CONSTITUTIVE EXPRESSION OF *Candida antarctica* LIPASE B (CALB) IN *Pichia pastoris* USING pGAPZα VECTOR

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Abstract

The synthetic gene of *CalBsyn* was previously constructed to encode *Candida antarctica* lipase B (CALB). Lipase of *CalBsyn* gene is slightly different from wild type CALB (CALB-wt) where it has three amino acids substitutions at different positions, i.e. V210I, A281E, and V221D, in order to improve its thermostability and catalytic efficiency. The *CalBsyn* gene was isolated from pJ912-*CalBsyn* vector by digestion using *Xho*I restriction enzyme. The 1136 bp fragment of *CalBsyn* gene was then ligated to pGAPZα expression vector and transformed into *Escherichia coli* TOP10F' to obtain recombinant vector pGAPZα-*CalBsyn*. The result show that pGAPZα-*CalBsyn* recombinant vector was successfully transformed into *E. coli* TOP10F' with transformation efficiency of 4.11 x 10^3 cfu/µg plasmid DNA. The pGAPZα-*CalBsyn* recombinant plasmid was successfully introduced into *Pichia pastoris* SMD1168H using electroporation method with transformation efficiency of 1.01 x 10^2 cfu/µg DNA. Recombinant protein expression was analyzed in several selected *P. pastoris* recombinant strains. Qualitative lipase activity assays results show that transformed *P. pastoris*-produced extracellular recombinant lipase (CALB) showing lipolytic activity; while results of quantitative lipase activity assays show that this *Pichia*-derived lipase achieved an activity of 6.35 Units/mL within 48 hours. SDS-PAGE analysis confirms the succesfull expression of CALB protein with molecular size was approximately 45 kDa.

Keywords: *Candida antarctica* lipase B (CALB), pGAPZα, constitutive expression, *Pichia pastoris*

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Introduction

Lipases catalyze the hydrolysis and the synthesis of esters formed from glycerol and long-chain fatty acids (Sharma *et al*., 2001). Lipases belong to an important group of biotechnologycally relevant enzymes and they find immense applications in food, dairy, detergent and pharmaceutical industries (Gupta, 2004). Lipase also has various enzymatic characteristic and substrate specificity in industrial uses (Blank *et al*., 2006). Lipases are produced by various microbes, including bacteria, fungi, and yeast. They also have been reported to be produced in higher plants, such as castor bean (*Ricinus communis*) and rapeseed (*Brassica napus*) (Gunasekaran & Das, 2005). Microbial enzymes are often more useful compared to other source such as plants or animals because of the great variety of their catalytic activities, the possible high yields protein expression, ease on genetic manipulation, and rapid growth of microorganisms on inexpensive media (Hasan *et al*., 2006).

Literature reviews reveal that the Antarctic yeast (*C. antarctica*) is the most extensively studied microorganism with respect to its lipase secretion. There are two lipase variants, *C. antarctica* lipase A (CALA) and *C. antarctica* lipase B (CALB). CALA shows interfacial activation, while CALB does not show such behavior and is therefore not considered to be true lipase. CALB has been extensively used in various industries and applications, such as food, feed, pharmaceuticals, environmental, bioremediation, and other molecular biology applications. It is mostly due to the nature of CALB which can be applied at low temperatures, has a good thermostability to the increased temperature and has a high stability toward organic

solvents (Joseph *et al*., 2008; Pfeffer *et al*., 2006).

Recombinant protein derived from eukaryotes is widely expressed in yeast expression system. Yeast has several advantages as a host cell for the production of heterologous proteins as it allows post-translational protein modification and disulfide bond formation, in addition to correct protein folding that increases resistance against protease degradation. Post-translational protein modification in yeast allows proteins to form a biologically active structure (Cho *et al*., 1998). *Pichia pastoris* has become highly popular expression host for production of various intracellular and extracellular recombinant proteins (Balamurugan *et al*., 2006). Yeast *P. pastoris* is a single-cell microorganism that is easy to manipulate and culture. *P. pastoris* is methylotropic yeast capable of utilizing methanol as sole carbon source. Recombinant protein production using *P. pastoris* has several advantages, including high level expression of recombinant protein and genetic stability. These advantages are mostly due to the introduction and integration of DNA vector into the yeast genome (Daly and Hearn, 2005).

