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Full Length Research Paper

A novel vector for expression cloning of large numbers of polymerase chain reaction products in *Pichia pastoris*

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We described construction of a novel vector, pAOX-HT, for direct cloning of polymerase chain reaction (PCR) amplified fragments and expression in *Pichia pastoris*. The pAOX-HT serves both as a T-vector and expression vector and can be generated from a parent plasmid by digestion with restriction enzyme *Xcm*I. To minimize the non-recombinant background, the parent vector pAOX-HT-BSK was designed to contain a large insert. The cloning efficiency was above 95% when tested with PCR products. The linearized pAOX-HT was engineered to harbor a potential *Afl*II site (CT) upstream of the T/A cloning site. An *Afl*II site was reconstructed when the PCR product with 5'-TAAG sequence was ligated into the T/A cloning site. Taking advantage of this property, we digested the ligated products by restriction enzyme *Afl*II before transformation to eliminate the clones containing inserts with undesired orientation. By using pAOX-HT vector, the lipase B gene from *Candida Antarctica* was efficiently cloned and expressed in *P. pastoris* and the recombinant protein was purified by affinity chromatography. These results demonstrate that the pAOX-HT might serve as a useful tool for gene function study.

Key words: T vector, expression vector, yeast expression, restriction endonuclease digestion.

INTRODUCTION

Recently, the rapid progress of genome sequencing projects has produced large amounts of Open Reading Frames (ORFs) whose function need to be elucidated (Liolios et al., 2008). To characterize these ORFs, cloning of them into a plasmid for subsequent investigation such as sequencing and expression is necessary. However, it is a challenge for cloning and expression of a large number of genes simultaneously.

The traditional cloning method based on restriction enzyme digestion is not efficient since it need to check the each individual sequence for the presence or absence of restriction enzyme recognition sites in the designed primers, which is time and labor consuming. T/A cloning is a promising cloning strategy. The advantage of T/A cloning is that the polymerase chain reaction (PCR) products do not need to be digested before cloning, allowing parallel cloning numbers of PCR products. PCR products with 3' adenine residual overhang can ligate with linearized T vector which harbor 5' thymine residual overhang at both end (Zhou and Gomez-Sanchez, 2000). Many kinds of T vectors kits such as pGEM-T (Promega) and pT7Blue (Novagen) are commercial available, however, they suffer a problem of high cost. Two economic methods can be use to generate T vectors in an ordinary laboratory. The first method involves tailing with dT or ddT residues to a linearized, blunt-ended vector by terminal deoxynucleotidyl transferase or Taq DNA polymerase (Holton and Graham, 1991). Alternative, to generate single dT overhangs in the both end of the vector, vector can be digested by suitable restriction enzyme, such as *Xcm*I (Jo and Jo, 2001), *Ahd*I (Jeung et al., 2002) and *Hph*I (Mead et al., 1991). The latter method was more attractive since the preparation procedure is simple. However, one obstacle is that partial digestion of the parent vector will increase the non-recombinant

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background (Jo and Jo, 2001).

For high throughput cloning and expression of target genes, researchers have developed a series of T/A-cloning-based expression vector to rapidly clone and express genes of interest in *Escherichia coli* (Kwak and Kim, 1995; Goda et al., 2004; Reisinger et al., 2007). Unfortunately, heterologous expression of genes in *E. coli* will tend to produce protein in inclusion bodies form, limiting the downstream investigation. In contrast, the *Pichia pastoris* is considered as a useful host to produce heterologous proteins in large quantity (Cregg et al., 2000). It has reported that many genes have been functionally expressed in *P*. *pastoris* (Daly and Hearn, 2005).

For reasons above, in this study, we reported development of a novel T/A-cloning-based expression vector, named pAOX-HT. Using the vectors described here, the highly efficient cloning of target genes and immediate protein expression in *P. pastoris* could be achieved.

