies (Wang et al. 2010). However, the expression level of this recombinant protein was very low, too low to allow it to be used in practical immunoassay applications (Li et al. 2008). Another disadvantage for expression of a mouse scFv in *E. coli* is that the refolding of the inclusion body from bacteria is intricate and time-consuming (Lange et al. 2001; Li et al. 2008). To overcome these disadvantages, a system is needed that can produce high expression of soluble active scFv useful for downstream applications.

Pichia pastoris, a methylotrophic yeast, has been utilized as an efficient expression system to produce heterologous proteins (Macauley-Patrick et al. 2005). Compared to traditional bacteria systems, the *P. pastoris* expression system has many attractive advantages, such as rapid growth on low-cost media, high cell densities, genetic stability, and scale-up without loss of yield (Romanos 1995). Furthermore, *P. pastoris* has the potential to perform many necessary posttranslational modifications such as processing of pre- and prepro-type signal sequences, folding, disulfide bridge formation, lipid addition, and glycosylation (Cereghino and Cregg 2000).

Although the CBL-scFv was expressed in *P. pastoris* successfully (Chen et al. 2011), the yield of the CBL-scFv was too low to be used on an industrial scale. A promising technique for increasing the protein expression level is codon optimization. Many researchers have shown that the different bias of codon usage between the exogenous gene sequence and the expression host has a considerable influence on the expression level of the recombinant protein (Hu et al. 2006; Teng et al. 2007; Wu et al. 2008). Codon optimization is a significant strategy to overcome the bias on codon usage between different species and to achieve a high level of expression.

In this paper, we redesigned and constructed a full-length synthetic gene by changing the codon usage to that preferred by *P. pastoris* for improving the expression of scFv against CBL. After determining the gene copies by real-time quantitative polymerase chain reaction (PCR), the recombinants were investigated for the effects of codon optimization and culture conditions on CBL-scFv accumulation. The bioactivity of the recombinant CBL-scFv antibody was characterized by competitive indirect enzyme-linked immunoassay (ciELISA).

Materials and methods

Strains, plasmid, and media

P. pastoris GS115 and the *P. pastoris* integrative expression vector (pPICZαA) were purchased from Invitrogen Biotechnology Co. (Shanghai, China). *E. coli* was grown in Luria–Bertani medium (1 % peptone, 0.5 % yeast extract, and 1 % sodium chloride), and *P. pastoris* was cultivated in yeast peptone dextrose (YPD) medium (1 % yeast extract, 2 % peptone, and 2 % glucose).

Reagents

Gel extraction kits were obtained from Tiangen (Beijing, China). Yeast genome extraction kits were obtained from Beijing ComWin Biotech Co., Ltd (Beijing, China). PrimerSTAR DNA polymerase, restriction enzymes, and dNTP were obtained from Takara Biotechnology Co. Ltd. (Dalian, China). Primers were synthesized by Shanghai Sangon Biotechnology (Shanghai, China). The mouse anti-His monoclonal antibody was obtained from Sigma-Aldrich (St. Louis, MO, USA). CBL standards were obtained from the National Center of Standard Material (Beijing, China).

Synthesis of the gene and construction of expression vector

The nucleotide sequence of CBL-scFv (GenBank accession no. FJ597759) was re-encoded by selecting the preferred codons in *P. pastoris* (http://www.kazusa.or.jp/codon) (Sinclair and Choy 2002), and codons with a low usage rate (<15 %) were replaced by ones with a high usage rate. The redesigned gene was synthesized by Shanghai Shengong Biotechonology (Shanghai, China).

The modified CBL-scFv gene (GenBank accession no. KF494185) was amplified with the primers PSH-CBL-syn-1 /PSH-CBL-syn-2 (Table 1). Both the modified and unmodified genes were cloned into the *Eco* R I-*Xba* I sites of the pPICZαA vector to generate pPICZαA-CBL-syn and pPICZαA-CBL, respectively. The recombinant vectors were transformed into competent *E. coli* DH5α cells for propagation of the recombinant plasmids. The recombinant plasmid pPICZαA-CBL-syn was confirmed by restriction enzyme digestion and DNA sequencing.

Transformation and selection of CBL-scFv expressing clones

The constructed pPICZαA-CBL-syn expression vector was linearized with *Bst* X I and then transformed into competent cells of *P. pastoris* strain GS115 by electroporation (1,500 V cm^{-1}, 25 μF, 400 Ω; Gene Pulser MXcell Electroporation System, Bio-Rad Laboratories, Hercules, CA, USA). After pulsing, electrocompetent cells were placed in ice-cold 1 M sorbitol

Table 1 Primers used for plasmid construction and quantitative PCR analysis

Name	Sequence (5′-3′)	Annotation
PSH-CBL-syn-1	CGCGAATTCATGGCTC AAGTCAAG	*Eco* R I site (underline)
PSH-CBL-syn-2	CGCTCTAGATCTCTTA ATCTCCAAT	*Xba* I site (underline)
FQ-TEST (F)	CGATGGCTCAAGTCAA GTTG	qPCR for pPICZαA-CBL and pPICZαA-CBL-syn
FQ-TEST (R)	GTCTAGATACTTATCAT CGTCGTCT	qPCR for pPICZαA-CBL and pPICZαA-CBL-syn
GAP (F)	CTCATCACTGCTCCATC CAA	qPCR for GAPDH gene
GAP (R)	CTTCCGAAAGTGTCGT TGAC	qPCR for GAPDH gene

(1 mL) and regenerated at 30 °C for 2 h. Transformants were plated (100 μL) on yeast extract peptone dextrose sorbitol medium plates containing 100 mg mL^{-1} Zeocin and grown at 30 °C for 3–5 days to isolate Zeocin-resistant transformants.

Single colonies were selected and transferred to 100 mL buffered glycerol-complex medium (BMGY) in a 250-mL baffled flask. After growing at 28 °C in a shaking incubator (260 rpm) over night, the cell pellets were harvested after centrifugation at 3,000×*g* for 5 min at 25 °C. The pellets were resuspended using 10 mL buffered methanol-complex medium (BMMY) and 100 μL of 100 % methanol was added every day to induce expression. The expression level of recombinant protein was determined using the Bradford method (Bradford 1976). Culture supernatants were analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The expression level of CBL-scFv was quantitated by scanning the gray level of samples on gels and comparing them with protein standards. The differences of expression among colonies were statistically analyzed in SPSS, version 16.0 (SPSS, Inc., Chicago, IL, USA). $P<0.05$ was considered statistically significant. Differences between the means of continuous parametric data were analyzed with the use of Duncan's test.