The *CalBsyn* gene used in this study had been modified at three amino acid residues according to Zhang *et al*. (2003). Modification has been done by three amino acid mutations at V210I (Valine *et al.* 210^{th} residue was mutated to Isoleucine), A281E, and V221D. These mutations were carried out to improve enzyme thermal stability through increasing temperature. This study was aimed to evaluate enzyme characteristics of *Pichia*-derived CALB that was overexpressed constitutively from *P. pastoris* SMD1168H.

Materials and Methods

Plasmid and Chemicals. DNA plasmid used in this study was pGAPZα (Invitrogen, USA). The synthetic gene of *CalBsyn* gene was previously constructed by Fuad (unpublished) to encode *C. antarctica* lipase B (CALB). The gene was mutated at 3 amino acid residues (V210I, A281E, and V221D). The *CalBsyn* gene was cloned into pJ912 vector [DNA 2.0]. *E. coli* TOP10F' (Invitrogen, USA) was used for vector cloning while *P. pastoris* SMD1168H (Invitrogen, USA) was used to express CALB. *E. coli* TOP10F' was grown in Low Salt Luria Bertani (LSLB) media (Sigma Chemical, Singapore) at 37°C, 150 rpm, overnight, while *P. pastoris* SMD1168H was grown in YPD (Yeast Peptone Dextrose) media (Sigma Chemical, Singapore) at 30°C, 250 rpm, 24h. To facilitate clonal selection, 25 µg/mL zeocin was added to LS-LB media for *E. coli* TOP10F'.

Construction of pGAPZα-*CalBsyn* plasmid. All procedures for plasmid isolation, gene cloning, ligation and transformation of recombinant plasmid into *E. coli* followed Ausubel *et al*. (2002). The *CalBsyn* gene was isolated from pJ912-*CalBsyn* based on PCR method using primers CalB forward (5'-TTG CCTTCAGGTTCAGACC-3') and CALB reverse (5'-GTCTAGAATCGATAGGAGTA ACTATACC-3'). Both *CalBsyn* gene and pGAPZα plasmid were digested using *Xho*I and incubated at 37°C for 18 hours. After digestion, *Fast Alcaline Phosphatase* enzyme (Thermo scientific, USA) was added into digested pGAPZα plasmid to prevent re-ligation. Both *CalBsyn* gene and plasmid were then purified using Gel DNA Extraction Kit (Gene Aid, Taiwan) and the result from which was analyzed using 1% agarose gel electrophoresis. Purified *CalBsyn* gene was cloned into pGAPZα vector using T4 DNA ligase (KAPA), followed by transformation into *E. coli* TOP10F' using heat shock method. The transformation mix was plated on LSLB-zeo medium and incubated overnight at 37°C. Transformed *E. coli* were analyzed using colony PCR method, using primers P_{GAP} forward (5'-GTCCCTATTTCAATCAATTG AA-3') and AOX_1 reverse (5'-GCAAATGGC ATTCTGACATCC-3'). Recombinant plasmids from positive colonies were isolated and characterized using PCR (CalB forward and AOX_1 reverse primers) and restriction analyses. Confirmed recombinant plasmids were then transformed into *P. pastoris* SMD1168H.

Expression of CALB in *P. pastoris*.