MATERIALS AND METHODS

Construction of parent plasmid

The frequently used *P. pastoris* expression vector pPIC9K (invitrogen), as an original vector, was chosen to construct the expression T-vector. The procedures were performed as following: (i) Silence of the *Xcm*I site. To silence a pre-existing *Xcm*I site located in the AOX1 promoter region, overlap extension PCR method was performed. First , a 498 bp fragment containing a mutated *Xcm* I site was generated by PCR using pPIC9k as template with the following primers: p1: (5'-ATTGGAGCTCGCTCATTCCAATTCCT-3', S*ac* I site (underlined)), p2: (GTATGCTTCGAAGATTCTGGTCGGAATACTGC-3'-3', mutated *Xcm*I site (underlined)). Second, a 640 bp fragment containing overlapping sequence with the former resulting fragment was generated by PCR using the following primers: p3 :(5'-GCAGTATTCCGACCAGAATCTTCGAAGCATAC-3'); 3'AOX primer (5'- GCAAATGGCATTCTGACATCC-3'). And then, fusion of the two fragments was performed by PCR using the primers P1 and 3'AOX primer. The resulting 1100 bp fragments were digested by *Sac*I and *EcoR* I (TaKaRa, Dalian, China) and cloned into the same site of the pPIC9k to generate a mutated pPIC9K vector. (ii) Preparation of inserts. We designed primers P5: (5'-CCGGAATTCCAT*CATCATCATCATCATCAT*TCCAGGTCTAGACTGG ATGAGTAAAGGAGAAG-3', with *EcoR*I (underlined) and *Xcm*I (bold) sites as well as a hexahistidine tag coding sequence (*italic*)) and P6: (5'-TAAAGCGGCCGCCCAGTCTTGACCTGGTCACTTGTACAGCTC G-3', with *Not*I (unerlined) and *Xcm*I site (bold)) to amplify Green Fluorescent Protein (GFP) gene using pblusctrit-GFP vector as temple. For preparation of a large insert, we designed primer P7 (5'-AGACTGGGCTCCCTCGTGCGCTCTCCTGT-3', rear parts of *Xcm*I site (underlined)) and P8 (5'-GACCTGGGGGGGAAACGCCTGGTATCTTT-3', rear parts of *Xcm*I site (underlined)) to amplify the whole backbone of pbluscrit SK (BSK) using LA polymerase (Takara). All the resulting PCR products were purified by gel extraction. (iii) Constructions of vectors pAOX-HT-GFP and pAOX-HT-BSK. The GFP DNA fragment was digested by *EcoR* I and *Not* I and inserted into the modified pPIC9K to generate plasmid pAOX-HT-GFP. And then, the pAOX-HT-GFP was digested by *Xcm* I to release the GFP fragment and generate linearized T vector. The BSK DNA fragment was ligated with the linearized T vector to generate pAOX-HT-BSK.

Preparation of linearized pAOX-HT

To prepare the linearized t-vector, pPIC9K-HT-GFP and pPIC9K-HT-BSK was digested by *Xcm*I restriction enzyme (New England BioLabs, MA, USA) according to the manufacturer's instruction. The digestion product was separated by 1% gel electrophoresis and the linearized pAOX-HT (approximate 9,000 bp) was purified by gel extraction.