Real-time qPCR protocol

The copy numbers of the original CBL-scFv gene (Wang et al. 2009) and modified CBL-scFv gene from recombinant *P. pastoris* were detected according to the previous report (Livak and Schmittgen 2001). The real-time quantitative PCR (qPCR) assay was based on the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Two pair of primers were designed for the assay: First, FQ-TEST (F) and FQ-TEST (R) (Table 1) were designed according to the homologous sequence from both the original CBL-scFv gene and modified CBL-scFv gene to amplify the same size of genomic DNA fragment. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene from *P. pastoris* was used as the reference. Second, GAP (F) and GAP (R) (Table 1) were used to amplify the reference gene GAPDH from strain GS115. Genomic DNA from strain GS115, GS115/pPICZαA-CBL, and GS115/pPICZαA-CBL-syn recombinants were prepared using a yeast DNA extraction kit. A standard curve was prepared by diluting the genomic DNA of GS115 1:2 from 200 to 50 μg mL^{-1}. The DNA of GS115/pPICZαA-CBL and GS115/pPICZαA-CBL-syn was adjusted to 50 μg μL^{-1} for the qPCR assay. The yeast recombinant DNA and the standard sample were analyzed concomitantly using a real-time PCR instrument (AP800, TaKaRa, Japan).

For each gradient sample, the crossing points of the amplification curve with the threshold line (C_t) versus the GAPDH gene concentration were plotted to calculate the slope. The PCR efficiency (E) was calculated from the slope of each standard curve by the following equation: $E=10^{-1/\text{slope}}$. To rectify the starting copy quantity of the GS115/pPICZαA-CBL and GS115/pPICZαA-CBL-syn DNA, the reference gene GAPDH was quantified in parallel. Relative quantification of the copy number was performed according to the $2^{-\Delta\Delta Ct}$ method, where $\Delta\Delta C_t=\Delta C_t$ of target$-\Delta C_t$ of calibrator; $\Delta C_t=C_t$ of target or calibrator$-C_t$ of reference (GAPDH); Copy number $_{(\text{target gene})}=(1+E)^{-\Delta\Delta Ct}$.

Optimization of the expression conditions

The culture conditions, such as induction time, inoculum density, methanol concentration, and pH value, were optimized to increase CBL-scFv expression levels. To optimize protein expression following methanol induction, culture supernatants were collected every 24 h for five collections. To investigate the optimal inoculum density, a single recombinant *P. pastoris* colony was grown in BMGY medium, and then inoculated into fresh BMMY medium at six different inoculum densities ($A_{600\ nm}$=2.25, 4.5, 9, 18, 45, and 90). To determine the optimal methanol concentration, 0.5, 1.0, 1.5, 2.0, 2.5, and 3.0 % methanol was added to the BMMY medium. The optimal pH was investigated by using 100 mM potassium phosphate added to BMMY medium at different pH values (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0).

Scale-up for the expression and purification of the CBL-scFv antibody

To scale-up scFv production under optimized conditions, the colony with the highest CBL-scFv expression was inoculated into a 5-L baffled flask containing 1 L of BMGY medium. Fermentation was performed according to the basic fermentation methods used for *P. pastoris*. The fermentation

culture was filtered through 0.22 μm filters and concentrated by ultrafiltration (Minitan ultrafiltration membrane with NMWL: 20,000; Millipore, Bedford, MA, USA).

The 6× His-tagged recombinant CBL-scFv protein was purified by Ni-NTA affinity chromatography. The pH of the concentrated protein solution was adjusted to 8.0 by the addition of 1/10 volume of 10× lysis buffer (500 mM NaH_2PO_4, 3,000 mM NaCl, 100 mM imidazole, pH 8.0). The 50 % Ni–NTA slurry was added to the pH-adjusted concentrate at the ratio of 1:4 (*v*/*v*) and mixed gently by shaking for 30 min at 4 °C. Then, the concentrate–resin mixture was carefully loaded into an empty column (20 cm×1.2 cm) with the bottom cap still attached. After removing the bottom cap, the flow-through was collected. The column was further washed with 15 mL washing buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 8.0). The recombinant protein was then eluted with 8–1 mL aliquots of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). The flow-through of the wash fraction and the eight elution fractions were collected for SDS-PAGE analysis.

Reverse phase high-performance liquid chromatography of the recombinant protein

The purified recombinant protein was further analyzed by reverse phase high-performance liquid chromatography (RP-HPLC). The separation was performed at 30 °C on a column of C-18 (25 cm×4.6 mm 300 pore size, 5 μm particle size (Agilent Technologies, Santa Clara, CA, USA). The mobile phase of 0.1 % aqueous trifluoroacetic acid, pH 2.5/ chromatography-purity acetonitrile (80/20, *v*/*v*), was delivered at a flow rate of 1 mL min^{-1}. The detection was monitored by ultraviolet absorbance at a wavelength of 280 nm. Injection volume was set to 20 μL. The chromatogram was analyzed using the Empower program (Waters, Milford, MA, USA).

CBL-scFv protein-based ciELISA protocol

The antigen-binding activities of the purified soluble CBL-scFv antibodies were determined by ciELISA as described previously (Wang et al. 2010). Briefly, soluble CBL-conjugated ovalbumin (CBL-OVA) at 100 μg mL^{-1} was coated (100 μL per well) on microtiter plates at 37 °C overnight. The plate was washed two times with 0.01 M phosphate-buffered saline which contained 0.05 % Tween-20 (PBST, 136.9 mM NaCl, 7.9 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 2.7 mM KCl, and 0.05 % Tween-20, pH 7.4) and then blocked with 120 μL per well of 5 % (*w*/*v*) skim milk powder in PBST at 37 °C for 3 h. After washing, serial diluted CBL standards (50 μL) and fusion protein (50 μL) appropriately diluted in PBST were added to each well, and the plates were incubated at 37 °C for 1 h. After washing, 100 μL of anti-His mouse monoclonal antibody diluted 1:15,000 in PBST was added as the primary antibody and incubated at 37 °C for 30 min. After washing, 100 μL of horseradish peroxidase (HRP)-conjugated goat anti-mouse immunoglobulin G diluted 1:15,000 in TBST was added as the secondary antibody and incubated at 37 °C for 30 min. After washing again, 50 μL of the substrate solution used with HRP (10 mL of substrate buffer (102 mM Na_2HPO_4, 48.6 mM citrate, pH 5.4), 50 μL of 1 % (*w*/*v*) 3,30,5,50-tetramethylbenzidine in *N*, *N*-dimethylformamide, and 2.5 μL of 6 % (*w*/*v*) H_2O_2) was added to visualize the signal, and the reaction was stopped after 20 min with 2 M H_2SO_4. The plates were analyzed at 450 nm using a Multiskan MK3 96-well microplate reader (Thermo Scientific, Hudson, NH, USA). The absorbance data was fitted with a four-parameter logistic equation to determine the 50 % inhibition of binding (IC_{50}) value.