Preparation of *Pichia* for Electroporation. *Pichia pastoris* were grown in 5 mL of YPD at 30^0C, 250 rpm, 24h. The overnight cultures (100 µL) were inoculated into 50 mL of YPD and incubated at 30^0C, 250 rpm, overnight to reach an OD_{600}= 1.3 - 1.5. The cells were centrifuged at 1500 g for 5 minutes at 4^0C. The pellets were resuspended with 50 mL of ice-cold (0^0C) sterile water. The cells were

centrifuged (4000 g, 7 minutes, 4^0C), then the pellets were resuspended with 25 mL of ice-cold (4^0C) sterile water. The centrifugation and resuspension of the pellets were repeated twice, and the pellets volume were adjusted into 2mL and 200 µL with ice-cold 1M sorbitol for first and second repetition, respectively. Keep the cells on ice and use in the same day.

Transformation using electroporation. Selected pGAPZα-*CalBsyn* recombinant plasmid was linearized with *Bsp*HI (*Pag*I). Plasmid transformation into yeast cells was carried out using electroporation following manufacturer's protocol by Invitrogen and Biorad. A total of 0.5 µg linearized DNA was transferred into 70 µL of *P. pastoris* competent cells, SMD1168H strain in an electroporation cuvette (2mm gap, Biorad, USA). Transformed colonies were grown and selected on YPDS zeocin selection medium (1% yeast extract, 2% peptone, 2% dextrose, 1M sorbitol, 1.7% agar) containing 100 µg/mL zeocin (Invitrogen, USA).

Selection of transformed *P. pastoris*. Transformed *P. pastoris* were selected following manufacturer's procedures by Invitrogen. All transformed colonies that grew on the selection medium were transferred into plates with new YPD zeocin medium containing 100 µg/mL zeocin. The plates were incubated at 30°C for 2 to 4 days. Stable transformants that grew on this medium were then transferred into YPD zeocin agar medium containing higher zeocin concentrations (200, 500, and 1000 µg/mL) to acquire recombinant clones having multi-copy target gene.

Protein expression of *P. pastoris* transformants. Evaluation of recombinant protein expression was conducted by culturing 4 colonies following manual instructions of *Pichia* expression kit (Invitrogen, USA). Transformed and non-transformed *P. pastoris* cultivated in 2 mL YPD medium (with and without zeocin) were incubated at 30°C and 250 rpm for 24 hours. These cultures were used to inoculate another 50 mL YPD media in 250 mL erlenmeyer flasks. Cultures were incubated at 30°C and 250 rpm for 72 hours. Every 24 hours, cultures were sampled and transferred into 1.5 mL microtubes. Cultures were harvested using centrifugation (10.000 rpm, 15 minutes, 4°C) after 72 hours cultivation. Cell biomass was stored at -20°C while cell-free culture medium (supernatant) was stored at 4°C. Protein expression level was analyzed using SDS-PAGE and lipase activity assays.

Quantitative Lipase Activity Assay. Quantitative lipase activity assay was performed following Continuous Spectro-photometric Rate Determination method (Quinn *et al*., 1982; Sirai and Jackson, 1982). Lipase activity assay was performed using ρ-Nitrophenyl Butyrate (PNPB) as the substrate and *Candida rugosa* lipase (CRL) as the positive control, both were from Sigma. Reagents used were consisted of Reagent A with 100 mM Sodium Phosphate Buffer, 150 mM Sodium Chloride, and 0.5% Triton X-100. Substrate was made by mixing 50 mM PNPB with acetonitrile solution. As the positive control, *C. rugosa* Lipase (CRL) from Sigma was dissolved in Reagent A. Lipase activity was measured spectrometrically at 400 nm wavelength and counted every minute up to five minutes.

Results and Discussion

Construction of pGAPZα-*CalBsyn*. The *CalBsyn* gen was isolated from pJ912-*CalBsyn* vector through PCR amplification and digested with *Xho*I. The pGAPZα vector was isolated from *E. coli* and digested with *Xho*I as well. The *CalBsyn* fragment was cloned at the *Xho*I site, next to the alpha-factor signal peptide (MFα) (Figure 1).