Cloning of the PCR product

The lipase B gene from *Candida Antarctica* (*CalB*) was amplified by PCR using proof-reading polymerase Primerstar (Takara) with pYDI-*CalB* (previously constructed) as template and oligonucleotdes with following sequence as primers: CalB–F: 5'-TTACCTTCTGGATCAGACCC -3', CalB-R: 5'-TAAGTTAGGGTGTAACAATACCAGAA -3', rear part of the *Afl*II site (underlined)). The PCR products were purified by gel electrophoresis and extracted from the gel. Tailing of the PCR product with A was performed by incubation of 30 ul purified CalB fragment (approximately 20 ng/ul) with 5 ul PCR buffer, 2ul Taq DNA polymeras (Dingguo, Beijing, China), 1ul dATP(100 mM) (Fermentas, Vilnius, Lithuania) and 12 ul deionized water at 72°C for 20 min. The A-tailed product was purified by PCR cleanup kit (Axygen, CA, USA). And then, the A-tail PCR products were ligated with the linearized pAOX-HT at 16°C for overnight. The ligation products were incubated at 65°C for 30 min to inactive the ligase and digested by *Afl*II according to the manufacturer's instruction. The ligation mixtures were transformated to DH5 alpha and were screened at two plates containing kanamycin and Isopropyl β-D-1-thiogalactopyranoside (IPTG) as well as X-gal. The white and blue colonies on the two plates were counted after culture. The clones with inserts were indentified by colony PCR. Primers CalB–F and CalB–R were used to indentify the clones with insert; Primers CalB– F and 3'AOX were used to check the orientation of the inserts in the clones.

Protein expression and purification

The recombinant plasmid pAOX-HT-*CalB* was linearized by *Sal*I and transformed to the GS115 strain (Invitrogen) according to the standard protocol (Wu and Letchworth, 2004). The transformants were selected on MD plate at 28°C for three days. The transformants were grown in the MMH (1.34% YNB (W/V), 4×10^{-5}% biotin (W/V) and 0.5% methanol (V/V)) plate containing 1% tributyrin (Sigmar) at 28°C. The recombinant yeast colonies producing the largest halos were selected for subsequent expression studies.

For expression of CalB in the shake flask, the recombinant yeast was cultured in BMGY (100 mM potassium phosphate (pH 6.0), 1.34% YNB (W/V), 4×10^{-5}% biotin (W/V) and 1% glycerol (V/V)) until an OD600 of 2-5 at 30°C. The cells were spun down by centrifugation and resuspended by 100 ml BMMY (100 mM potassium phosphate (pH 6.0), 1.34% YNB (W/V), 4×10^{-5}% biotin (W/V) and 0.5% methanol) to adjusting an OD600 of 1. Methanol was added at a concentration of 0.5% (v/v) for induction everyday during the expression. The cells were spun down by centrifugation and the supernatant was analyzed by SDS-PAGE.

For purification of the recombinant CalB, the cells were spun down by centrifugation at a speed of 10,000 rpm. The supernatant was loaded onto a sepherdex G25 column previously equilibrated with 50 mM NaCl, 20 mM Tris-HCl, and pH 8.0 (Buffer A). The