Results

Codon modification and synthesis of the CBL-scFv gene

After codon modification the gene sequence, more than 124 non-preferred codons in the original gene were replaced by preferred codons in the synthetic gene, which represents 49.4 % of the total amino acid sequence. As a result, codon modification reduced the overall G+C content of the full-length gene from 53 to 47.2 %, which is closer to the G+C content of other high-expression genes in *P. pastoris*, but the new engineered CBL-scFv still shared 79.4 % similarity to the original CBL-scFv (Fig. S1). Moreover, to avoid premature termination, much of the potential DNA folding regions were eliminated by alternately changing the preferred codons. Compared with the original CBL-scFv gene, more than 3/4 of the potential DNA folding regions were eliminated (Fig. 1).

Transformation and selection of high-expressing clones

After selection of transformants on YPD-Zeocin plates, 43 positive pPICZαA-CBL-syn colonies were obtained. Eight of the 43 colonies were resistant to 1,000 μg mL^{-1} Zeocin. But this strategy is not universal because a number of genes have now been identified whose expression did not increase with increasing copies of the recombinant genes (Yang et al. 2012). Therefore, it is necessary to screen for high-yielding clones using an expression test. Eight colonies were grown and induced in BMMY medium at 30 °C for 120 h as described in the "Materials and Methods." A clear band with molecular weight of 41 kDa was detected in the supernatant (Fig. 2). Colony no. 6 (GS115/pPICZαA-CBL-syn-6) showed steady, strong expression of the 41 kDa protein and was selected for protein expression optimization.

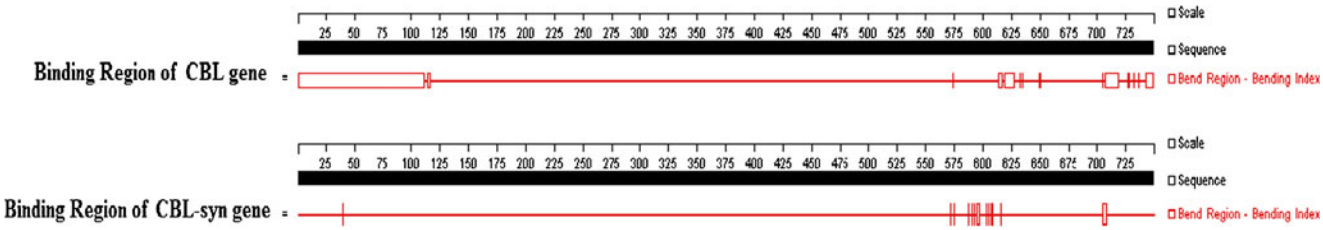

Fig. 1 Analysis of the potential folding region by DNAstar

Identification of copy number by qPCR

According to the qPCR test, the ΔC_t of GS115/pPICZαA-CBL was −0.95 and the ΔC_t of GS115/pPICZαA-CBL-syn-6 was −0.45. The $\Delta\Delta C_t$ was 0.50 and the PCR efficiency was 93 %. On the basis of the $2^{-\Delta\Delta Ct}$ method, the ratio of the copy number of GS115/pPICZαA-CBL-syn to GS115/pPICZαA-CBL was 1.38 (Table 2).

Optimization of expression conditions

The expression level was further improved by optimization of the induction conditions. To obtain the best time for CBL-scFv protein collection, a time course of the extracted recombinant protein GS115/pPICZαA-CBL-syn-6 from *P. pastoris* cells was conducted and analyzed by SDS-PAGE and ELISA (Fig. 3a). The novel 41-kDa recombinant protein appeared at 24 h post-induction and peaked at 96 h. The antigen-binding activity of CBL-scFv peaked at 72 h and decreased in the late stages of induction. Considering the expression and the quality of CBL-scFv, the supernatant was collected at 72 h post-induction to prevent significant degradation and maintain bioactivity of the CBL-scFv.

Cell growth is particularly important for bioreactor production of secreted proteins, and optimal inoculum density plays an important role in the production of recombinant proteins (Macauley-Patrick et al. 2005). For optimization of the inoculum density, the recombinant protein was inoculated at six different inoculum densities and was analyzed by both SDS-PAGE and ELISA (Fig. 3b). An inoculum density of $A_{600\ nm}$=45 expressed the most recombinant CBL-scFv. Also, it was found that the highest binding activity of the CBL-scFv was recorded at an inoculum density of $A_{600\ nm}$=45. Therefore, the overall optimal inoculum density was determined to be $A_{600\ nm}$= 45 for the best CBL-scFv expression.

During optimization of the methanol concentration required for the best expression, six different methanol concentrations were investigated, and the expression was analyzed by SDS-PAGE and ELISA (Fig. 3c). The expression yield of CBL-scFv improved over the range of 0.5–1 % methanol feeding (Fig. 3c, lanes 1–2). When the methanol concentration was increased between 1.5 and 3 % (Fig. 3c, lanes 3–6), the original 41 kDa protein band was greatly reduced compared with a methanol feeding of 0.5–1 %. In addition, at 1 % methanol feeding, the binding activity of CBL-scFv was stronger than that of other methanol levels. Therefore, the optimal concentration of methanol feeding was selected as 1.0 % *v*/*v*.

During traditional induction of *P. pastoris*, a growth culture pH of 5–6 is routinely used; however, when the media is buffered to lower pH values, the yield of recombinant proteins often increases due to reduced protease activity (País-Chanfrau et al. 2004). In the experiment determining the optimal pH values, inductions were carried out in BMMY culture medium at six pH values (3.0, 4.0, 5.0, 6.0, 7.0, and 8.0). The highest CBL-scFv production was observed at pH 4 (Fig. 3d). In addition, the production of CBL-scFv was reduced when the pH increased from 6.0 to 8.0. The binding activity of CBL-scFv was steady when the pH was between 3.0 and 6.0 and decreased above pH 6.0; therefore, pH 4.0 was selected as optimal due to the maximum CBL-scFv secretion and binding.

Scale-up of expression and purification of the CBL-scFv antibody

Large-scale expression of the CBL-scFv was accomplished by increasing the culture volume and then concentrating the CBL-scFv protein by ultrafiltration. The CBL-scFv antibody yield was 0.223 g L^{-1}, which is 2.34-fold higher than that from the original CBL-scFv gene in recombinant *P. pastoris* (0.095 g L^{-1}) (Chen et al. 2011). These results demonstrated that soluble CBL-scFv protein expression was functionally enhanced by optimization of the codon and expression conditions in *P. pastoris*. The culture supernatant was concentrated by ultrafiltration and then separated by Ni-NTA column chromatography for further purification. The purified recombinant CBL-scFv antibody had a retention time of 7 min in RP-HPLC analysis (Fig. 4). In addition, the purity of the CBL-scFv antibody was determined by SDS-PAGE (Fig. 5a, lane 2) and Western blot (Fig. 5b, lane 2) analysis. The recombinant CBL-scFv protein demonstrated a single 41-kDa band. The final scale-up yield of the CBL-scFv protein was approximately 3.2 g L^{-1} and the purity reached 95 %.