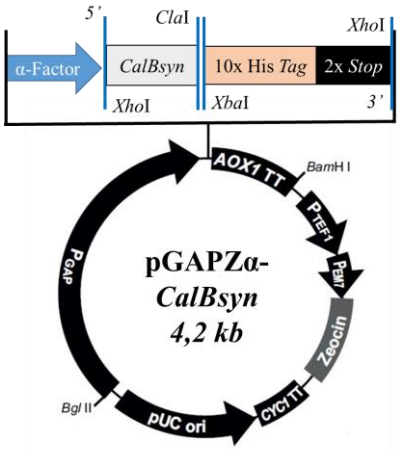

Figure 1. Construction of pGAPZα-*CalBsyn* recombinant plasmid

The pGAPZα-*CalBsyn* recombinant plasmid had been successfully obtained by a general ligation protocol using T4 DNA ligase. The cloned *CalBsyn* fragment contains a pre-protein sequence of CALB protein, which includes the native CALB signal peptide (first 25aa). This *CalBsyn* fragment was subcloned next to the MFα signal peptide already present in the vector. The length of the cloned *CalBsyn* gene was 1088 bp. Additional short linker and 10xHis-tag sequence at the C-terminal made the total gene length of 1136 bp. DNA ligase is an Mg^{2+} dependent and ATP dependent enzyme that seals DNA nicks in three steps, i.e. covalently binds AMP, transadenylates the nick phospate, and catalyses the formation of phosphodiester bond releasing AMP (Cherepanov, 2003). Recombinant plasmid was then introduced into *E. coli* TOP10F' using heat shock method and grew on LSLB agar medium with 25 µg/mL zeocin. A total of 13 recombinant *E. coli* clones were grown on LSLB medium + 25 µg/mL zeocin. Various potential factors may influence transformation efficiency, including incubation condition, concentration of the inserted recombinant plasmid, relative competence of the recipient cells, and presence of DNA contamination (Classen *et al*., 2002).

A key step in the construction of recombinant plasmids is verification of the successful cloning of insert DNA into the vector (Siekierke, 2003). Additional screening methods are required to confirm the presence and orientation of the insert. Screening is also necessary because not all colonies, which grow after transformation contained plasmid with the desired DNA insert (Woodman, 2008). There are several screening methods available to verify the insert gene, e.g. colony PCR, restriction digestion, and sequencing. This study utilized colony PCR method to screen transformants. Transformants were characterized using colony PCR method with P_{GAP} forward and AOX1 reverse primers. A total of five out of 13 colonies were found to carry *CalBsyn* insert.

To confirm correct recombinant plasmids, several positive colonies were picked up and the plasmids were characterized using PCR, plasmid restriction, and plasmid sequencing analyses. Using CalB forward and AOX1 reverse primers, the PCR analysis was performed to obtain the correct direction for the inserted gene. Positive colonies produced PCR product with the size of approximately 1358 bp (Figure 2A). Meanwhile, restriction analyses of recombinant plasmids using *Xho*I enzyme produced DNA bands with molecular size of 1136 bp and 3100 bp, which were in accordance with theoretical size of the insert DNA and the expression vector, respectively (Figure 2B).