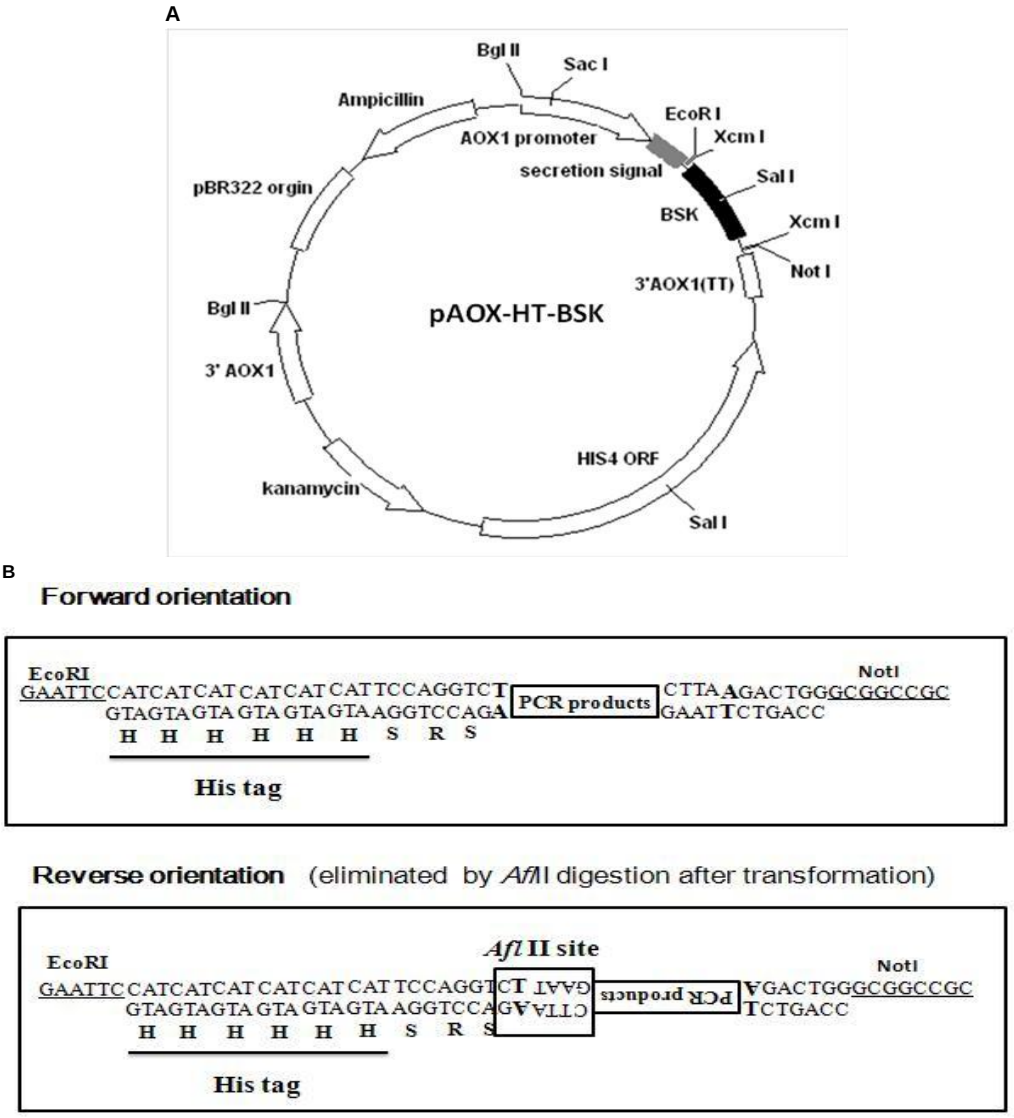

Figure 1. Construction of yeast expression "T-vector" and principle of cloning PCR products. (A) Physic map of the pAOX-HT-BSK. The restriction enzyme recognition site used in this paper was shown in the map. (B) Cloning of PCR products. pAOX-HT-BSK was digested by *Xcm* I to generate the 3' overhang T and a potential *Afl* II site (5'-CT-3'). PCR products with 3' overhanging A was ligated with the overhanging T in the vector. A new *Afl* II site will appear only when the PCR product with 5-TAAG sequence is ligated into the T/A cloning site in a reverse orientation. These clones was linearized by restriction enzyme *Afl*II digestion and eliminated after transformation.

desalted supernatant sample was then loaded onto the Ni^{2+}-NTA His Bind resin (GE) previously equilibrated with Buffer A. Protein was eluted by Buffer A containing 50, 100, 300 and 500 mM imidazole in order. Fractions eluted from the column were analyzed by SDS-PAGE.

RESULTS AND DISCUSSION

For preparation of linearized T-vector by restriction enzyme digestion, introduction of an inserts into the cleavage sites can improve the efficiency of digestion and make separation and purification of the completely digested DNA fragments from the undigested one more readily (Jo and Jo, 2001). Therefore, we introduced two kinds insert with various sizes into the vector to generate pAOX-HT-GFP and pAOX-HT-BSK (Figure 1). To test the efficiency of the T vectors prepared by these two parent vectors, we selected the well-known *CalB* gene as a model (Anderson et al., 1998; Rotticci-Mulder et al., 2000). High cloning rate (23/24) has been observed by using T vector prepared by pAOX-HT-BSK, and the ratio of blue colony on X-gal/IPTG plate was only 1/400, suggesting that the rate of self-ligation of partial digestion pAOX-HT-BSK was rare. The reason might be that the