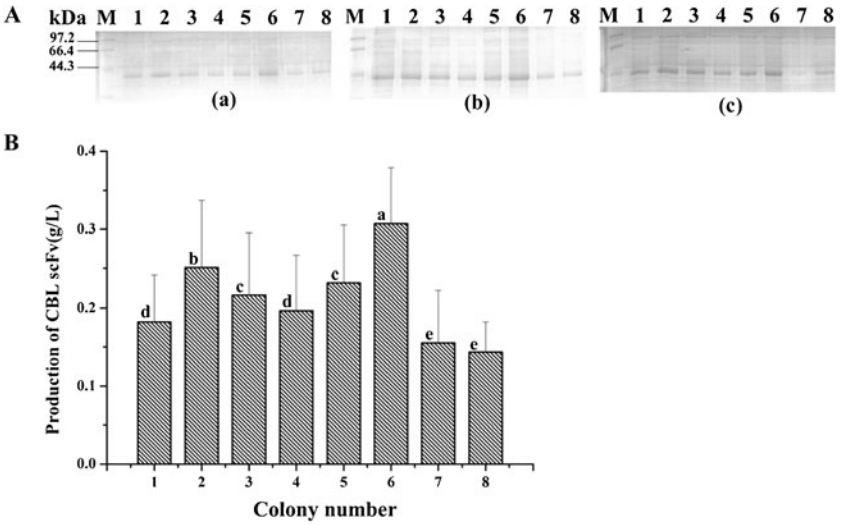

Fig. 2 SDS-PAGE and quantitative analysis of the recombinant protein expressed in different colonies. *Lane M*, protein molecular weight standards; *lanes 1–8*, secreted proteins from different pPICZαA-CBL-GS115-syn recombinant *P. pastoris* 120 h after methanol induction. The quantitative experiments of CBL-scFv were performed by scanning the gray level and comparing the results to protein standards. Statistical analysis of the results from the experiments was conducted using one-way analysis of variance. Results showed that there was a significant difference in protein concentration for colony 6 compared to all others ($P < 0.05$)

CBL-scFv protein-based ciELISA

To determine whether the obtained CBL-scFv protein presented characteristics of the parent antibody, a ciELISA was developed and the results are shown in Fig. 6. The linear range extended from 1.76 to 19.97 ng mL^{-1} and the limit of detection was 0.77 ng mL^{-1}. The IC_{50} value of the prepared CBL-scFv was 5.82 ng mL^{-1}. The results demonstrated that the CBL-scFv produced in *P. pastoris* can be used for the determination of CBL by a ciELISA.

Table 2 Treatment of replicate data where target and reference are amplified in separate wells

Object	CBL	CBL-syn
Ct ($n=3$)	17.64	17.63
	17.57	17.68
	17.61	17.66
GADPH C_t ($n=3$)	18.62	18.11
	18.48	18.09
	18.56	18.13
Average (Avg C_t − Avg GADPH C_t)	−1.01	−0.48
	−0.91	−0.41
	−0.92	−0.47
ΔC_t	−0.95±0.03	−0.45±0.02
$\Delta\Delta C_t$	0.50	
$(1+E)^{-\Delta\Delta Ct}$	1.38	

Discussion

Immunoassays are often developed using either a polyclonal antibody (PAb) or a monoclonal antibody (MAb) and standard immunochemical methods. PAbs are the easiest and quickest to produce, but they are not single molecular entities and sometimes cause nonspecific binding. MAbs are single molecular entities, and multiple clones are available for selection during the developmental process. But the preparation of MAbs is complex, and expensive cell culturing facilities are required for large-scale production. Recently, advances in the field of recombinant antibody (RAb) technology have provided an alternative means to engineer low-cost antibodies with desirable affinity and specificity (Chiu et al. 2000; Lauer et al. 2005). Moreover, the cost of RAb production can be lowered through fermentation of engineered strains, such as recombinant *P. pastoris*. The scFv is one of the most popular candidates for RAb production. Many researchers have described the preparation and application of scFvs against low molecular weight contaminants in food, water, or soil, such as aflatoxin, atrazine, chlorpyrifos, dioxin, sulfadiazine, and triazine (Ward et al. 1993; Lee et al. 1998; Alcocer et al. 2000; Moghaddam et al. 2001; Korpimäki et al. 2004; Lauer et al. 2005; Nishi et al. 2005).

This study focused on obtaining high-level expression of a recombinant scFv against CBL in *P. pastoris* and to develop a novel screening immunoassay for CBL using a RAb instead of a conventional PAb or MAb. *P. pastoris* has been widely used as a heterologous expression system because of its