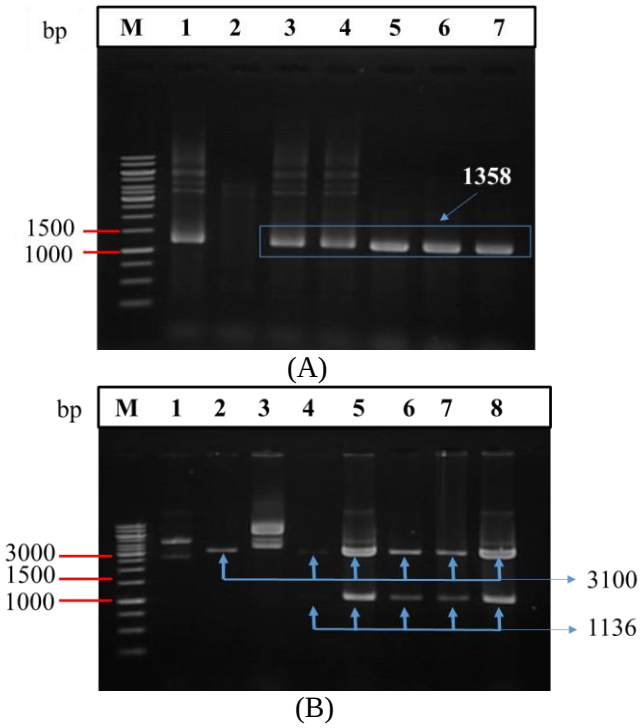

Figure 2. (A) PCR analysis of transformed *E. coli* using CalB forward and AOX1 reverse primers. M: 1 kb DNA Ladder; 1: pJ912-*CalBsyn* (positive control); 2: pGAPZα (negative control); 3-7: different clones of pGAPZα-*CalBsyn*. (B) Restriction analysis of pGAPZα-*CalBsyn* plasmid using *Xho*I. M: 1kb DNA Ladder; 1: uncut pGAPZα; 2: pGAPZα cut with *Xho*I; 3: uncut pGAPZα-*CalBsyn*; 4-8: different clones of pGAPZα-*CalBsyn* cut with *Xho*I.

Some clones of recombinant plasmid from selected transformants were sequenced. Based on the result of DNA sequencing analysis, it was found that there was no mutation in DNA encoding *CalBsyn* gene (Sequence data not shown). Conclusion drawn from all conducted analyses is the recombinant plasmid was successfully constructed.

Expression of CALB in *P. pastoris*. After plasmid confirmation by sequencing followed

by linearization of plasmid using *Bsp*HI, pGAPZα-*CalBsyn* recombinant plasmid (clone #11) was transformed into *P. pastoris* using electroporation method. The transformation process yielded 64 individual transformed *P. pastoris* colonies with transformation efficiency 1.01 x 10^2 cfu/µg DNA. The recombinant plasmid was inserted into the *P. pastoris* genome by homologous recombination mechanism that benefited from similarity between GAP promotor (P_{GAP}) sequence found in *P. pastoris* genome and pGAPZα vector (Figure 1 & 3). Low transformation efficiency due to difficult integration of the linearized plasmid into the *P. pastoris* genome. Integration of the DNA plasmid into the genome was based on homologous recombination event. There are various burdens for the plasmid to enter the nucleus, where the homologous recombination takes place. Some factors that may affect low transformation efficiency are, among others, inefficient integration site, troublesome insert DNA and non-optimal transformation condition (Wu & d Letchworth, 2004).

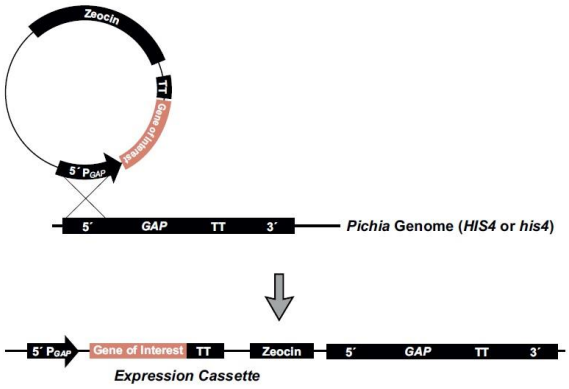

Figure 3. Integration map of a heterologous gene into *Pichia pastoris* genome for single copy integration (Adapted from Invitrogen, 2002)