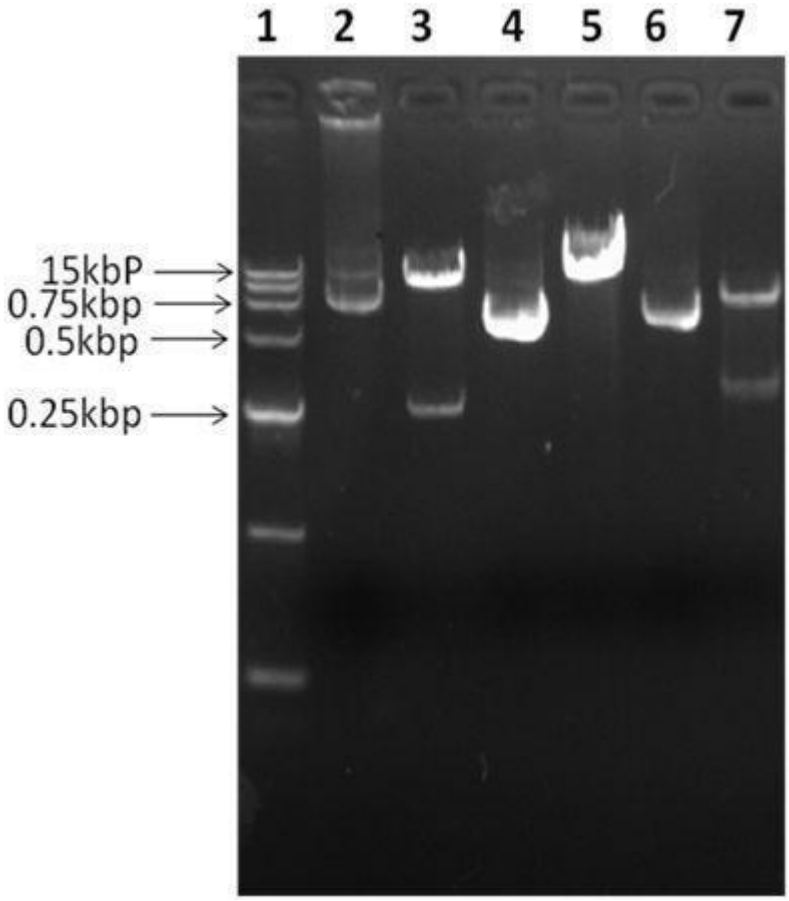

Figure 2. Restriction analysis of isolated plasmid DNA. Lane 1: DNA marker. Lane 2: isolated pAOX-HT-BSK plasmid. Lane 3: pAOX-HT-BSK digested by *Sal* I. Lane 4: isolated pAOX-HT-CALB. Lane 5: pAOX-HT-CALB (forward orientation) digested by *Afl*II and *Sal*I. Lane 6: isolated pAOX-HT-CALB (inverse orientation). Lane 7: pAOX-HT-CALB (inverse orientation) digested by *Afl*II and *Sal* I.

linearized vector pAOX-HT (about 9,000 bp) was easily separated from the partly digested pAOX-HT-BSK (about 12,000) and the released BSK fragment (about 3,000 bp) by electrophoresis. Moreover, blue/white screen could be used for depleting the non-recombinant clones since the insert BSK contained a *lacZ* gene. For this reason, the pAOX-HT-BSK was used as a parent vector for the subsequent study.