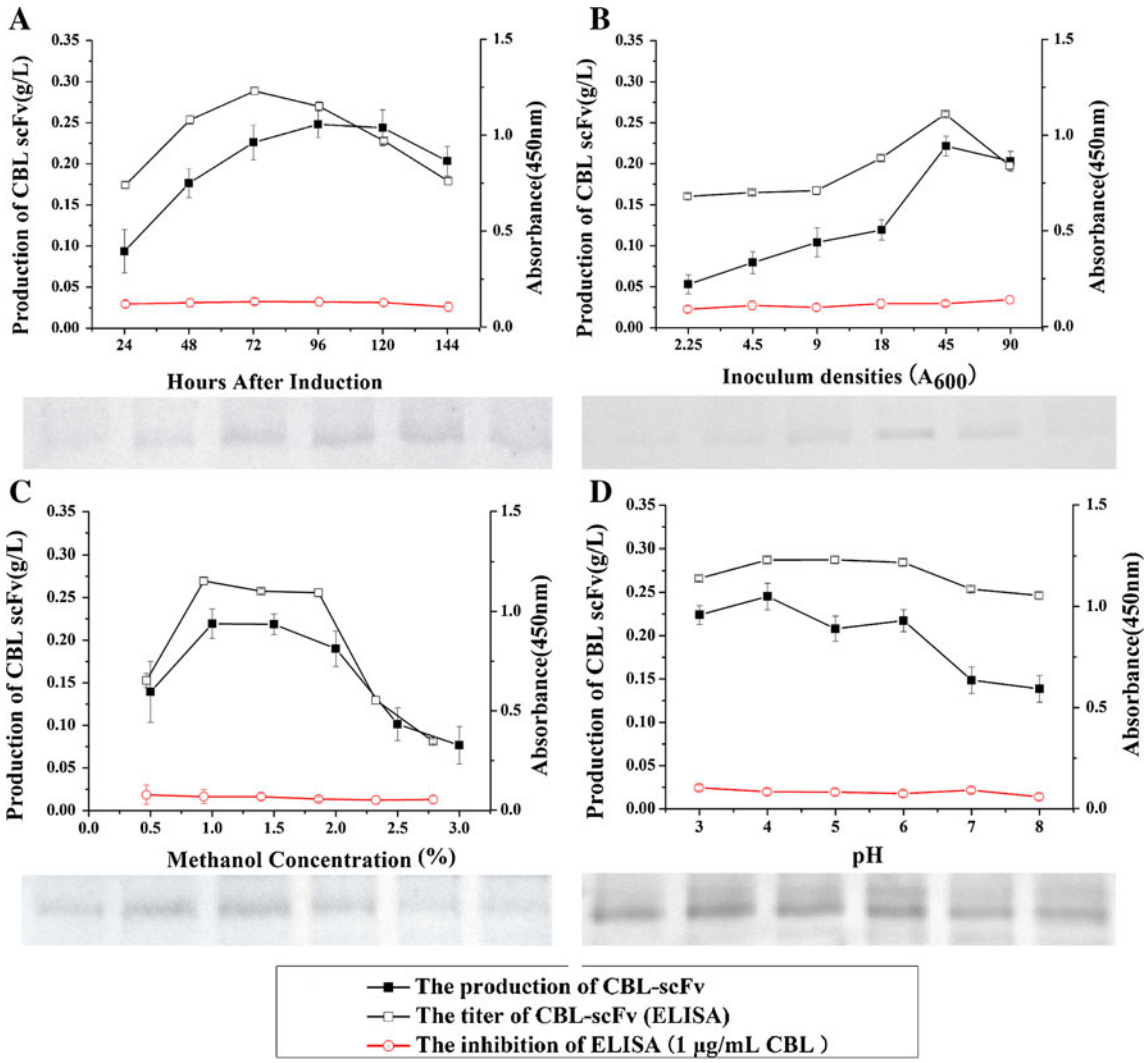

Fig. 3 Different influences on scFv expression and antigen-binding. **a** Different induction times of culture supernatants; **b** different inoculum densities; **c** different methanol concentrations; and **d** different pH values

efficient protein secretion, high-expression, proper protein folding ability, and high-density of growth. However, poor codon bias in the native CBL-scFv gene may be a possible cause of inefficient translation and, therefore, production in *P. pastoris*. Over the past decade, a number of different genes have achieved high-level or increased expression through codon optimization, such as *Bacillus licheniformis* beta-1,3-1,4-glucanase (Teng et al. 2007), internalizing anti-ErbB2 single-chain antibody (Hu et al. 2006), *Aspergillus sulphureus* endo-β-1,4-xylanase (Li et al. 2010), *Thermotoga maritima* β-fructosidase (Menéndez et al. 2013), capsid protein of human papillomavirus type 16 and 18 (Rao et al. 2011), *Penicillium notatum* glucose oxidase (Gao et al. 2012), eukaryotic membrane proteins (Öberg et al. 2011), and *Drosophila melanogaster* acetylcholinesterase (Wu et al. 2008). Some human genes including glucocerebrosidase (Sinclair and Choy 2002), adenosine A2A receptor (Singh et al. 2008), activin A (Fredericks et al. 2010), and Zbtb7A (Wang et al. 2008) were also highly expressed in *P. pastoris* through codon optimization. To achieve a high-level of expression for the CBL-scFv gene in *P. pastoris*, we used a novel strategy of optimizing the codons of the scFv according to the following two steps: First, the CBL-scFv gene was redesigned by changing the codons to those that *P. pastoris* preferred (Hoekema et al. 1987). When encountering several identical amino acids, we changed the codons to the most-preferred and the second-preferred codons alternatively to eliminate stable secondary structures like hairpin turns. The G+C content was adjusted to an appropriate range based on the content of highly expressed genes in *P. pastoris*. Second, the DNAStar program (Burland 2000) was used to predict the potential DNA folding positions. The potential folding positions were then interrupted by changing to the preferred codons.

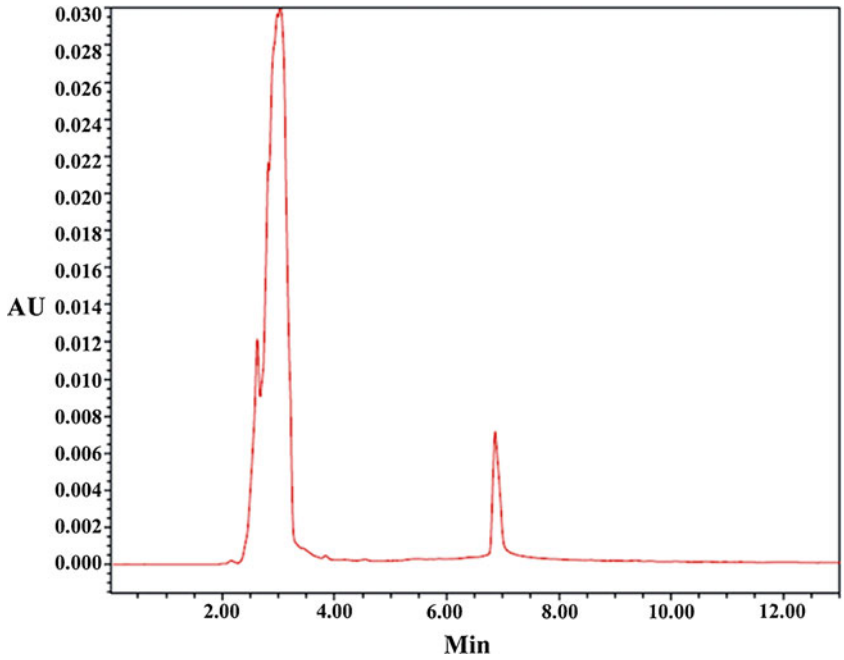

Fig. 4 RP-HPLC analysis of purified inclusion bodies

Studies have revealed that the gene copy number of foreign proteins is one of the vital factors for recombinant protein production (Yu et al. 2009; Zhu et al. 2009). Athmaram et al. (2012) demonstrated that an increase in copy number resulted in a proportional increase in expression level of the H1N1HA recombinant protein. Previous reports indicated that determining the copy number of strains for both wild-type and codon optimized genes and comparing their expression levels were necessary to confirm whether codon optimization was a good strategy for improving expression levels (Hu et al. 2013; Menéndez et al. 2013). In our study, the copy number of GS115/pPICZαA-CBL and GS115/pPICZαA-CBL-syn-6 was at the same level. Therefore, the observed improvement in expression level of the redesigned CBL-scFv was due to codon modification. Mechanistically, associations between mRNA concentrations (Coghlan and Wolfe 2000) and aminoacyl-tRNA (aa-tRNA) abundances (Akashi 2003) could contribute to relationships between gene expression and codon bias. As previously reported, codon bias affects the transcriptional rate and mRNA stability and therefore may contribute to the mRNA concentration (Kim et al. 2010). The increase of mRNA concentration is a necessary and sufficient cause for the improvement of protein expression (Maertens et al. 2010). In addition, the codon bias is co-adapted with aa-tRNA pools to enhance the efficiency of protein synthesis. The pool of