The vector contains a gene encoding resistance against zeocin, namely *Sh ble* gene from *Streptoalloteichus hindustanus* (Invitrogen, 2002). This transformation selection marker allowed us to screen and select transformed cells with ease. Selection of transformed cells in zeocin medium, in most cases, resulted in cells which is genetically stable and eventually allowed us to screen individual cells showing multicopy genes. Zeocin concentrations used for screening were 100, 200, 500, and 1000 µg/mL (Figure 4). Non-transformed *P. pastoris* was included as control to observe zeocin effectiveness in selecting transformed *Pichia*. At 100 µg/mL zeocin, approximately 90% of all colonies were well grown. However, 40% only of those colonies were able to grow at 200 and 500 µg/mL of zeocin. It was found that no colony was able to grow well at 1000 µg/mL zeocin. Non-transformed *P. pastoris* was unable to grow on zeocin medium as well. Level of resistance against zeocin indirectly reflects number of recombinant genes transformed into the *P. pastoris* genome. According to Norden *et al*. (2011), a transformant cell which is resistant at 100 µg/mL zeocin has at least one copy of plasmid integrated within its genome; at 500 µg/mL zeocin the cell has at least 4 copies of plasmid; and at 1000 µg/mL zeocin the cell has at least 9 copies of plasmid in its genome. Increasing number of genes integrated in the genome would increase the expression of heterologous proteins (Vassileva *et al*., 2001; Mansur *et al*., 2005).

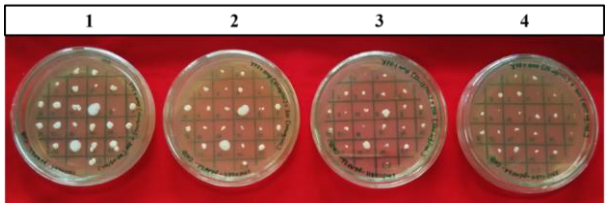

Figure 4. Screening of transformed yeast in YPDzeo selection medium with various zeocin concentration. 1: 100 µg/mL zeocin; 2: 200 µg/mL zeocin; 3: 500 µg/mL zeocin; 4: 1000 µg/mL zeocin.

Genetically stable transformed *P. pastoris* was acquired through homologous recombination mechanism between GAP promoter sequence in the vector and the same promoter in the *P. pastoris* genome. The integration process was started through crossover process of DNA recombination. Linearized DNA vector stimulated the recombination process at the homologous locus. Linearized DNA fragment acquired contained expression cassette and marker gene (zeocin) that was flanked by both 5'- and 3'-GAP sequences. Such condition stimulates gene replacement at the GAP genome, making the *GAP* gene replaced by the expression cassette and marker gene (Li *et al*., 2007).

One of the transformed *P. pastoris* was selected (clone #21) for quantitative lipase

activity assay. The clone was selected among those that were grown well at 500 µg/mL zeocin plate. Prior to the assay, the cell growth was observed. The cell growth of this selected *P. pastoris* clone stably increased up to 72 hours of culture. The growth of *P. pastoris* with YPD media reached logarithm final phase at 48 hours and reached stationary phase after 48 hours upto 72 hours (Tawfeek *et al*., 1989; Kolleva *et al*., 2008).

The growth curve of *P. pastoris* can be used to observe and describes the stages of its growth cycle. The growth of transformed *P. pastoris* increased along the increase in cell biomass, and reached its maximum point after 48 hours. The growth curve can also be used to predict the optimum period of enzyme production. To observe the optimum period, enzyme activities from the cell culture at different period had to be analyzed. Lipase activity assay was carried out from different time samplings up to 72 hours. It was found that the highest lipase activity was observed in the second day (48 hours) of culture with activity of 6.35 Units/mL (Figure 5). Unfortunately, we do not have the *wt*CALB yet and it's enzyme activity data that we could compare of. However, Zhang *et al.* (2003) has reported that this mutated variant of CALB, called as strain 195F1, has specific enzyme activity of 3.5 times and 4.2 times greater than the *wt*CALB with p-NB and DiFMU as substrates respectively. More over, the 195F1 strain or the mutated CALB variant used in this study has been reported to have a $T^1/_2$ of 211 minutes at 70°C compared to only 8 minutes for the *wt*CALB (Zhang *et al*., 2003). It suggests that this mutated CALB has much greater thermostability compared to the *wt*CALB.