Although, the T/A cloning is an efficient strategy for PCR product cloning, one obstacle is that the cloning is not directional, thus needing more efforts to indentify the correct orientation of cloned PCR products for subsequent gene expression study. We employed a restriction enzyme digestion method to eliminate the clones containing inserts in an undesired orientation. This method was also applied for directional cloning and analysis promoter sequences (Wang et al., 2007). As described in the materials and methods section, the reverse primer of *CalB* was designed to contain four additional nucleotides (5'-TAAG-3'), while the linearized pAOX-HT was engineered to harbor a potential *Afl*II site (CT) upstream of the T/A cloning site. As shown in Figure 1B, an *Afl* II recognition site was reconstructed only when the PCR products were ligated into the pAOX-HT vector in a reverse orientation. These unwanted clones were linearized by *Afl* II digestion and eliminated after transformation into the bacteria host. In our case, transformation of the ligation product after restriction enzyme digestion yielded an efficiency of 8×10^6 CFU/mg DNA. The recombinant clones containing insert with correct orientation were identified by colony PCR and the positive clones at a rate above 80% (20/24) were observed. The clones were further identified by *Sal* I and *Afl* II digestion. The clones containing inserts with forward orientation were only linearized by *Sal*I (Figures 2 lane 4, 5), while the clones containing inserts with reverse orientation would release a DNA fragment after digestion by *Sal*I and *Afl*II (Figure 2 lane 6, 7).

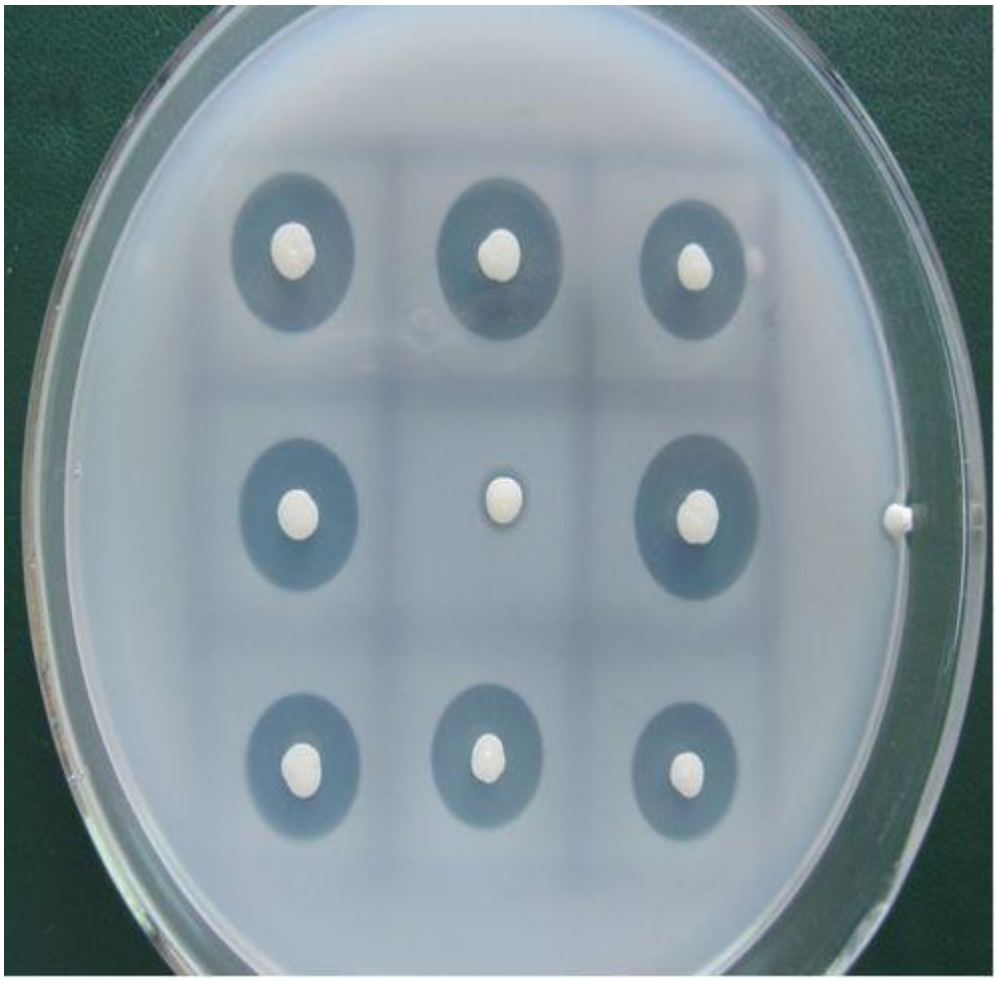

Figure 3. Identity of the transformants secreting lipase by the MM-tributyrin plate. The control strain, wild type strain X-33, was located in the middle of the plate. The transformants harboring CALB expression vector surrounded the X-33. Functional expression of lipase would yield a halo around the colony.