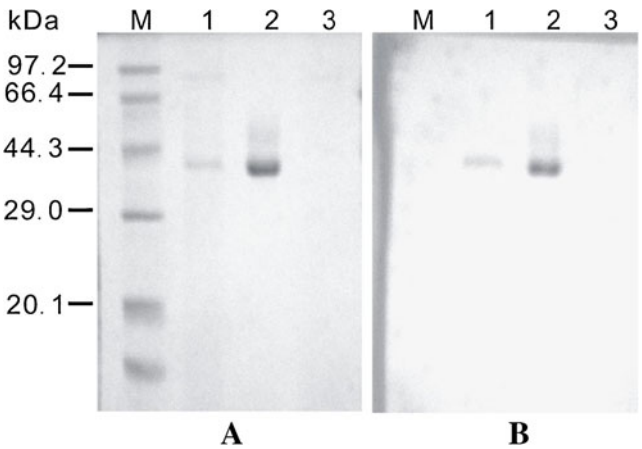

Fig. 5 SDS-PAGE and Western blotting analysis of the CBL-scFv antibody: **a** SDS-PAGE analysis and **b** Western blotting. *Lane M*, protein molecular weight standards; *lane 1*, secreted proteins from GS115/pPICZαA-CBL-syn-6 recombinant *P. pastoris*; *lane 2*, purified CBL-scFv antibody; and *lane 3*, secreted proteins from pPICZαA-GS115 recombinant *P. pastoris*

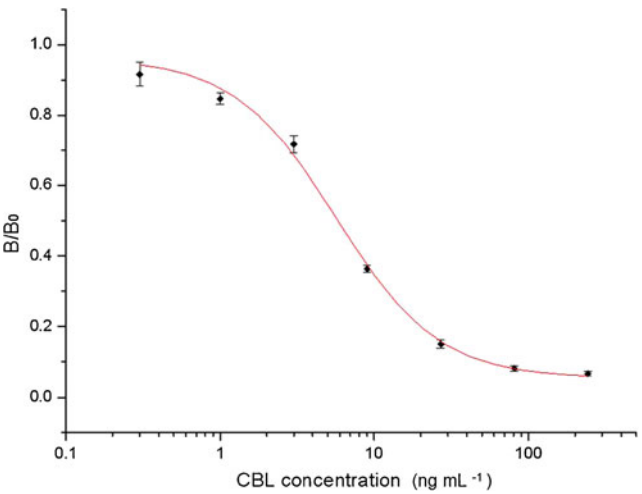

Fig. 6 ciELISA for CBL using CBL-scFv antibodies

available aa-tRNAs is kept rate limiting for accuracy and efficiency of gene translation (Zhao et al. 2005). Replacement of the less-preferred codons with those codons that are more commonly used can greatly increase gene expression in living cells, resulting in improvement in translational efficiency attributed to correction of a mismatch between the aa-tRNA pools and gene codon bias between different species.

To further improve scFv production, we examined parameters such as induction time, inoculum densities, methanol concentration, and culture medium pH. In the induction time assay, the recombinant CBL-scFv can be detected by SDS-PAGE at 24 h following methanol induction. But a decrease in CBL-scFv expression levels was observed after 96 h, which was most likely due to the presence of proteases. Previous reports indicated that the presence of endogenous or extracellular proteases may cause partial degradation of the protein over a long induction period (Minning et al. 2001). We chose 96 h as the optimal induction time to prevent further degradation and maintain bioactivity of the CBL-scFv. The production and bioactivity of the protein were increased when inoculum densities were between 2.25 and 45 (Fig. 3b), which indicated that higher densities were more effective for protein secretion. Expression decreased at an inoculum density of $A_{600\ nm}$=90, which coincided with the known damage from proteases and also the negative effect of oxygen limitation at high cell densities (Kang et al. 2000; Koganesawa et al. 2002). The amount of added methanol in the BMMY medium has significant effects on heterologous protein production because the methanol-induced AOX1 promoter is used in *P. pastoris* (Zhang et al. 2009). In our study, we found the peak scFv expression and binding activity at a methanol concentration of 1 %. But a decrease was observed when methanol feeding was increased to 1.5–3 %, which might be due to either limited carbon availability or to a toxic effect of accumulated methanol (Boettner et al. 2002; Mayson et al. 2003). In the traditional induction step of *P. pastoris*, a pH value of 5–6 has most often been used for the growth medium. However, the yield of recombinant protein often increases when there is reduced protease activity because of low media pH values (País-Chanfrau et al. 2004). As seen in Fig. 3d, the amount of CBL-scFv produced at pH 7.0 was only 51 % of that produced at pH 4.0. This result was probably due to degradation by protease activity at pH 7. Therefore, proteolysis of the recombinant protein may be reduced by adjusting the pH of the culture medium to a lower value.

In our studies, *P. pastoris* released comparatively few endogenous proteins into the culture medium while secreting large amounts of recombinant protein. This characteristic greatly facilitates subsequent protein purification (Sarramegna et al. 2003; Idiris et al. 2010). To further characterize the purified protein, we performed ciELISA to determine the CBL-binding activity of the purified scFv. The *P. pastoris*-expressed scFv showed comparable CBL recognition activity to the parent MAb (Pan et al. 2006). The recombinant scFv was then used in the determination of CBL by a ciELISA.

The methylotrophic yeast *P. pastoris* is particularly suited for scFv expression because it cannot only express high levels of protein at the intra- or extracellular level but also can perform higher eukaryotic protein modifications, such as glycosylation and disulfide bond formation (Macauley-Patrick et al. 2005). In general, the conserved intrachain disulfide bond is one of the main structural features of immunoglobulin folding and is seen as an important factor in the folding of the native antibody domain structure. ScFv fragments normally contain two intrachain disulfide bridges: one in the VH domain and one in the VL domain (de Jaeger et al. 1999). Thus, the eukaryotic protein modification of disulfide bond formation would be helpful to retain the recognition characteristics of an antibody.

Preparation of a high quality antibody plays an important part in immunoassay development. In this study, the expression efficiency of recombinant CBL-scFv was functionally enhanced by optimization of the codon and expression conditions. This work suggests that *P. pastoris* is a suitable host for high level production of the CBL-scFv antibody. Soluble CBL-scFv can be useful for developing a high throughput, rapid, and cost-effective immunoassay for the detection of CBL in food and in the animal production industry.