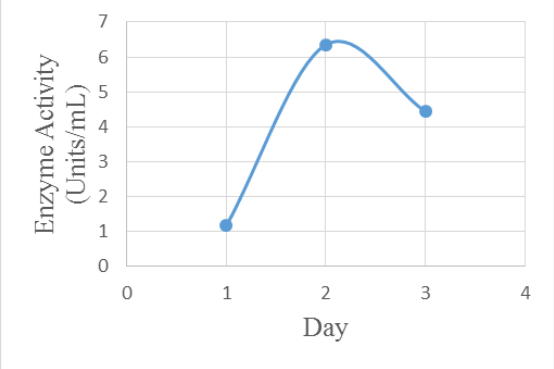

Figure 5. Lipase Activity Assay (*P. pastoris* clone #21). Day 1: 1.172 Unit/mL, Day 2: 6.35 Units/mL; Day 3: 4.44 Units/mL.

In this study, GAP promoter was used in *Pichia* expression system. This promoter regulates the expression of glyceraldehyde 3-phosphate dehydrogenase (GAP) in yeast cell. GAP promoter in *P. pastoris* regulated protein expression constitutively in glucose medium (Gaffar, 2010). The promoter allowed cell to grow without any additional inducer other than glucose. Using GAP promoter instead of AOX promoter has several advantages, e.g. methanol is not required for induction and transferring the culture from one carbon source to others is not necessary (Li *et al*., 2007).

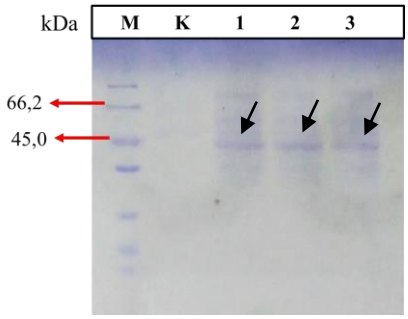

Figure 6. SDS-PAGE analysis of CALB protein (shown by arrows) from crude culture of transformed yeasts. M: Protein Marker; K: Negative control; 1-3: different clones of transformed yeasts.

Protein expression analysis of *Pichia*-derived CALB shows that the recombinant enzyme had been successfully produced constitutively and has a molecular size of approximately 45 kDa (Figure 6). Vadhana (2013) showed that recombinant CALB expression in *P. pastoris* had been improved just simply using the CALB native signal peptide (nsB-CALB) instead of MFα signal peptide (MFα-CALB). The expressed CALB using inducible vector pPICZα and pPICZ in *P. pastoris* GS115 showed a molecular size of slightly above 35 kDa (Vadhana *et al*., 2013). The *CalBsyn* gene sequence used in this study was the pre-protein sequence, which includes the first 25aa CALB natural signal peptide (nsB). It suggests that higher molecular size of recombinant CALB found in this study was due to the size of pre-protein sequence of CALB, where the pre-protein sequence was not cleaved.

Conclusion

The *CalBsyn* gene was successfully cloned into pGAPZα vector and fused with MFα signal peptide. This recombinant plasmid was confirmed by DNA sequencing. The plasmid was successfully transformed into *P. pastoris* SMD1168H with transformation efficiency 1.01 x 10^2 cfu/µg DNA. Lipase activity assay from crude culture achieved its maximum lipolytic activity at 6.35 Units/mL within 48 hours. The recombinant CALB was secreted constitutively as expected. SDS-PAGE analyses show that CALB protein has a molecular size of approximately 45 kDa. Its molecular size was most probably matched the size of CALB pre-protein.

Acknowledgements

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