For expression CalB in *P. pastoris,* the pAOX-HT-*CalB* was linearized by *Sal*I and transformed into the GS115 strain. Transformants were screened by tributyrin plate assay after transformation. The Figure 3 shows that all of the selected transformants in the plate can yield clear halo, suggesting that the selected transformants functionally produced CalB lipase. Purification of recombinant lipase with reasonable amount was important for subsequent biochemical properties study and application. As shown in Figure 4A, high level expression of recombinant CalB with an expected size was observed and the recombinant CalB was purified by affinity chromatography with the help of the artificial his tag which we designed at the vector (Figure 4B).

In summary, we develop a T/A cloning based expression vector, pAOX-HT, for expression genes of interest in *P. pastoris* yeast. Our vector has several obvious advantages: First, a large insert (BSK) located between the two *Xcm*I sites attributes to decrease the non-recombinant background. Second, pre-digestion of ligation products by *Afl*II before transformation could eliminate the unwanted clones, which permits directional cloning of PCR products. Finally, the artificial His tag in the vector facilitate the purification of recombinant protein by affinity chromatography. Therefore, the pAOX-HT vector has numerous potential for gene function study in *P. pastoris*.

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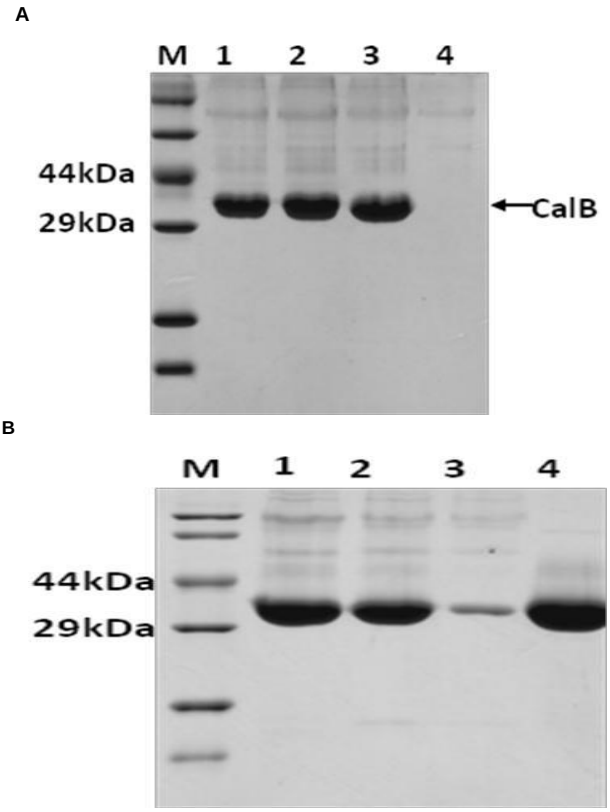

Figure 4. SDS-PAGE analysis of sample from the expression and purification of the recombinant CalB. (A) Expression of CalB in *P. pastoris*. Lane M: marker, lane 1-3: cultural supernatant samples from three selected transformants, lane 4: sample from control cell. Arrow denotes the recombinant CalB with expected size. (B) Purification of CalB using nickel-chelate chromatography. Lane M: marker, lane 1: cultural supernatant sample, lane 2: desalted cultural superntant after G25 column, Lane 3, flow-through sample from nickel-chelate column. Lane 4: purified CalB after affinity chromatography.

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