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References

Akashi H (2003) Translational selection and yeast proteome evolution. Genetics 164:1291–1303

Alcocer MJC, Doyen C, Lee HA, Morgan MRA (2000) Properties of polyclonal, monoclonal, and recombinant antibodies recognizing the organophosphorus pesticide chlorpyrifos-ethyl. J Agric Food Chem 48:4053–4059

Athmaram TN, Saraswat S, Singh AK, Rao MK, Gopalan N, Suryanarayana VVS, Rao PVL (2012) Influence of copy number on the expression levels of pandemic influenza hemagglutinin recombinant protein in methylotrophic yeast *Pichia pastoris*. Virus Genes 45:440–451

Barbosa J, Cruz C, Martins J, Silva JM, Neves C, Alves C, Ramos F, Noronha da Silveira MI (2005) Food poisoning by clenbuterol in Portugal. Food Addit Contam 22:563–566

Boettner M, Prinz B, Holz C, Stahl U, Lang C (2002) High-throughput screening for expression of heterologous proteins in the yeast *Pichia pastoris*. J Biotechnol 99:51–62

Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem 72:248–254

Burland TG (2000) DNASTAR's lasergene sequence analysis software. In: Misener S, Krawetz SA (eds) Methods in molecular biology: bioinformatics methods and protocols, vol 132. Human, Totowa, pp 71–91

Cereghino JL, Cregg JM (2000) Heterologous protein expression in the methylotrophic yeast *Pichia pastoris*. FEMS Microbiol Rev 24:45–66

Chan CP-y, Cheung Y-c, Renneberg R, Seydack M (2008) New trends in immunoassays. In: Advances in biochemical engineering/ biotechnology: biosensing for the 21st century, vol 109. Springer, Berlin, pp 123–154

Chen S-C, He Y-S, Xu X-Y, Lu T-Z, Wang H (2011) Expression of a single chain Fv antibody against clenbuterol in *Pichia pastoris*. Chin Sci Technol Food Ind 7:217–219

Chiu Y-W, Chen R, Li QX, Karu AE (2000) Derivation and properties of recombinant Fab antibodies to coplanar polychlorinated biphenyls. J Agric Food Chem 48:2614–2624

Coghlan A, Wolfe KH (2000) Relationship of codon bias to mRNA concentration and protein length in *Saccharomyces cerevisiae*. Yeast 16:1131–1145

Damasceno L, Ventura R, Cardoso J, Segura J (2002) Diagnostic evidence for the presence of β-agonists using two consecutive derivatization procedures and gas chromatography–mass spectrometric analysis. J Chromatogr B 780:61–71

De Jaeger G, Buys E, Eeckhout D, de Wilde C, Jacobs A, Kapila J, Angenon G, van Montagu M, Gerats T, Depicker A (1999) High level accumulation of single-chain variable fragments in the cytosol of transgenic *Petunia hybrida*. Eur J Biochem 259:426–434

Fredericks D, Clay R, Warner T, O'Connor A, de Kretser DM, Hearn MTW (2010) Optimization of the expression of recombinant human activin A in the yeast *Pichia pastoris*. Biotechnol Prog 26:372–383

Gao Z, Li Z, Zhang Y, Huang H, Li M, Zhou L, Tang Y, Yao B, Zhang W (2012) High-level expression of the *Penicillium notatum* glucose oxidase gene in *Pichia pastoris* using codon optimization. Biotechnol Lett 34:507–514

Hoekema A, Kastelein RA, Vasser M, de Boer HA (1987) Codon replacement in the *PGK1* gene of *Saccharomyces cerevisiae*: experimental approach to study the role of biased codon usage in gene expression. Mol Cell Biol 7:2914–2924

Hu S, Li L, Qiao J, Guo Y, Cheng L, Liu J (2006) Codon optimization, expression, and characterization of an internalizing anti-ErbB2 single-chain antibody in *Pichia pastoris*. Protein Expr Purif 47: 249–257

Hu H, Gao J, He J, Yu B, Zheng P, Huang Z, Mao X, Yu J, Han G, Chen D (2013) Codon optimization significantly improves the expression level of a keratinase gene in *Pichia pastoris*. PLoS ONE 8(3): e58393. doi:10.1371/journal.pone.0058393

Idiris A, Tohda H, Kumagai H, Takegawa K (2010) Engineering of protein secretion in yeast: strategies and impact on protein production. Appl Microbiol Biotechnol 86:403–417

Kang HA, Choi E-S, Hong W-K, Kim J-Y, Ko S-M, Sohn J-H, Rhee S-K (2000) Proteolytic stability of recombinant human serum albumin secreted in the yeast *Saccharomyces cerevisiae*. Appl Microbiol Biotechnol 53:575–582

Kim H-J, Lee S-J, Kim H-J (2010) Optimizing the secondary structure of human papillomavirus type 16 L1 mRNA enhances L1 protein expression in *Saccharomyces cerevisiae*. J Biotechnol 150:31–36

Koganesawa N, Aizawa T, Shimojo H, Miura K, Ohnishi A, Demura M, Hayakawa Y, Nitta K, Kawano K (2002) Expression and purification of a small cytokine growth-blocking peptide from armyworm *Pseudaletia separata* by an optimized fermentation method using the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif 25:416–425

Korpimäki T, Brockmann E-C, Kuronen O, Saraste M, Lamminmäki U, Tuomola M (2004) Engineering of a broad specificity antibody for simultaneous detection of 13 sulfonamides at the maximum residue level. J Agric Food Chem 52:40–47

Lange S, Schmitt J, Schmid RD (2001) High-yield expression of the recombinant, atrazine-specific Fab fragment K411B by the methylotrophic yeast *Pichia pastoris*. J Immunol Methods 255: 103–114

Lauer B, Ottleben I, Jacobsen H-J, Reinard T (2005) Production of a single-chain variable fragment antibody against fumonisin B1. J Agric Food Chem 53:899–904

Lee N, Holtzapple CK, Stanker LH (1998) Cloning, expression, and characterization of recombinant Fab antibodies against dioxin. J Agric Food Chem 46:3381–3388

Li T, Cheng J, Hu B, Liu Y, Qian G, Liu F (2008) Construction, production, and characterization of recombinant scFv antibodies against methamidophos expressed in *Pichia pastoris*. World J Microbiol Biotechnol 24:867–874

Li Y, Zhang B, Chen X, Chen Y, Cao Y (2010) Improvement of *Aspergillus sulphureus* endo-β-1,4-xylanase expression in *Pichia pastoris* by codon optimization and analysis of the enzymatic characterization. Appl Biochem Biotechnol 160:1321–1331

Livak KJ, Schmittgen TD (2001) Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta Ct}$ method. Methods 25:402–408

Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. Yeast 22:249–270

Maertens B, Spriestersbach A, von Groll U, Roth U, Kubicek J, Gerrits M, Graf M, Liss M, Daubert D, Wagner R, Schäfer F (2010) Gene optimization mechanisms: a multi-gene study reveals a high success rate of full-length human proteins expressed in *Escherichia coli*. Protein Sci 19:1312–1326

Mayson BE, Kilburn DG, Zamost BL, Raymond CK, Lesnicki GJ (2003) Effects of methanol concentration on expression levels of recombinant protein in fed-batch cultures of *Pichia methanolica*. Biotechnol Bioeng 81:291–298

Melwanki MB, Huang S-D, Fuh M-R (2007) Three-phase solvent bar microextraction and determination of trace amounts of clenbuterol in human urine by liquid chromatography and electrospray tandem mass spectrometry. Talanta 72:373–377

Menéndez C, Martínez D, Trujillo LE, Mazola Y, González E, Pérez ER, Hernández L (2013) Constitutive high-level expression of a codon-optimized β-fructosidase gene from the hyperthermophile *Thermotoga maritima* in *Pichia pastoris*. Appl Microbiol Biotechnol 97:1201–1212

Minning S, Serrano A, Ferrer P, Solá C, Schmid RD, Valero F (2001) Optimization of the high-level production of *Rhizopus oryzae* lipase in *Pichia pastoris*. J Biotechnol 86:59–70

Moghaddam A, Løbersli I, Gebhardt K, Braunagel M, Marvik OJ (2001) Selection and characterisation of recombinant single-chain antibodies to the hapten Aflatoxin-B1 from naive recombinant antibody libraries. J Immunol Methods 254:169–181

Mörtl M, Németh G, Juracsek J, Darvas B, Kamp L, Rubio F, Székács A (2013) Determination of glyphosate residues in Hungarian water samples by immunoassay. Microchem J 107:143–151

Nishi K, Ishiuchi M, Morimune K, Ohkawa H (2005) Molecular and immunochemical characteristics of monoclonal and recombinant antibodies selective for the triazine herbicide simetryn and application to environmental analysis. J Agric Food Chem 53: 5096–5104

Öberg F, Sjöhamn J, Conner MT, Bill RM, Hedfalk K (2011) Improving recombinant eukaryotic membrane protein yields in *Pichia pastoris*: the importance of codon optimization and clone selection. Mol Membr Biol 28:398–411

País-Chanfrau JM, García Y, Licor L, Besada V, Castellanos-Serra L, Cabello CI, Hernández L, Mansur M, Plana L, Hidalgo A, Támbara Y, del Abrahantes-Pérez CM, del Toro Y, Valdés J, Martínez E (2004) Improving the expression of mini-proinsulin in *Pichia pastoris*. Biotechnol Lett 26:1269–1272

Pan K, Wang H, Zhang H-b, Liu H-w, Lei H-t, Huang L, Sun Y-m (2006) Production and characterization of single chain Fv directed against β_2-agonist clenbuterol. J Agric Food Chem 54:6654–6659

Rao NH, Balou BP, Rajendra L, Sriraman R, Pang Y-YS, Schiller JT, Srinivasan VA (2011) Expression of codon optimized major capsid protein (L1) of human papillomavirus type 16 and 18 in *Pichia pastoris*; purification and characterization of the virus-like particles. Vaccine 29:7326–7334

Romanos M (1995) Advances in the use of *Pichia pastoris* for high-level gene expression. Curr Opin Biotechnol 6:527–533

Sarramegna V, Talmont F, Demange P, Milon A (2003) Heterologous expression of G-protein-coupled receptors: comparison of expression systems from the standpoint of large-scale production and purification. Cell Mol Life Sci 60:1529–1546

Sinclair G, Choy FYM (2002) Synonymous codon usage bias and the expression of human glucocerebrosidase in the methylotrophic yeast, *Pichia pastoris*. Protein Expr Purif 26:96–105

Singh S, Gras A, Fiez-Vandal C, Ruprecht J, Rana R, Martinez M, Strange PG, Wagner R, Byrne B (2008) Large-scale functional expression of WT and truncated human adenosine A_{2A} receptor in *Pichia pastoris* bioreactor cultures. Microb Cell Factories 7:28

Sommaruga S, Lombardi A, Salvadè A, Mazzucchelli S, Corsi F, Galeffi P, Tortora P, Prosperi D (2011) Highly efficient production of anti-HER2 scFv antibody variant for targeting breast cancer cells. Appl Microbiol Biotechnol 91:613–621

Sporano V, Grasso L, Esposito M, Oliviero G, Brambilla G, Loizzo A (1998) Clenbuterol residues in non-liver containing meat as a cause of collective food poisoning. Vet Hum Toxicol 40:141–143

Teng D, Fan Y, Yang Y-L, Tian Z-G, Luo J, Wang J-h (2007) Codon optimization of *Bacillus licheniformis* β-1,3-1,4-glucanase gene and its expression in *Pichia pastoris*. Appl Microbiol Biotechnol 74:1074–1083

Wang H, Wang Q, Zhang F, Huang Y, Ji Y, Hou Y (2008) Protein expression and purification of human Zbtb7A in *Pichia pastoris* via gene codon optimization and synthesis. Protein Expr Purif 60: 97–102

Wang H, Yang J, Liu X, Liang Y, Lei H, Shen Y, Xu X, Sun Y, Xu Z, He Y (2009) Cloning, expression, and identification of anti-carbofuran single chain Fv gene. Biotechnol Prog 25:1018–1024

Wang H, Liu X, He Y, Dong J, Sun Y, Liang Y, Yang J, Lei H, Shen Y, Xu X (2010) Expression and purification of an anti-clenbuterol single chain Fv antibody in *Escherichia coli*. Protein Expr Purif 72:26–31

Ward VK, Schneider PG, Kreissig SB, Hammock BD, Choudary PV (1993) Cloning, sequencing and expression of the Fab fragment of a monoclonal antibody to the herbicide atrazine. Protein Eng Des Sel 6:981–988

Wu AB, Chen HD, Tang ZZ, Ye BW, Liu WJ, Jia HY, Zhang DB (2008) Synthesis of *Drosophila melanogaster* acetylcholinesterase gene using yeast preferred codons and its expression in *Pichia pastoris*. Chem Biol Interact 175:403–405

Yang M, Johnson SC, Murthy PPN (2012) Enhancement of alkaline phytase production in *Pichia pastoris*: influence of gene dosage, sequence optimization and expression temperature. Protein Expr Purif 84:247–254

Yu H, Yan X, Shen W, Hong Q, Zhang J, Shen Y, Li S (2009) Expression of methyl parathion hydrolase in *Pichia pastoris*. Curr Microbiol 59:573–578

Zhang T, Gong F, Peng Y, Chi Z (2009) Optimization for high-level expression of the *Pichia guilliermondii* recombinant inulinase in *Pichia pastoris* and characterization of the recombinant inulinase. Process Biochem 44:1335–1339

Zhao K-N, Gu W, Fang NX, Saunders NA, Frazer IH (2005) Gene codon composition determines differentiation-dependent expression of a viral capsid gene in keratinocytes in vitro and in vivo. Mol Cell Biol 25:8643–8655

Zhu T, Guo M, Sun C, Qian J, Zhuang Y, Chu J, Zhang S (2009) A systematical investigation on the genetic stability of multi-copy *Pichia pastoris* strains. Biotechnol Lett 31:679–684